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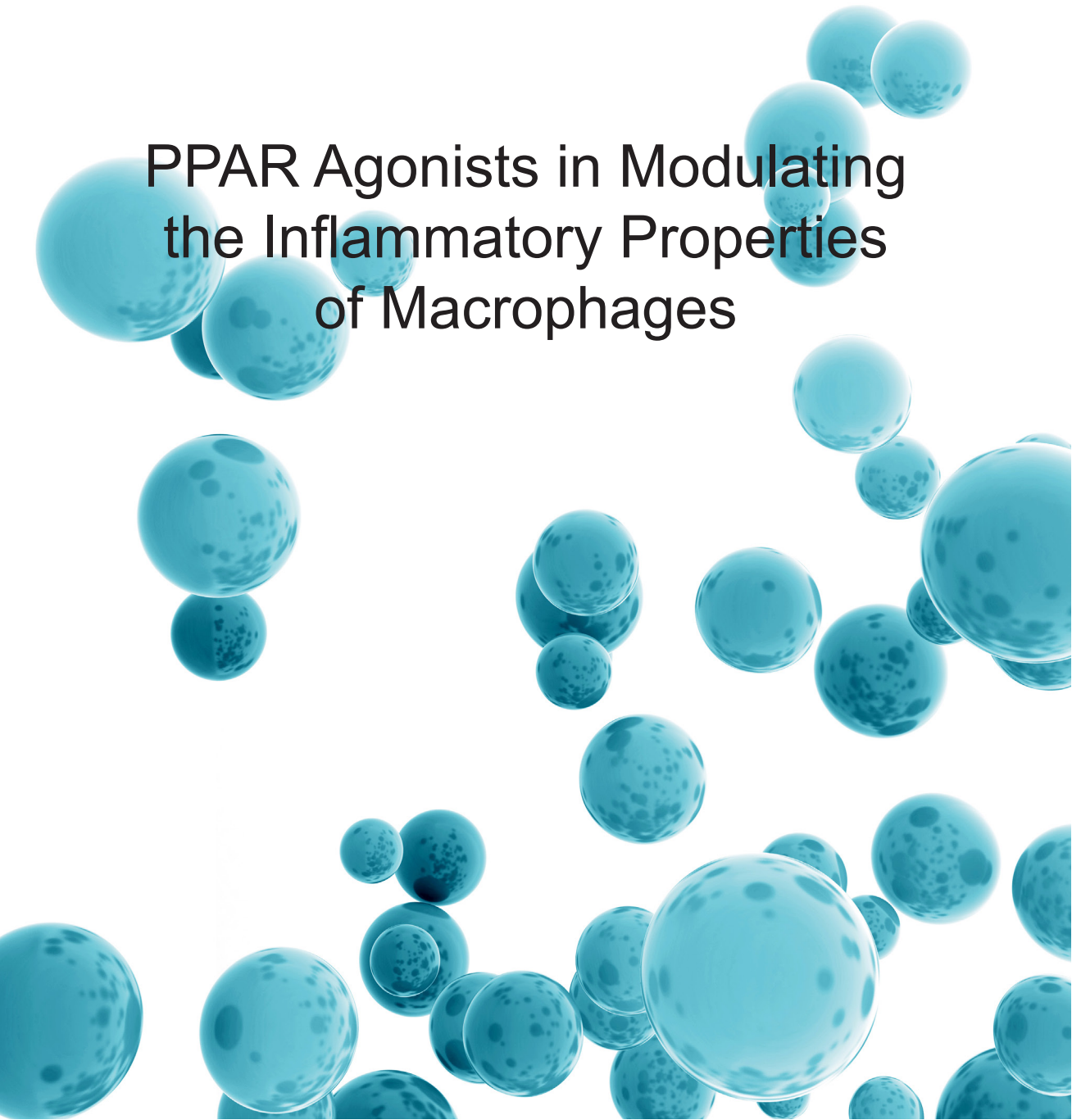
ERJA-LEENA PAUKKERI

PPAR Agonists in Modulating the Inflammatory Properties of Macrophages



ERJA-LEENA PAUKKERI

PPAR Agonists in Modulating the Inflammatory Properties of Macrophages

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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

ERJA-LEENA PAUKKERI

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II Paukkeri EL, Leppänen T, Lindholm M, Yam MF, Asmawi MZ, Kolmonen A, Aulaskari PH, Moilanen E (2013): Anti-inflammatory properties of a dual PPARgamma/alpha agonist muraglitazar in in vitro and in vivo models. Arthritis Res Ther. 15:R51.

III Paukkeri EL, Pekurinen A, Moilanen E (2015): Peroxisome proliferator-activated receptor α and γ agonists differently regulate classical and alternative macrophage activation. Immunometabolism. 2:1-11.

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ABBREVIATIONS

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
ACPA	anti-citrullinated protein antibody
ARE	AU-rich element
cAMP	cyclic adenosine 3'-5'-monophosphate
CBP	CREB binding protein
CCL	(C-C motif) ligand
CCR	C-C chemokine receptor
C/EBP	CCAAT-enhancer-binding protein
CHIP	COOH terminus of heat shock protein 70-interacting protein
CLA-1	CD36 and LIMPII analogous 1
CPT	carnitine palmitoyl transferase
CREB	cAMP responsive element binding protein
CRP	C-reactive protein
CTGF	connective tissue growth factor
CX_3CR1	CX_3C-chemokine receptor 1
DAS28	disease activity score 28
DM	diabetes mellitus
DMARDs	disease-modifying antirheumatic drugs
dcSSc	diffuse cutaneous systemic sclerosis
eIF	eukaryotic initiation factor
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal regulated kinase
FDA	Food and Drug Administration
fizz1	found in inflammatory zone 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLP-1	glucagon-like peptide-1
HDL	high-density lipoprotein
HMG-CoA	hydroxymethylglutaryl CoA
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IFNGR	interferon γ receptor
IGF-1	insulin-like growth factor 1
IκB	inhibitor of κB
IKK	inhibitor of κB kinase

IL	interleukin
IL-4R	interleukin 4 receptor
iNOS	inducible nitric oxide synthase
IRAK	interleukin 1 receptor-associated kinase
IRF	interferon regulatory factor
IRS	insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LBP	lipopolysaccharide binding protein
lcSSc	limited cutaneous systemic sclerosis
LDL	low-density lipoprotein
L-NIL	N^6-(1-iminoethyl)-L-lysine hydrochloride
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MAPK	mitogen-activated protein kinase
MAP2K	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MCP-1	monocyte chemotactic protein 1
MMP	matrix metalloproteinase
mrc-1	mannose receptor 1
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response gene 88
N-CoR	nuclear receptor corepressor
NF-κB	nuclear factor-κB
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCI	percutaneous coronary intervention
PDGF	platelet-derived growth factor
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1α
PI3K	phosphatidylinositol 3-kinase
PLA	polylactic acid
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor κB ligand
Rel	reticuloendotheliosis viral oncogene homolog
RF	rheumatoid factor
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate

SEM	standard error of mean
SHC	(Src homology 2 domain containing) transforming protein
SOCS1	suppressor of cytokine signalling 1
SPSB	SPRY domain-containing suppressor of cytokine signalling box protein
SSc	systemic sclerosis
STAT	signal transducers and activators of transcription
T_c lymphocyte	cytotoxic T lymphocyte
TGF	transforming growth factor
T_h lymphocyte	T helper lymphocyte
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein
TLR	toll-like receptor
TNF	tumour necrosis factor
TRAF	TNF receptor associated factor
TRAM	translocating chain-associating membrane protein
T_{reg} lymphocyte	regulatory T lymphocyte
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTP	tristetraprolin
TZD	thiazolidinedione
Ub	ubiquitin
VAS	visual analogue value
VCAM-1	vascular cell adhesion molecule-1

ABSTRACT

Inflammation plays a central role in the pathophysiology of several chronic diseases. Although the role of inflammation in traditional inflammatory diseases, like in rheumatoid diseases, is better understood, it is believed that inflammation is also involved in other chronic conditions, for example in several metabolic diseases. In rheumatoid diseases, the overwhelming inflammation causes tissue damage, joint erosion and subsequent pain or abnormal tissue healing with fibrosis, both of which often lead to disability and represent a serious burden both to the individual patient and to society. Although a wide range of immunomodulating therapeutics are available, a significant number of patients with rheumatoid diseases do not obtain even adequate relief with the current treatments.

The concept of the interaction between metabolism and inflammation has revealed new avenues in inflammation research. In metabolic diseases, the intensity of inflammation is lower than in traditional inflammatory diseases, but over time, it insidiously impairs the function of tissues and organs. Although the close relationship between metabolism and inflammation is now becoming clear, very little is known about the mechanisms that are underlying this interaction.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that were originally found to regulate glucose and fatty acid metabolism in cells. The PPARα agonists, fibrates, are old lipid-lowering drugs that are currently used in the treatment of hypertriglyceridemia. The PPARγ agonists, thiazolidinediones, are a group of insulin sensitizing drugs that became available for the treatment of type II diabetes mellitus in the late 1990s. Interestingly, PPARs have later been found to affect inflammatory responses. The aim of the present study was to investigate the immunomodulating properties of PPAR agonists with the focus on macrophage activation.

Both PPARα and PPARγ agonists suppressed lipopolysaccharide (LPS)-induced classical activation of macrophages as shown by reduced production of proinflammatory mediators interleukin 6 (IL-6) and nitric oxide and protein levels of the inducible nitric oxide synthase (iNOS) by PPAR agonists in J774 murine macrophages. The effects of PPAR agonists on iNOS expression were studied in more detail and PPARα and PPARγ agonists were found to suppress iNOS

expression via different mechanisms. PPARγ agonists reduced the transcription of iNOS, whereas PPARα agonists had no effect on iNOS transcription. Instead, PPARα agonists increased the degradation of iNOS protein through the proteasome pathway.

PPARα and PPARγ agonists also had different effects on IL-4 + IL-13 –induced alternative activation of J774 macrophages, a phenomenon linked to fibrotic diseases, for example. PPARγ agonists increased the levels of three alternative activation markers, arginase 1, found in inflammatory zone 1 (fizz1) and mannose receptor 1. In contrast, PPARα agonists decreased the levels of these markers. Similarly to murine J774 cells, PPARα agonists also reduced the levels of alternative activation markers in human THP-1 monocyte-macrophages.

This is the first study where the anti-inflammatory properties of a dual PPARα/γ agonist muraglitazar were characterized. Similarly to both PPARα and PPARγ agonists, muraglitazar suppressed the production of IL-6 and nitric oxide and the expression of iNOS in classically activated macrophages. Muraglitazar also attenuated the development of carrageenan-induced paw oedema in mice. In parallel to the results *in vitro*, IL-6, iNOS and tumour necrosis factor (TNF) mRNA levels were also lower in carrageenan-induced paw inflammation in muraglitazar-treated than in control mice *in vivo*.

Finally, as an example of novel applications that would combine the benefits of muraglitazar in both metabolism and inflammatory pathways, a muraglitazar-eluting degradable vascular stent was manufactured. In the experiments reported in this study, the biological efficacy of muraglitazar was retained during the manufacturing process of the drug-coated stent. The drug-elution kinetics of the muraglitazar-coated stent was also promising thus representing a promising basis for further studies.

Although the current anti-inflammatory therapeutic agents have significantly improved the prognosis of the patients with rheumatoid diseases, novel pharmacological approaches are needed to treat those patients who do not appropriately respond to the medications or are not able, for some reason, to use the drugs. The link between inflammation and metabolism may offer a new option to treat the traditional inflammatory diseases. This study supports the hypothesis of PPARs as important players or channels between metabolism and inflammation. PPARs are promising targets in the development of new antirheumatic pharmaceutics.

TIIVISTELMÄ

Tulehdus on mukana useiden kroonisten sairauksien patofysiologiassa. Perinteisten tulehdussairauksien, kuten reumasairauksien, lisäksi tulehdusta pidetään nykyisin merkittävänä tekijänä myös muissa kroonisissa sairaustiloissa, kuten aineenvaihduntasairauksissa. Reumasairauksissa liiallinen tulehdusreaktio aiheuttaa kudostuhoa ja kipua tai fibroosiin johtavaa epänormaalia kudoksen parantumista. Sekä kudostuho että fibroosi usein heikentävät potilaan toimintakykyä ja aiheuttavat siten merkittävää haittaa sekä potilaan henkilökohtaisessa elämässä että yhteiskunnallisesti. Nykyisin on useita tulehdusta hillitseviä lääkeaineita saatavilla, mutta silti merkittävä osa reumasairauksia sairastavista potilaista ei saa käytössä olevista lääkkeistä riittävää apua sairauteensa.

Aineenvaihduntasairauksiin liittyvän tulehduksen havaitseminen on mullistanut tulehduksen tutkimusta ja aihetta tutkitaan vilkkaasti. Tulehduksen voimakkuus on aineenvaihduntasairauksissa pienempi kuin perinteisissä tulehdussairauksissa, mutta hiljainenkin tulehdus vähitellen heikentää kudosten ja elinten toimintaa. Vaikka aineenvaihdunnan ja tulehduksen läheinen yhteys alkaa olla selvä, yhteyden taustalla olevista mekanismeista tiedetään edelleen erittäin vähän.

Peroksisomin proliferaattoriaktivoidut reseptorit (PPAR:t) ovat tumareseptoreja, joiden alun perin havaittiin säätelevän solujen glukoosi- ja rasvahappoaineenvaihduntaa. Eräät PPARα-agonistit, fibraatit, ovat vanhoja, veren rasvapitoisuutta ja kolesterolia vähentäviä lääkkeitä, joita nykyisin käytetään hypertriglyseridemian hoidossa. PPARγ:aa aktivoivat tiatsolidiinidionit ovat ryhmä insuliiniherkkyyttä lisääviä lääkkeitä, jotka tulivat markkinoille tyypin II diabeteksen hoitoon 1990-luvun lopulla. Myöhemmin PPAR:ien on havaittu vaikuttavan aineenvaihdunnan lisäksi myös tulehdusvasteisiin. Tämän tutkimuksen tavoitteena oli tutkia PPAR-agonistien vaikutuksia tulehdusreaktiossa keskittyen makrofagien aktivaatioon.

Sekä PPARα- että PPARγ-agonistit vähensivät lipopolysakkaridilla (LPS:lla) stimuloitua interleukiini 6:n (IL-6:n) ja typpioksidin tuottoa sekä indusoituvan typpioksidisyntaasin proteiinitasoa hiiren J774-makrofageissa. PPARα- ja PPARγ-agonistit siis vähensivät makrofagien ns. klassista aktivaatiota. PPAR-agonistien vaikutuksia indusoituvan typpioksidisyntaasin ilmenemiseen tutkittiin tarkemmin ja

PPARα- ja PPARγ-agonistien havaittiin vähentävän indusoituvan typpioksidisyntaasin ilmenemistä eri mekanismeilla. PPARγ-agonistit vähensivät indusoituvan typpioksidisyntaasin transkriptiota, kun taas PPARα-agonistit lisäsivät indusoituvan typpioksidisyntaasin hajoamista proteasomin kautta ilman vaikutusta indusoituvan typpioksidisyntaasin transkriptioon.

PPARα- ja PPARγ-agonisteilla havaittiin olevan myös erilaiset vaikutukset IL-4:lla ja IL-13:lla stimuloituun J774-makrofagien ns. vaihtoehtoiseen aktivoitumiseen. Makrofagien on havaittu olevan vaihtoehtoisesti aktivoituneita erityisesti fibroottisissa sairauksissa. PPARγ-agonistit lisäsivät kolmen vaihtoehtoista aktivaatiota kuvaavan merkkiaineen, arginaasi 1:n, fizz1:n (found in inflammatory zone 1:n) ja mannoosireseptori 1:n, määrää. Sitä vastoin PPARα-agonistit vähensivät näiden merkkiaineiden ilmenemistasoja. Kuten J774-soluissa, PPARα-agonistit vähensivät vaihtoehtoista aktivaatiota kuvaavien merkkiaineiden ilmenemistä myös ihmisen THP-1-monosyytti-makrofageissa.

Tämä tutkimus on ensimmäinen PPARα/γ-kaksoisagonistin, muraglitatsaarin, tulehdusta hillitseviä ominaisuuksia kartoittava tutkimus. Kuten sekä PPARα- että PPARγ-agonistit, myös muraglitatsaari vähensi IL-6:n, typpioksidin ja indusoituvan typpioksidisyntaasin määrää klassisesti aktivoiduissa makrofageissa. Muraglitatsaari myös vähensi karrageenilla indusoitua tassuturvotusta hiirillä. Sopien yhteen soluviljelmästä saatujen tulosten kanssa, muraglitatsaari-hoito vähensi IL-6:n, indusoituvan typpioksidisyntaasin ja tuumorinekroositekijän (TNF:n) lähetti-RNA-tasoja karrageenilla indusoidussa tulehduksessa.

Uusien, muraglitatsaarin hyödyllisiä vaikutuksia sekä aineenvaihduntaan että tulehdukseen hyödyntävien käyttöaiheiden tutkimiseksi tässä tutkimuksessa valmistettiin lopuksi muraglitatsaaria vapauttava biohajoava verisuonistentti. Tutkimuksessa näytettiin, että muraglitatsaarin biologiset vaikutukset säilyivät stentin valmistusprosessin aikana. Lisäksi muraglitatsaarilla päällystetyn stentin lääkettä vapauttava kinetiikka oli lupaava, mikä rohkaisee jatkamaan stentin ominaisuuksien tutkimista.

Nykyiset tulehdusta hillitsevät lääkeaineet ovat merkittävästi parantaneet reumasairauksia sairastavien potilaiden ennustetta. Kuitenkin uusia farmakologisia lähestymistapoja tarvitaan, koska osa potilaista ei saa nykyisistä lääkkeistä riittävää lääkevastetta tai ei pysty käyttämään niitä. Tulehduksen ja aineenvaihdunnan yhteys voi tarjota uuden tavan hoitaa perinteisiä tulehdussairauksia. Tämä tutkimus vahvistaa hypoteesia PPAR:sta välittäjänä aineenvaihdunnan ja tulehduksen välillä. PPAR:t ovat lupaavia uusien antireumaattisten lääkkeiden vaikutuskohteita.

INTRODUCTION

In 1638, the Flemish artist, Peter Paul Rubens, painted a portrait of three women. At the first glance, the painting looks like a typical baroque work of art, but if the painting is studied more carefully, one can notice that the fingers on the right hand of one of the ladies are flexed and in hyperextension. This realistic detail in a famous painting makes a startling point. The artist named the painting *the Three Graces* and it is often regarded as one of the first representations of rheumatoid arthritis in fine art (Appelboom et al. 1981, Entezami et al. 2011).

At the time *the Three Graces* was painted, different inflammatory conditions in a joint were considered as one single disease. Joint inflammation was often thought to be caused by a substance (or humor) that "flows" (*rheuma* in Greek), settles in joints and causes arthritis. This theory, called humoralism, was developed by Greek physician Hippocrates already in the fourth century B.C. and in this connection, Hippocrates also introduced the word *rheuma* (i.e. flowing) (Joshi 2012). Remarkably, this theory resembles to some extend our current understanding of pathophysiology of one type of arthritis, gout, in which blood urate crystallizes in joint and causes joint inflammation.

If the woman with the deformities in *the Three Graces* was a real person, she probably became seriously disabled later in her life, since there was no effective treatment for rheumatoid arthritis in the 17th century. Available treatment may have included blood letting, warm spas, different diets and spiritual techniques (Hart 1976). The first non-steroidal anti-inflammatory drug –type pharmaceutic pain killer, acetylsalicylic acid, did not become commercially available until 1899 (Mottram 2003).

The humoral theory of inflammation was started to be criticized in the 19th century. Louis Pasteur (1822-1895) created a hypothesis that bacteria were causing infections. Rudolph Virchow described the cellular pathology at the background of inflammation in 1871. Elie Metchnikoff characterized lymphocytes and macrophages and described their role in tissue homeostasis and in the fight against pathogens in 1908. In 1974, Rocha e Silva identified inflammatory cytokines that mediate the development of inflammatory symptoms (Scott et al. 2004). The view of inflammation widened in 1978 when immune responses were found to increase

insulin resistance in sepsis and later in rheumatoid arthritis. Little by little it also became evident that excessive adipose tissue feeds inflammatory responses in obese people (Gregor, Hotamisligil 2011).

Elsewhere, Alfred B. Garrod named and diagnostically distinguished rheumatoid arthritis from gout and osteoarthritis in 1858-1859, which, together with increasing knowledge of inflammation, led to the development of understanding and treatment of rheumatoid arthritis (Aceves-Avila, Medina & Fraga 2001, Joshi 2012). In 1925, the first effective antirheumatic drug containing gold salts was introduced for rheumatoid arthritis (Joshi 2012). A quarter of a century later, in 1950, cortisone was isolated and started to be used to treat rheumatoid arthritis. The discovery of cortisone revolutionized the life of the patients suffering from rheumatoid arthritis, but it was soon found that cortisone evoked serious side effects if it was overdosed or used for a long time (Hart 1976). A huge improvement in the treatment of rheumatoid arthritis occurred in 1980s when the cytostatic drug methotrexate, was established to clearly improve the symptoms and prognosis of rheumatoid arthritis (Joshi 2012). Finally, neutralizing antibodies or soluble receptors that selectively bind to cytokines or to inflammatory cells and prevent their action, started to appear in 1998.

In contrast to the tragedy of the patients suffering from rheumatoid arthritis in 17th century, now several effective antirheumatic drugs are available and the prognosis of patients has improved. Still, our understanding of inflammation is tenuous although it is expanding. The driving force of this study was the desire to increase, at least to some extent, the knowledge of this amazing life-sustaining system.

REVIEW OF LITERATURE

1 Inflammation

Inflammation is a physiological response in which the body reacts to pathogen or cell or tissue damage. The immune system plays a central role in this response and it consists of both nonspecific defence mechanisms that protect the body from pathogens and other foreign factors and extremely specific mechanisms that are only turned on in the presence of highly selected antigens.

The nonspecific part of the immune system is often called innate immunity since it does not need any prior exposure to its targets but it exists already at birth. Innate immunity includes physical barriers, chemical defence mechanisms and phagocytic cells. The physical barriers, like skin and mucous membranes, prevent foreign particles from gaining access to the body. The chemical defence system detoxifies pathogens in chemical reactions; it includes gastric acid, lytic enzymes and the complement system, but also natural killer cells, basophils and eosinophils since these cells secrete toxic compounds. Macrophages, neutrophils and dendritic cells are phagocytic cells that phagocyte foreign particles and destroy them through the generation of reactive oxygen species (ROS) and lytic enzymes. (Colburn 2012, Abbas, Lichtman & Pillai 2012)

The specific defence system is called adaptive immunity since it has to adapt itself to each new pathogen encountered. Once an encounter has occurred, the adaptive immunity remembers the pathogen and on a subsequent encounter it can attack the pathogen rapidly and specifically. Adaptive immunity is characterized by the action of T and B lymphocytes. B lymphocytes are specialized at producing antibodies against a specific pathogen. T lymphocytes can be further divided into T helper cells (T_h cells) that assist other leukocytes in immunological processes, cytotoxic T cells (T_c cells) that recognize the cells that express foreign molecules and induce the apoptosis of the target cell and regulatory T cells (T_{reg} cells) that suppress T cell-

mediated immunity at the termination of the immune reaction. (Colburn 2012, Abbas, Lichtman & Pillai 2012)

According to our common understanding today, the main function of immune system and inflammation is to destroy pathogens and once activated, the immune system responds in a way that is regardless of the nature of stimulus. When the stimulus is eradicated, the immune system becomes dampened and inflammation proceeds to the phase of tissue repair. During the repair process, the tissues which have suffered inflammation-caused damage become healed. (Abbas, Lichtman & Pillai 2012, Colburn 2012)

The acute inflammatory reaction starts with the activation of macrophages and mast cells. The activated cells release many inflammatory mediators, including chemokines, eicosanoids and cytokines. These mediators activate endothelial cells, and the endothelium becomes penetrable to neutrophils, which then migrate to the site of inflammation. The neutrophils become activated either by the cytokines or through direct contact with pathogens and when activated, they release ROS, reactive nitrogen species and proteases from their granules to kill the target, either the real or imaginary. When the stimulus which triggered the inflammatory response, has been eliminated macrophages orchestrate the resolution and repair phases of inflammation. (Colburn 2012, Abbas, Lichtman & Pillai 2012)

If the acute inflammatory reaction fails to eliminate the stimulus of the inflammatory response, inflammation persists and there are changes in the pattern of the reaction. The neutrophils are replaced with macrophages and T cells and finally macrophage clusters and granulomas are formed. The aim of granulomas is to create a cellular wall against the inducer of inflammation and thus protect the host. (Colburn 2012, Abbas, Lichtman & Pillai 2012)

1.1 Chronic inflammatory diseases

Sometimes the control of inflammation fails and inflammation itself leads to a disease. Diseases characterized by the attack of immune system against the host's own tissues are called autoimmune diseases. There are many examples of these kinds of diseases e.g. rheumatoid diseases (becomes manifest in joints and connective tissue), multiple sclerosis (in the myelinated nerves in the brain), type I diabetes mellitus (type I DM) (in pancreatic β-cells) and inflammatory bowel diseases (in small intestine and colon). This review will focus on the characteristics of inflammation in two rheumatoid diseases, rheumatoid arthritis (RA) and systemic sclerosis (SSc).

1.1.1 Rheumatoid arthritis

Rheumatoid arthritis is the most common of the rheumatoid diseases and it is characterized by joint inflammation and, if not treated, by cartilage and bone destruction. The aetiology of RA is unknown, but it seems to be associated with multifactorial background factors with both genes and environment being involved in the development of RA. However, understanding the pathogenesis of the disease has progressed remarkably during the last decade due to new therapeutics that have revolutionized the treatment of RA. Still, despite the wide range of drug options currently available (Table 1), some patients do not respond to the treatments sufficiently or they cannot use the effective drugs due to their adverse effects.

The typical appearance of RA is inflamed joint synovium with hyperplasia and inflammatory cell accumulation. In the histological examination, the affected synovium is occupied by macrophages, dendritic cells, T_h1 and T_h17 lymphocytes, B cells and plasma cells with other immune cells being in the minority. According to the current understanding, macrophages are the most important effectors in synovitis. Those cells release proinflammatory cytokines, like tumour necrosis factor (TNF), interleukin 1 (IL-1), IL-6, IL-12, IL-18 and IL-23, ROS, nitrogen intermediates, like nitric oxide, and matrix-degrading enzymes, but also growth factors like transforming factor β (TGF-β). TGF-β, IL-1, IL-6, IL-21 and IL-23 support T_h17 differentiation and inhibit T_{reg} differentiation in humans (Choy 2012, McInnes, Schett 2011). In mice, T_h17 activation may need the concurrent presence of both IL-6 and TGF-β (Choy 2012, Azizi, Jadidi-Niaragh & Mirshafiey 2013). Since T_{reg} lymphocytes suppress the activity of inflammatory T_h17 cells, the reduced differentiation of T_{reg} cells further promotes T_h17 activation (Cooles, Isaacs & Anderson 2013). Activated T_h17 cells produce IL-17A, IL-17F, IL-21 and TNF. TNF, which is produced by both macrophages and T_h17 lymphocytes, activates fibroblasts and chondrocytes to produce cartilage- and bone-destroying enzymes, such as matrix metalloproteinases (MMPs), in addition to proinflammatory or osteoclastogenic mediators such as receptor activator of nuclear factor κB ligand (RANKL) (McInnes, Schett 2011). The erosion process is boosted by the invasive growth of synovial tissue at cartilage and bone interfaces (Pablos, Canete 2013).

Rheumatoid inflammation also induces an acute-phase response, in which liver secretes certain proteins, such as C-reactive protein (CRP), hepcidin, serum amyloid A, haptoglobin and fibrinogen. It has been hypothesized that these proteins, especially CRP, may exacerbate the tissue damage in RA (Choy 2012).

Table 1. Pharmaceuticals used in the treatment in rheumatoid arthritis

Pharmaceutical	The main anti-inflammatory mechanism(s)
Conventional synthetic DMARDs	
azathioprine	Inhibition of ATase and purine synthesis (Case 2001, Colburn 2012)
ciclosporin	Inhibition of calcineurin and transcription factor NF-AT (Rang et al. 2012, Colburn 2012)
cyclophosphamide	Alkylation of DNA and apoptosis of rapidly dividing cells (Colburn 2012)
gold compounds	Hypothesis: inhibition of AP-1 (Case 2001)
hydroxy-chloroquine	Hypothesis: impairment of phagosomal/lysosomal function (Katz, Russell 2011, Colburn 2012), inhibition of TLRs (Katz, Russell 2011)
leflunomide	Inhibition of DHODH and pyrimidine synthesis (Sanders, Harisdangkul 2002, Colburn 2012)
methotrexate	Hypothesis: increasing intracellular adenosine (Colburn 2012)
mykophenylate mofetil	Inhibition of IMPDH and purine synthesis (Colburn 2012)
penicillamine	Hypothesis: inhibition of angiogenesis, synovial fibroblast proliferation, AP-1 (Case 2001)
sulfasalazine	Hypothesis: increasing intracellular adenosine (Case 2001), scavenger of toxic oxygen metabolites (Rang et al. 2012)
Biological DMARDs	
abatacept	Inhibition of T lymphocyte activation (Conti et al. 2013, Rang et al. 2012)
anakinra	Inhibition of the action of IL-1 (Conti et al. 2013, Rang et al. 2012)
rituximab	Depletion of B lymphocytes (Conti et al. 2013, Rang et al. 2012)
TNF blocking agents	Inhibition of the action of TNF (Conti et al. 2013, Rang et al. 2012)
adalimumab	
certolizumab pegol	
etanercept	
golimumab	
infliximab	
tocilizumab	Inhibition of the action of IL-6 (Conti et al. 2013, Rang et al. 2012)

AP-1, activator protein 1; ATase, amidophosphoribosyltransferase; DHODH, dihydroorotate dehydrogenase; DMARDs, disease-modifying antirheumatic drugs; IMPDH, inosine-5'-monophosphate dehydrogenase; IL, interleukin; NF-AT, nuclear factor of activated T-cells; TLRs, toll-like receptors; TNF, tumour necrosis factor

The key roles of IL-6 and TNF in rheumatoid inflammation have been confirmed by the effectiveness of selective IL-6 receptor inhibitor tocilizumab and TNF blockers in the treatment of RA. However, in addition to TNF blockers and tocilizumab, also the anti-CD20 antibody, rituximab, a drug that blocks the action of B lymphocytes, has been found to be effective in the treatment of RA. This was not a surprise since some autoantibodies have long been linked with the risk of RA. The oldest of the known autoantibodies is rheumatoid factor (RF) that recognizes the Fc portion of immunoglobulin G (McInnes, Schett 2011). However, RF is not very sensitive or specific for RA, since only 80% of patients suffering from RA and 10% of general population (10-30% of aged people) express this factor (Moeez, John & Bhatti 2013, Mewar, Wilson 2006). Anti-citrullinated protein antibody (ACPA) is equally sensitive (80% of RA patients express it), but more specific than RF with 98% specificity (Moeez, John & Bhatti 2013). Protein citrullination, a process where arginine present in an amino acid chain is converted into citrullin, is a normal physiological event occurring usually during apoptosis (McInnes, Schett 2011, Moeez, John & Bhatti 2013). However, it is not known why some patients with RA develop antibodies against the citrullinated proteins.

The importance of T lymphocyte activation in the pathogenesis of RA has been clearly demonstrated after abatacept, a fusion protein that prevents the costimulatory signal of $CD28^+$ T lymphocytes, became available for the treatment of RA. The full activation of $CD28^+$ T lymphocytes requires the binding of CD80 or CD86 expressed at the cell membrane of antigen presenting cells in addition to the activating signal through T cell receptor. Abatacept binds to CD80 and CD86 and thus prevents their interaction with CD28 (Abbas, Lichtman & Pillai 2012). The downregulation of T lymphocyte activation by abatacept has been shown to decrease disease activity and to increase the physical function in patients with RA (Maxwell, Singh 2010).

It seems evident that the rheumatoid inflammation starts long before the onset of symptoms. Several studies have demonstrated that levels of autoantibodies (especially ACPA) and circulating proinflammatory cytokines often tend to increase gradually 2-4 years before an RA diagnosis is made (Schaeverbeke, Truchetet & Richez 2012).

RA does not affect synovial joints only, but it is also associated with extra-articular and systemic effects. Extra-articular manifestations include rheumatoid nodules, vasculitis, pericarditis, uveitis, pleuritis and rheumatoid lung. Patients suffering from RA have an increased incidence of suffering cardiovascular diseases, anaemia, osteoporosis, fatigue and depression. It has been postulated that the elevated

circulating levels of IL-6, TNF and IL-1 contribute to these systemic effects. (Choy 2012)

1.1.2 Systemic sclerosis

Systemic sclerosis is a connective tissue disease that is characterized by effusive fibrosis in skin and the internal organs such as lungs and intestine. SSc has customarily been divided into two subgroups according to the involvement of the internal organs: the limited cutaneous subset (lcSSc) and the diffuse cutaneous subset (dcSSc). Skin thickening with immune cell infiltration and fibrosis and vascular abnormalities are a shared feature in the both subsets of the disease (Katsumoto, Whitfield & Connolly 2011). In comparison to RA, very little is known about molecular mechanisms of SSc and few medicine options are available for these patients.

In early SSc lesions, both in skin and in internal organs, the small arteries become permeable to monocytes and lymphocytes and the amount of collagen in the surrounding tissue is increased. Over time, the amount of the extracellular matrix including collagen increases, finally replacing the resident tissue cells. In arteries, the intima proliferates and thickens and this narrows the lumen, which exposes tissues to hypoxia. In addition, when the function of endothelium becomes impaired, vasospastic episodes become common which further worsen the transport of oxygen. The activation of T_h2 lymphocytes has been linked to SSc in several studies, and the levels of T_h2 cytokines IL-4 and IL-13 are increased in SSc patients (Katsumoto, Whitfield & Connolly 2011). However, macrophages also play a crucial role in the disease pathogenesis (Aliprantis et al. 2007). Especially in the early phases of SSc, macrophages are the prominent immune cell type detected in the lesions (O'Reilly 2014). Further evidence for the importance of macrophages in fibrosis was received when the deficiency of C-C chemokine receptor 2 (CCR2), a receptor needed for the signalling of chemokine monocyte chemotactic protein 1 (MCP-1, also known as chemokine (C-C motif) ligand 2 (CCL2), and small inducible cytokine A2), was shown to reduce the lung remodelling in bleomycin-induced pulmonary fibrosis in mice (Okuma et al. 2004).

The reason why SSc is classified as an autoimmune disease is the appearance of autoantibodies in SSc patients. Ninety percent of patients express antinuclear antibodies with the three major subclasses being anticentromere, anti-topoisomerase 1 and anti-RNA polymerase III antibodies. The high frequency of autoantibodies strongly indicates that B cells are also important in the pathogenesis of SSc. Indeed,

treatment of SSc patients with B lymphocyte inhibitor rituximab has led to promising results in few preliminary clinical studies. (Katsumoto, Whitfield & Connolly 2011)

Fibrosis is a condition where there is an excessive amount of extracellular matrix. The extracellular matrix is mainly produced by fibroblasts and their activation is associated with aberrant immune response with increased growth factor production (Katsumoto, Whitfield & Connolly 2011, O'Reilly 2014). The pathogenesis of SSc is linked to the elevated levels of TGF-β, connective tissue growth factor (CTGF), endothelin 1 and platelet-derived growth factor (PDGF) (Katsumoto, Whitfield & Connolly 2011). Major evidence for the importance of PDGF and CTGF in the pathogenesis of SSc has emerged from drug studies. Imatinib, an inhibitor of PDGF signalling pathway, as well as CTGF-neutralizing antibodies ameliorated fibrosis in mice models (Akhmetshina et al. 2009, Ikawa et al. 2008). Although TGF-β1 is thought to be a key regulator of fibrosis, anti-TGF-β1 antibody therapy did not show any efficacy against the fibrosis in SSc patients (Denton et al. 2007).

1.2 Inflammation in metabolic diseases

Hypertension, central obesity, hypertriglyceridemia, low high-density lipoprotein (HDL) levels and elevated blood glucose are metabolic abnormalities that increase the risk of cardiovascular diseases and type II DM. The so called metabolic syndrome is a condition, where these risk factors have accumulated and the expression of three of the five abnormalities is usually considered as diagnostic criteria for metabolic syndrome (Alberti et al. 2009). Obesity plays a central role as a risk factor for the other metabolic abnormalities and the subsequent appearance of the metabolic syndrome (Monsalve et al. 2013, Johnson, Milner & Makowski 2012).

In a situation with chronic positive energy balance, one ultimately exceeds the capacity of adipose tissue to expand. This leads to the necrosis of adipocytes in visceral adipose tissue and to the increase in the amount of free fatty acids in the circulation. The necrotic adipocytes release lipids, which together with other compounds, attract macrophages and the macrophages gather around necrotic adipocytes to phagocyte the exposed lipids. The necrotic debris activates macrophages to take on a proinflammatory appearance and they start to secrete inflammatory mediators like TNF, IL-1 and IL-6 (Prieur, Roszer & Ricote 2010, Johnson, Milner & Makowski 2012, Patel, Buras & Balasubramanyam 2013). In addition to the conventional cytokines, adipose tissue secretes many other inflammatory mediators, adipokines, in response to inflammatory stimulus.

Adipokines include e.g. adiponectin, leptin, resistin, retinol-binding protein 4 and angiopoietin-like protein 2, the last four of which have been linked to proinflammatory action, while adiponectin has both pro- and anti-inflammatory effects (Ouchi et al. 2011).

Thus, obesity leads to the secretion of proinflammatory mediators from adipose tissue. Under normal physiological conditions insulin mediates the glucose transport into cells by binding to its receptor at the cell membrane (Figure 1). The binding of insulin leads to the phosphorylation of three tyrosine molecules on the β chain of insulin receptor. This enables the insulin receptor substrate 1 (IRS-1) to be translocated to the plasma membrane where one of its tyrosine moieties becomes phosphorylated. Tyrosine phosphorylation leads to activation of phosphatidylinositol 3-kinase (PI3 kinase) and Akt, transportation of glucose into the cell and the stimulation of multiple intracellular metabolic processes (Eldor, DeFronzo & Abdul-Ghani 2013). However, the proinflammatory mediators related to obesity activate pathways that lead to the activation of c-Jun N-terminal kinase-1 (JNK1) and inhibitor of κB kinase β (IKKβ). Both IKKβ and JNK1 can directly phosphorylate the serine residues of IRS-1, which leads to its inactivation. When IRS-1 is inactivated, the actions of insulin are attenuated. This situation is called insulin resistance (Patel, Buras & Balasubramanyam 2013, Eldor, DeFronzo & Abdul-Ghani 2013).

As mentioned above, in obesity also the amount of free fatty acids in the circulation is increased. According to *in vitro* studies, saturated fatty acids can activate toll-like receptor 4 (TLR4) (Nguyen et al. 2007, Shi et al. 2006). The activation of TLR4 leads to the activation of JNK1 and IKKβ and subsequent IRS-1 inactivation and insulin resistance along with the activation of inflammatory gene expression. Accordingly, Tsukomo et al. reported TLR4 deficient mice to be resistant to diet-induced obesity and insulin resistance (Tsukumo et al. 2007). In addition to the effects through TLR4, high free fatty acid levels can also lead to other problems. The released lipids are deposited in crucial organs like liver, kidney, muscle and pancreas (Eldor, DeFronzo & Abdul-Ghani 2013, Prieur, Roszer & Ricote 2010). Once inside cells, fatty acids are metabolized and toxic lipid metabolites, like fatty acyl CoA, diacylglycerol and ceramides, are formed. In addition, accumulation of fatty acids in tissues promotes the infiltration of macrophages to the site and the activation of tissue resident macrophages. In liver, the accumulation of lipids activates Kupffer cells, the resident macrophages in liver, and they start to release ROS, prostanoids and cytokines. These inflammatory mediators cause surrounding hepatocytes to become insulin resistant (Prieur, Roszer & Ricote 2010, Gregor, Hotamisligil 2011)

(Figure 1). This hepatic manifestation of the metabolic syndrome increases the apoptosis of hepatocytes and evokes steatohepatitis (Gregor, Hotamisligil 2011). In pancreas, both the fat accumulation and the macrophage activation reduce insulin secretion and trigger β cell apoptosis (Gregor, Hotamisligil 2011). A similar kind of macrophage-mediated insulin resistance and inflammation occurs also in kidney (Eldor, DeFronzo & Abdul-Ghani 2013, Prieur, Roszer & Ricote 2010, Lumeng, Saltiel 2011).

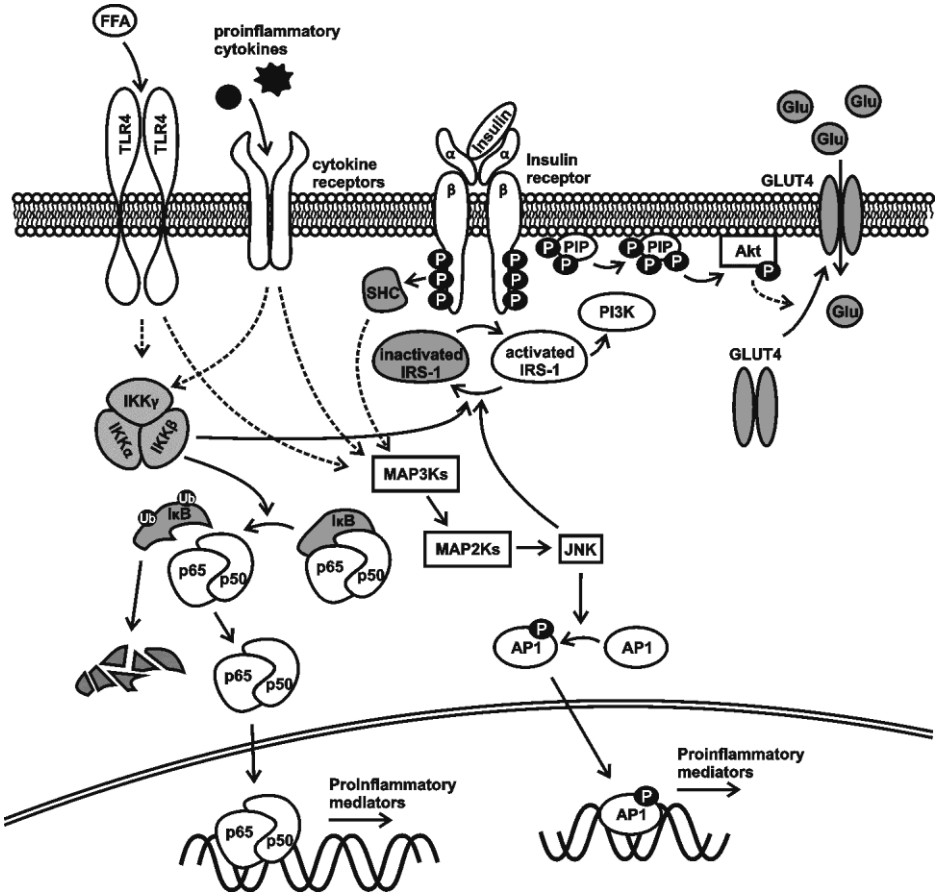

Figure 1. Pathways involved in inflammatory gene expression in metabolic diseases. AP1, activator protein 1; FFA, free fatty acid; Glu, glucose; GLUT4, glucose transporter type 4; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; MAP2(3)K, mitogen-activated protein kinase kinase (kinase); P, phosphate; PI3K, phosphatidylinositol 3-kinase; PI2(3)P, phosphatidylinositol 2(3)-phosphate; SHC, (Src homology 2 domain containing) transforming protein; TLR4, toll-like receptor 4; Ub, ubiquitin

In addition to lipids, hyperinsulinemia also promotes inflammation. This is mediated by the mitogen-activated protein kinase (MAPK) pathway, which is responsive to insulin and which in turn activates multiple intracellular pathways involved in inflammation, cellular proliferation and atherogenesis. Although the insulin-stimulated glucose transport into cells is impaired in type II DM, the responsiveness of MAPK to insulin is not changed. This is one reason why hyperinsulinemia causes aberrant activation of intracellular pathways involved in inflammation and artherogenesis in type II DM. (Eldor, DeFronzo & Abdul-Ghani 2013)

The link between low-grade inflammation and insulin resistance has been demonstrated in several studies in humans. It has been revealed that there is a strong correlation between insulin resistance and the levels of TNF in adipose tissue (Hotamisligil et al. 1995). In addition, elevated level of high-sensitivity CRP is a risk factor for diabetes (Pfutzner, Forst 2006). Interestingly, the extent of insulin resistance can be reduced during treatment with TNF blockers or IL-1 receptor inhibitor in patients suffering from the metabolic syndrome or type II DM, respectively (Stanley et al. 2011, Larsen et al. 2007).

As mentioned above, metabolic abnormalities increase the risk of vascular complications. In hyperlipidaemia, low-density lipoprotein (LDL) particles are retained in the walls of the arteries and the endothelium is activated. The activated endothelium expresses adhesion molecules that bind monocytes, and produces chemokines that attract monocytes to migrate across the endothelium into the LDL plaque. In the tissue, the recruited monocytes differentiate into macrophages or dendritic cells. The macrophages phagocyte LDL particles until they become so-called foam cells, which secrete proinflammatory mediators. Some of the mediators, like MCP-1, CCL5 and CXC-chemokine ligand 1, attract more monocytes to the plaque while others, like nitric oxide and ROS oxidize LDL particles, which helps the macrophages to phagocyte them. Furthermore, many of the secreted mediators, like IL-6, TNF and IL-1, establish a state of chronic inflammation in the vessel wall, which in turn increases the proliferation of smooth muscle cells. The long-lasting inflammation finally leads to apoptosis of the macrophages. If recruited macrophages are not capable of clearing all their dying counterparts, then toxic enzymes can be released. The toxic enzymes induce necrosis of macrophages and, due to lack of functioning macrophages, to the formation of lipid cores. The necrotic cell debris makes the plaque vulnerable and expose it to plaque rupture and subsequent blockage of the artery. (Moore, Sheedy & Fisher 2013)

Several pharmacological compounds are used in the clinic in attempts to prevent the risks associated with these metabolic abnormalities. Antidiabetic, antihypertensive and lipid-lowering drugs have been shown to reduce the complications associated with metabolic abnormalities.

2 Macrophages

Macrophages are mononuclear phagocytes that have a wide range of roles in several physiological conditions: cleaning of apoptotic cells and tissue debris, remodelling of tissues, destruction of microbes and the regulation of inflammatory responses. In the steady state, most of the macrophages are located in tissues where they are involved in ensuring homeostasis. In certain tissues, the macrophages have been endowed with specific names. For instance, macrophages are called Kupffer cells in liver, microglia in central nervous system, osteoclasts in bone, Hofbauer cells in placenta and intraglomerular mesangial cells in kidney. According to current understanding, most of the tissue macrophages are derived from embryonic precursors and they can maintain themselves by self-renewal (Ginhoux, Jung 2014). When there is inflammation, the activities of tissue macrophages are supported by colonization of bone marrow –derived monocytes that differentiate into macrophages (Ginhoux, Jung 2014).

2.1 The phenotypes of macrophages

Macrophages change their phenotype according to their microenvironment. For instance, lipopolysaccharide (LPS) and T_h1 cytokine interferon γ (IFNγ) induce a strong proinflammatory phenotype in macrophages, resulting in the production of IL-6, TNF and nitric oxide. On the other hand, T_h2 cytokines IL-4 and IL-13 promote the formation of a macrophage phenotype characterized by effective killing of extracellular parasites and the by production of high levels of factors linked to homeostatic functions and generation of extracellular matrix such as arginase 1, mannose receptor 1 and found in inflammatory zone 1 (fizz1, also known as resistin-like molecule α and hypoxia-induced mitogenic factor). These two phenotypes, classically activated macrophages or M1 and alternatively activated macrophages or M2 are the best-established macrophage phenotypes. The M1/M2 nomenclature was established in mouse macrophages and it reflects the T_h1/T_h2 paradigm (Lawrence,

Natoli 2011, Van Dyken, Locksley 2013). It is not clear yet to what extent the M1/M2 division reflects the characteristics of human macrophages. At least, the activation markers of alternatively activated macrophages are somewhat different from those in mice since human macrophages do not express certain genes e.g. fizz1 (Lawrence, Natoli 2011). In addition, arginase 1 is expressed only in certain situations in humans (Pourcet, Pineda-Torra 2013, Munder 2009). Some of the proposed human markers of alternative activation include CCL13 (also known as MCP-4), PDGF, TGF-β and insulin-like growth factor 1 (IGF-1) all of which play a role in the healing process and fibrosis (Murray, Wynn 2011).

Macrophages express the alternative phenotype especially in parasite infections, but also in other, physical and pathological, conditions. In murine models, alternatively activated macrophages have been shown to be present in glomerulonephritis, autoimmune encephalomyelitis, asthma, lung fibrosis, sepsis, trauma and tumours (Munder 2009). In humans, alternatively activated macrophages are documented to appear in arthritis, asthma, psoriasis and tuberculosis (Munder 2009).

2.2 Markers of classical macrophage activation

2.2.1 Inducible nitric oxide synthase and nitric oxide

Nitric oxide can be synthesized in almost all cells by the nitric oxide synthase (NOS) enzymes. Three isoforms of NOS have been characterized: endothelial (e)NOS, neuronal (n)NOS and inducible (i)NOS. According to their names, eNOS and nNOS were primarily found to be expressed in endothelial cells and neurons, respectively. They are constitutively expressed and they synthesize low amounts of nitric oxide in response to elevations in intracellular Ca^{2+} levels. In contrast, iNOS is expressed ubiquitously in different tissues and its expression is highly inducible e.g. by several pathogen-associated molecules and proinflammatory cytokines. All of the NOS enzymes synthesize nitric oxide by catalysing the conversion of L-arginine to L-citrulline. (Kobayashi 2010, Korhonen et al. 2005)

From the molecular point of view, nitric oxide is an oxygen radical that has the ability to damage several cell structures if it is secreted at high doses. This is a beneficial characteristic e.g. when a macrophage is combatting an invading pathogen. However in chronic inflammation, the prolonged secretion of nitric oxide is deleterious for the host tissues. For this reason the expression of iNOS needs to be

stringently controlled. According to the results gathered by intensive research during the last three and half decades, the expression of iNOS is regulated not only at the transcriptional, but also at the posttranscriptional levels. The two most important transcription factors regulating the transcription of iNOS gene are nuclear factor κB (NF-κB) and signal transducers and activators of transcription 1α (STAT1α), but also other transcription factors, like MAPK, CCAAT-enhancer-binding protein β (C/EBPβ) and interferon regulatory factor 1 (IRF-1), are involved in iNOS transcription. In addition, iNOS mRNA contains AU-rich element (ARE) motifs through which several proteins and compounds regulate the degradation of iNOS mRNA. iNOS activity has been found to be rather constant regardless of environmental signals and the action of iNOS is usually terminated by its degradation in proteasome. (Korhonen et al. 2005, Pautz et al. 2010)

2.2.2 Other markers of classical activation

In addition to nitric oxide, classically activated macrophages produce a wide variety of proinflammatory cytokines. The most important, or at least the most extensively studied, of these are IL-6, IL-1, TNF and chemokines, like MCP-1. All of these cytokines act both in autocrine and paracrine manner, but also at the systemic level. IL-6 and IL-1 activate T cells and induce the differentiation of B cells. IL-1 and TNF increase vascular permeability and MCP-1, IL-8 and other chemotactic factors attract circulating immune cells, both of which facilitate the migration of immune cells into inflammatory tissue. At the systemic level, IL-6, IL-1 and TNF promote liver to secrete acute phase proteins, which opsonize pathogens and facilitate their elimination. These three cytokines also act on hypothalamus and induce fever and promote the mobilization of neutrophils from bone marrow. (Burmester, Pezzutto 2003, Abbas, Lichtman & Pillai 2012)

2.3 Markers of alternative macrophage activation

2.3.1 Arginase 1

Arginase has long been known as the final enzyme in the urea cycle. It catalyses L-arginine hydrolysis to urea and L-ornithine and thus participates in removing the harmful ammonia compound, from the body. In mammals, there are two arginase

isoenzymes: arginase 1 and arginase 2. Both of them are inducible and catalyse the same reaction, but their expression in tissues, regulation and subcellular localization differ. Arginase 1 is prominently expressed in liver and macrophages, while arginase 2 is expressed ubiquitously. (Munder 2009, Morris 2009)

Recently, arginase 1 has been found to be highly expressed in murine alternatively activated macrophages, and in some pathological conditions also in human alternatively activated macrophages (Munder 2009, Pourcet, Pineda-Torra 2013). It has been speculated that the production of ornithine increases the formation of polyamines and proline (Munder 2009, Pourcet, Pineda-Torra 2013). Since collagen synthesis requires proline (Dunn et al. 1977), this might further increase the collagen synthesis and fibrosis. Arginase 1 has been thought to possess anti-inflammatory properties through different mechanisms. Firstly, arginase 1 competes for the substrate with NOS and thus reduces nitric oxide production and the nitric oxide - mediated cytotoxic and proinflammatory effects (Pourcet, Pineda-Torra 2013). Secondly, the depletion of arginine via activation of arginase 1 interrupts cell cycle and prevents the division of quickly proliferating cells like T cells during inflammation (Munder 2009).

2.3.2 Mannose receptor 1

Mannose receptor 1 is a C-type lectin receptor, which can bind to and subsequently promote the endocytosis of several protein-attached glycans that carry mannose residues. The mannose receptor 1 is widely expressed in tissue macrophages, dendritic cells and endothelial cells. Mannose-rich proteoglycans can be detected on the surface of certain microorganisms, like helminths (Van Dyken, Locksley 2013), and in several endogenous proteins (Martinez-Pomares 2012).

In contrast to the phagocytosis mediated by many other receptors, the phagocytosis after mannose receptor 1 activation does not promote inflammatory responses in other cells (Gazi, Martinez-Pomares 2009). There is some evidence to suggest that mannose receptor 1 might also have immunomodulating effects, but the mechanisms are not clear. Mannose receptor 1 gene variations have been claimed to be associated with asthma (Hattori et al. 2009) and sarcoidosis (Hattori et al. 2010). In addition, the activation of mannose receptor promoted anti-inflammatory cytokine profile in tumour-associated macrophages (Allavena et al. 2010).

2.3.3 Growth factors

Alternatively activated macrophages produce growth factors, most importantly PDGF and TGF-β, which stimulate fibroblasts and epithelial cells (Murray, Wynn 2011). PDGF is an important promoting factor for fibroblast proliferation, survival and migration during the wound healing process and fibrosis (Wynn 2008, Wynn, Barron 2010). It is also one of the direct mediators of wound healing and profibrotic activity of macrophages (Wynn, Chawla & Pollard 2013) and a central pathophysiological factor in fibrosing diseases, like SSc (Trojanowska 2008, Liakouli et al. 2011). Four different PDGF proteins exist: PDGF-A, PDGF-B, PDGF-C, PDGF-D. These proteins act as homo- or heterodimers and bind with different affinities to three PDGF receptors. PDGF receptors consist of homo- or heterodimers of PDGF receptor α and/or PDGF receptor β subunits (Andrae, Gallini & Betsholtz 2008). Baroni et al. demonstrated in 2006 that patients with SSc expressed autoantibodies against PDGF receptor in their serum. The antibodies activated both PDGF receptor α and PDGF receptor β and were able to convert human fibroblasts into myofibroblasts that expressed collagen 1 (Baroni et al. 2006) emphasizing the importance of PDGF in the pathophysiology of SSc.

Macrophages also produce TGF-β. TGF-β family consists of three isoforms, and these proteins bind to a heteromeric complex of type I and type II receptors. TGF-β strongly promotes the proliferation of fibroblasts and the synthesis of extracellular matrix, like collagen 1. TGF-β suppresses the proliferation of several immune cells, but it has also proinflammatory effects by for example supporting the differentiation of T_h17 lymphocytes. (Farkas et al. 2011, Biernacka, Dobaczewski & Frangogiannis 2011)

Alternatively activated macrophages also produce IGF-1 as a response to IL-4 and IL-13 (Wynes, Riches 2003). IGF-1 further promotes the collagen synthesis of fibroblasts (Goldstein et al. 1989), the survival of myofibroblasts (Wynes, Frankel & Riches 2004) and is associated with pulmonary fibrosis (Pala et al. 2001, Hamaguchi et al. 2008).

2.3.4 Other markers of alternatively activated phenotype

Fizz1 is induced during helminth infection in mice. It is primarily expressed in epithelial cells, eosinophils and alternatively activated macrophages (Van Dyken, Locksley 2013). Fizz1 stimulates differentiation and collagen production of

myofibroblasts (Liu et al. 2004). In addition, fizz1 exposure has induced airway eosinophilia and peribronchial fibrosis in mice (Doherty et al. 2012).

In addition to mannose receptor 1 and growth factors, several potential markers of alternative activation in human cells have been proposed. In an oligonucleotide microarray published by Martinez et al., CCL13 was one of the most extensively induced genes in response to IL-4 in macrophages (Martinez et al. 2006). The involvement of CCL13 in alternative macrophage activation is supported by several reports showing that CCL13 expression is increased in the diseases that are associated with alternative activation of macrophages. CCL13 expression was demonstrated to be induced in atopic dermatitis (Taha et al. 2000) and sputum of asthmatic patients (Taha et al. 2001) suggesting that CCL13 is linked to T_h2 response. In addition, serum levels of CCL13 were higher in patients with helminth infection (Lechner et al. 2013) and in SSc (Yanaba et al. 2010) than in controls.

2.4 The regulation of macrophage activation

Lipopolysaccharide and IFNγ are the most important environmental signals for macrophages to become activated in the classical manner. Lipopolysaccharides are large molecules that have been found in the outer membrane of Gram-negative bacteria. Macrophages bind LPS by TLR4, which is one of the receptors that enable macrophages to recognize pathogenic structures (Figure 2). TLR4 cooperates with three other proteins: CD14, LPS binding protein (LBP) and MD-2. After ligand detection, four cytosolic proteins, myeloid differentiation primary response gene 88 (MyD88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and translocating chain-associating membrane protein (TRAM) bind to the intracellular part of TLR4 (Tan, Kagan 2014). The MyD88 and TIRAP complex activates interleukin-1 receptor-associated kinase 4 (IRAK4) after which IRAK1 and IRAK2 bind to the complex and recruit TNF receptor associated factor 6 (TRAF6). TRAF6 activates further NF-κB and MAPK pathways (Tan, Kagan 2014, Napetschnig, Wu 2013). TRAM and TRIF pair activates the kinases TANK-binding kinase 1 and IKKε which in turn activates IRF-3 pathway and type I IFN expression (Tan, Kagan 2014). According to a recent study, the activation of the TRAM and TRIF –mediated pathway requires the endocytosis of LPS-bound TLR4 (Kagan et al. 2008).

IFNγ, the sole member of type II interferons, binds to the interferon γ receptor (IFNGR), which consists two chains: IFNGR1 and IFNGR2. Upon IFNγ binding,

two IFNGR1 chains homodimerize, which is followed by the association of IFNGR2 chains to both IFNGR1 chains resulting in the formation of a tetramer receptor complex of two IFNGR1 and two IFNGR2 molecules (Figure 2). Since inactive Janus kinase 1 (JAK1) and JAK2 are bound to IFNGR1 and IFNGR2 chains, respectively, the formation of IFNGR complex brings JAK1 and JAK2 together. JAK1 and JAK2 transactivate each other and phosphorylate IFNGR1. After the phosphorylation, two STAT1 molecules bind to the complex and become activated by tyrosine phosphorylation. The homodimerized and activated STAT1 acts as a transcription factor for several proinflammatory genes. (Sikorski et al. 2012)

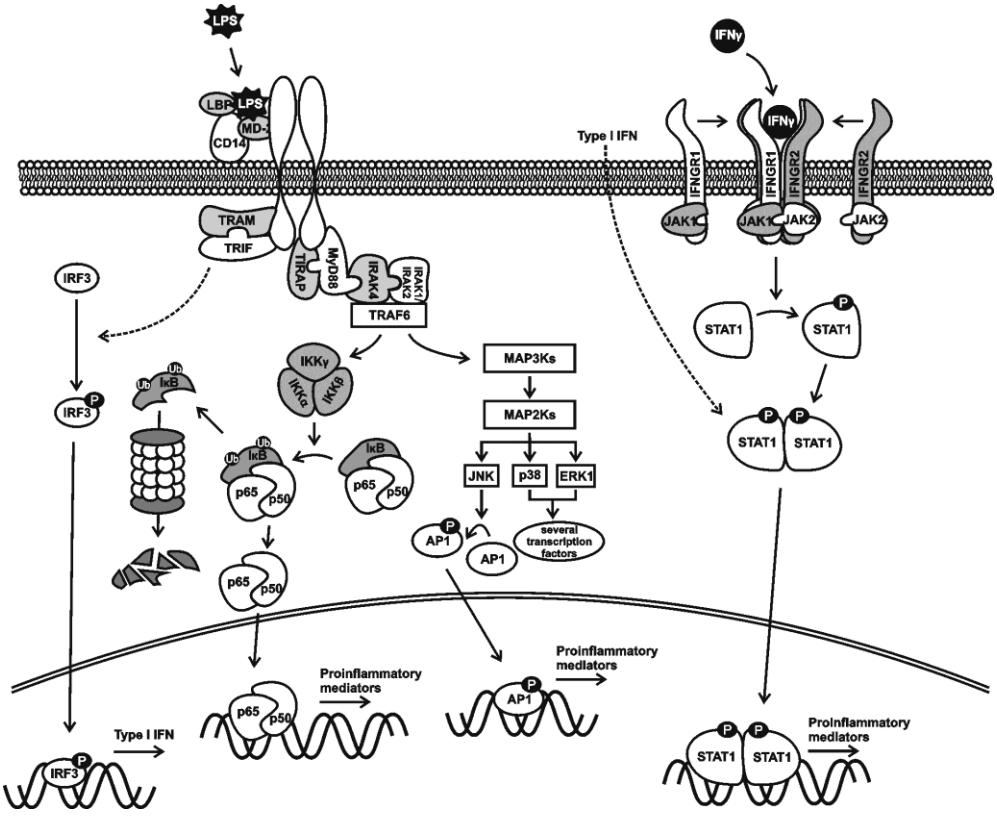

Figure 2. Schematic representation of main signalling pathways mediating classical macrophage activation. AP1, activator protein 1; IFN, interferon; IFNGR, interferon γ receptor; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; IRAK, interleukin-1 receptor-associated kinase; IRF3, interferon regulatory factor 3; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MAP2(3)K, mitogen-activated protein kinase kinase (kinase); MyD88, myeloid differentiation primary response gene 88; P, phosphate; STAT, signal transducers and activators of transcription; TIRAP, toll-interleukin 1 receptor domain containing adaptor protein; TLR4, toll-like receptor 4; TRAF, TNF receptor associated factor; TRAM, translocating chain-associating membrane protein; TRIF, TIR-domain-containing adapter-inducing interferon-β; Ub, ubiquitin

Alternative activation is induced by IL-4 and IL-13. Macrophages recognize IL-4 and IL-13 by two types of transmembrane receptor complexes (Figure 3). The type I receptor is composed of IL-4 receptor α (IL-4Rα) and γ_c subunits and the type II receptor of IL-4Rα and IL-13Rα1 subunits. The type I receptor binds IL-4 only, but the type II receptor binds both IL-4 and IL-13 (Van Dyken, Locksley 2013). Similarly to IFNGR, the binding of the ligand to either IL-4Rα or IL-13Rα1 leads to the association of the ligand binding chain to the other subunit of the receptor. This further activates cytosolic Janus kinases to phosphorylate the ligand binding chain. IL-4Rα associates with JAK1, IL-13Rα1 with JAK2 or tyrosine kinase 2 and γ_c with JAK3 (Van Dyken, Locksley 2013, LaPorte et al. 2008) and when activated IL-4Rα or IL-13Rα1 phosphorylates STAT6 (LaPorte et al. 2008).

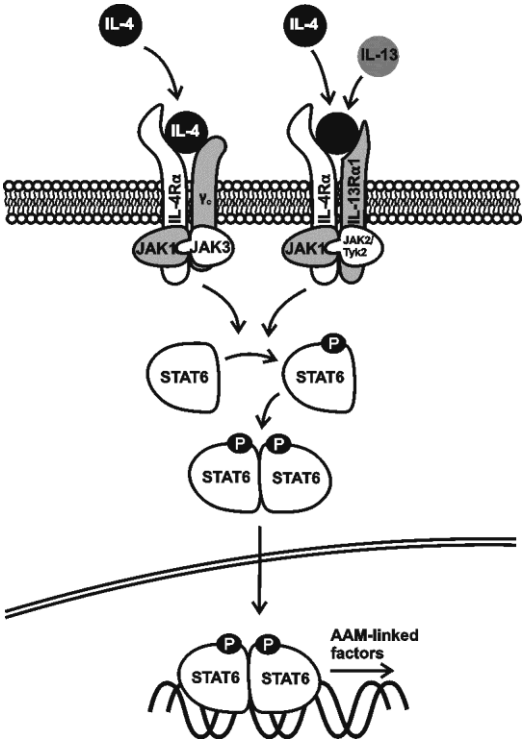

Figure 3. Schematic representation of IL-4 and IL-13 –responsive signalling pathway mediating alternative macrophage activation. AAM, alternatively activated macrophage; IL, interleukin; IL-4/13R, interleukin 4/13 receptor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; Tyk, tyrosine kinase

The differences in the spectrum of the cytokines produced by the two macrophage phenotypes are regulated in amazingly sophisticated ways. The

pathways that regulate the activity of macrophages are mainly known with regards to classical activation. However, understanding of the regulation of the activity of alternatively activated macrophages is also increasing.

2.4.1 Transcription factors as regulators of macrophage activation

As described earlier, TLR4-mediated NF-κB and MAPK pathways and IFNγ receptor -mediated STAT1 pathway are the principal pathways that activate the transcription of the genes linked to classical macrophage activation. In addition, LPS-induced type I IFN production activates the STAT1 pathway thus linking TLR4-induction to STAT1 activation (Hu, Ivashkiv 2009). On the other hand, IL-4/IL-13-induced alternative activation is also mediated through STAT family, although it involves another member of the family, i.e. STAT6, which is not engaged in classical activation. The activity of all of these pathways is stringently regulated at all steps from receptor-mediated activation to the induction of transcription.

NF-κB is the best studied proinflammatory transcription factor. The NF-κB family consists of five members: reticuloendotheliosis viral oncogene homolog A (RelA, also known as p65), RelB, c-Rel, NF-κB1 (also known as p50) and NF-κB2 (also known as p52). These subunits form homodimers and heterodimers, all of which can bind to certain DNA elements. However, only p65, RelB and c-Rel possess a transactivator domain, which is needed for induction of transcription. Normally in resting cells, NF-κB members are bound to one of three κB inhibitory proteins (IκBs): IκBα, IκBβ and IκBε (Ruland 2011, Sun, Chang & Jin 2013). Upon TLR4 stimulation, a complex of IκB kinases (IKKs) phosphorylates IκB, which exposes IκB to ubiquitinylation and further to proteosomal degradation (Figure 2). The degradation of IκB releases p65 and p50, which can then heterodimerize, translocate to nucleus and bind to the promoter of target genes. The transcription of IκBα is also induced, and newly synthetized IκBα translocates to nucleus, binds to DNA-bound NF-κB, relocates it to cytosol and thus terminates the transcriptional activity of NF-κB (Hoesel, Schmid 2013, Haddad, Abdel-Karim 2011).

The activity of NF-κB is regulated in very complex ways; p65 alone can undergo over 300 documented interactions with other proteins (Hoesel, Schmid 2013). NF-κB activity is known to be induced by phosphorylation and acetylation of p65. In fact, both phosphorylation and acetylation seem to be essential to the transcriptional activity of p65 (Huang, Hung 2013). In addition to IκB, ubiquitinylation and nuclear degradation of p65 is another important mechanism to shorten NF-κB activity. The ubiquitinylation of p65 is known to be mediated by two E3 ubiquitin ligases. One of

these is PDZ and LIM domain protein 2, the other is Cullin-RING ubiquitin ligase 2 that contains suppressor of cytokine signalling 1 (SOCS1), Cullin-2 and COMM domain-containing protein 1 (Sun, Chang & Jin 2013, Ruland 2011). In addition, alternative NF-κB signalling pathways, induced by different stimuli other than LPS, and other transcription factors interfere and regulate the canonical NF-κB activity (Hoesel, Schmid 2013, Ruland 2011).

The mitogen-activated protein kinase signalling cascade (Figure 2) is another intracellular pathway that strongly responds to TLR4 stimulus in addition to other potentially harmful stimuli like oxidative stress, DNA damage and abnormal osmolarity. The cascade is composed of a core of kinases in three levels, in which the upstream kinases phosphorylate and activate the downstream kinases. When activated, MAP kinases phosphorylate a number of substrates needed for inflammation and the maintenance of tissue homeostasis. Most is known about the so-called classical MAP kinases: extracellular signal-regulated kinases (ERKs), JNKs and p38 kinases. However today, no fewer than fourteen mammalian MAP kinases have been identified. MAP kinases become activated by phosphorylation mediated by MAPK kinases (MAP2Ks), and MAP2Ks are activated by MAPK kinase kinases (MAP3Ks). The activation of MAP3K involves several regulated steps (Kyriakis, Avruch 2012, Arthur, Ley 2013). MAPKs have an important role in creating the activation phenotypes of macrophages after TLR4 stimulus (Arthur, Ley 2013). MAPKs activate several downstream proteins, which further change the expression of a wide range of genes. A well-known target of JNK is AP-1, which acts as a transcription factor for the proinflammatory cytokines (Meng, Xia 2011). Furthermore, at least ERK1, ERK2 and p38α increase the transcription of proinflammatory cytokines. Interestingly, it has been shown that JNK deficiency generally impairs the macrophage polarization to classically activated macrophage phenotype (Arthur, Ley 2013).

Janus kinase -induced activation of STAT1 leads to its homodimerization and translocation into the nucleus. In the nucleus, STAT1 binds to its promoter-response element in DNA and increases the transcription of several inflammatory genes, like iNOS and IL-12. (Lawrence, Natoli 2011). Transcriptional activity of STAT1 can be increased by a MAPK-mediated phosphorylation of a serine residue. On the other hand, SOCS1 suppresses IFNγ-mediated macrophage activation (Hu, Ivashkiv 2009, Rauch, Muller & Decker 2013). In addition to phosphorylation, there is evidence showing that STAT1 can also be acetylated and the acetylation of STAT1 decreases its phosphorylation and thus mediates inactivation (Kramer et al. 2009). When dephosphorylated, STAT1 is translocated back to cytoplasm (Hu, Ivashkiv 2009).

STAT6 is the key transcription factor to mediate the effects of IL-4 and IL-13. After JAK-mediated phosphorylation, STAT6 homodimerizes, is translocated into the nucleus where it binds to promoter elements of IL-4/IL-13-responsive genes, such as arginase 1, mannose receptor 1 and fizz1 (Lawrence, Natoli 2011). At least two other transcription factors are believed to regulate the efficacy of STAT6: peroxisome proliferator-activated receptor γ (PPARγ) and cyclic adenosine 3'-5'-monophosphate (cAMP) response element-binding protein (CREB) –C/EBPβ. PPARγ-deficient macrophages are resistant to polarization into the alternative activation phenotype (Odegaard et al. 2007). According to a recent study, PPARγ-mediated gene regulation is dependent on the presence of STAT6 (Szanto et al. 2010). However, the exact mechanisms behind the effects of PPARγ are not clear and there are several hypotheses attempting to explain the action of PPARγ in macrophage polarization (Lawrence, Natoli 2011, Pourcet, Pineda-Torra 2013).

The activation of C/EBPβ is also required for the IL-4-induced transcription of fizz1 and arginase 1 (Stutz et al. 2003, Pauleau et al. 2004, Gray et al. 2005). Perhaps more interestingly, M2-associated, but not M1-associated, genes were inhibited in mice whose CREB-induced C/EBPβ expression was prevented (Ruffell et al. 2009). In LPS-induced macrophages, CREB was shown to activate cascades that downregulated the expression of multiple proinflammatory genes (Lawrence, Natoli 2011). These results are interesting especially due to the well-documented fact that C/EBPβ activation increases the expression of proinflammatory cytokines in macrophages. It has been postulated that the proinflammatory action of C/EBPβ is independent from CREB (Lawrence, Natoli 2011). According to a recent study, the effect of C/EBPβ on TLR-induced arginase 1 expression may be mediated through STAT3 activation (Qualls et al. 2010).

It is well demonstrated that macrophages are able to change their phenotype according to the environmental signals *in vitro*. However, it is less clear if a similar phenotype shifting occurs *in vivo*. In tissue necrosis -induced inflammation in mice, classically activated macrophages (characterized by low expression of CX_3C-chemokine receptor 1 (CX_3CR1) and high expression of LY6C) are the first cells to be recruited into the damaged area. These proinflammatory cells are slowly replaced by $CX_3CR1^{hi}LY6C^-$ cells that represent alternatively activated macrophages. Whether non-inflammatory $CX_3CR1^{hi}LY6C^-$ macrophages are derived from original classically activated macrophage population or recruitment of new macrophages from the blood is not known yet (Lawrence, Natoli 2011).

2.4.2 Other factors regulating macrophage activation

Classical activation of macrophages is regulated also at posttranscriptional level in multiple manners. Most of the regulators affect the processing steps of mRNA: splicing, polyadenylation and translation, but some regulating factors can also modify the stability of mRNA or protein.

During mRNA splicing, the intronic sequences of mRNA are removed. However, under certain circumstances, exon sequences can be removed, introns can be retained or alternative splicing sites at intron ends can be used. Furthermore, the poly(A) tail at the end of mature mRNA can be cleaved by different deadenylation enzymes. Deadenylases are often recruited by regulatory proteins. Alternative splicing and alternative polyadenylation may lead to the translation of truncated proteins that may increase or decrease the activity of certain signalling pathways (Carpenter et al. 2014). On the other hand, a zinc-finger protein ZCCHC11 has been found to stabilize mRNA, possibly by adding uridine residues to the 3' ends of RNA (Mino, Takeuchi 2013).

The presence of ARE at the 3'UTR of mRNA strongly exposes the mRNA to deadenylation and subsequent degradation. AREs recruit ARE-binding proteins that define the stability of the target transcript; the proteins can either stabilize or destabilize the transcript. There are at least 20 different ARE-binding proteins, and two of the best characterized of them are tristetraprolin (TTP) and AU-rich element RNA-binding protein 1. The activation of TTP or AU-rich element RNA-binding protein 1 enables these proteins to bind to the AREs of a target mRNA, which recruits degradation factors to the transcript. (Ivanov, Anderson 2013)

Two proteins are under specific control at the initiation of translation. The first is the initiation factor eIF2 (eukaryotic initiation factor 2), which recruits initiator tRNA to the ribosome. The phosphorylation of eIF2 leads to a global reduction of mRNA translation. The other important protein is the cap-binding protein eIF4E, which is required for recruitment of 43S pre-initiation complex to the mRNA. Some proteins, such as mammalian target of rapamycin (mTOR) can bind to eIF4E and prevent its action. (Carpenter et al. 2014, Ivanov, Anderson 2013)

The ubiquitin-proteasome system is the primary proteolytic system in the cell. Proteins are flagged for proteasomal degradation by ubiquitinylation of lysine residue(s). The ubiquitinylation cascade involves three distinct classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s); the activity of all of these enzymes is tightly regulated. After ubiquitinylation, the 19S structure of the proteasome recognizes the substrate, removes its ubiquitin chain and unfolds the protein. Next, the amino acid chain

enters the cylindrical-shaped 20S structure, which contains subunits with three different types of proteolytic activity: the first cleaves after acidic residues, the second after basic residues and the third after hydrophobic residues of the amino acid chain (caspase-like, trypsin-like and chymotrypsin-like activity, respectively) (Groettrup, Kirk & Basler 2010, Qureshi, Morrison & Reis 2012). Several proteins have been shown to induce the activity of 20S proteasome, and those include PA700, activators of the PA28 family and Blm10/PA200 activators. For example, PA28αβ is induced by cytokines (Schmidt, Finley 2014).

2.5 The role of macrophages in inflammatory and metabolic diseases

Macrophages are key players in the pathology of RA. It has been shown that the number of macrophages in synovial tissue correlates with the severity of symptoms, the disease activity and the subsequent erosive damage in patients suffering from RA (Isaacs 2010, Strehl et al. 2014). TNF and IL-6 promote the differentiation and TNF also helps in the survival of macrophages. Treatment with TNF blockers can induce apoptosis of monocytes and macrophages in both synovial fluid and peripheral blood (Li, Hsu & Mountz 2012). In addition, the depletion of inflammatory macrophages in death receptor 5 -transgenic mice also reduced the number of T_h17 cells and osteoclasts and increased the number of T_{reg} cells during collagen-induced arthritis (Li et al. 2012).

Macrophages contribute to the bone and cartilage destruction in RA by inducing osteoclastogenesis in the synovial membrane and by activating synoviocytes and chondrocytes. Osteoclasts are cells involved in bone destruction. The activation of synoviocytes and chondrocytes leads to the secretion of cartilage degrading MMP enzymes and inflammatory factors by these cells. The MMP enzymes and inflammatory factors also decrease the synthesis of matrix components in cartilage. (Choy 2012)

In the typical histological view of SSc, there is prominent macrophage infiltration in addition to lymphocytes (O'Reilly 2014). Nonetheless, the role of macrophages in SSc has been much less extensively studied than that of lymphocytes. However, since SSc is a fibrosing disease, the pathophysiological role of macrophages can be assumed to be similar to other fibroproliferative diseases.

Macrophages play a crucial role in the early events in the pathogenesis of fibrosis. Inflammation always precedes fibrosis and during inflammation macrophages produce several proinflammatory cytokines, like TNF and IL-1 that activate

fibroblasts to produce extracellular matrix proteins. At the same time they produce MMPs that degrade extracellular matrix (Wick et al. 2013). Higashi-Kuwata et al. demonstrated in 2010 that the proportion of alternatively activated macrophages between collagen fibres was increased in the skin of SSc patients when compared to that of healthy subjects (Higashi-Kuwata et al. 2010). In addition, the expression of CD163, a marker of alternative macrophage activation, was increased in $CD14^+$ peripheral blood mononuclear cells in patients with SSc (Higashi-Kuwata et al. 2010). Additionally, patients with either limited or diffuse cutaneous SSc display elevated serum levels of cytokines that are related to alternative activated macrophages: IL-4, IL-10 and IL-13 (Christmann et al. 2011, Hasegawa et al. 1997). Confirming the pathological role of IL-13, IL-13 receptor antagonists attenuated skin and lung fibrosis in experimental models (Aliprantis et al. 2007, Fichtner-Feigl et al. 2006). Accordingly, treatment with IFNγ has been shown to improve skin scores in patients with SSc (Grassegger et al. 1998), suggesting that driving macrophages towards classical activation is beneficial in SSc. This is logical since alternatively activated macrophages produce immunosuppressive cytokines like IL-10 and growth factors, like TGF-β1 and PDGF, which would support the production of extracellular matrix by fibroblasts and subsequent fibrosis (Wick et al. 2013).

Under physiological conditions, the free fatty acids stored in adipose tissue and LDL particles in the arterial wall become modified and released to HDL by macrophages. In the arterial wall, oxidized LDL particles are phagocytosed by macrophages through the activation of two scavenger receptors, CD36 and scavenger receptor A. Once inside the macrophage, oxidized LDL is hydrolyzed in late endosomes, lysosomes and phagolysosomes to yield free cholesterol. In the presence of pre-HDL, free cholesterol can be excreted from macrophages by reverse cholesterol transport mediated by ATP binding cassette A1 and G1. Macrophages can also use free cholesterol as a source of energy. The excess of free cholesterol can be esterified to inflammatory inactive cholesterol esters. (Prieur, Roszer & Ricote 2010)

In metabolic diseases, the lipid overload exceeds the capacity of adipocytes to store free fatty acids and macrophages to process oxidized LDL particles in the vessel wall. Free fatty acids directly promote the classical activation of macrophages. Free cholesterol can exert a lipotoxic effect in macrophages by changing membrane fluidity, inducing apoptosis and increasing the secretion of TNF and IL-6. The macrophage death encountered in atherosclerotic lesions promotes lesion necrosis, which leads to plaque instability and possibly to plaque rupture (Prieur, Roszer &

Ricote 2010). In atherosclerotic plaques, alternatively activated macrophages typically surround the lipid core (Pourcet, Pineda-Torra 2013). Alternatively activated macrophages attempt to stabilize atherosclerotic plaque via the secretion of anti-inflammatory cytokines and furthermore, they strive to reduce the level of inflammation, by induction of collagen formation and by their effective housekeeping functions to clear dying cells and debris (Moore, Sheedy & Fisher 2013). It has also been postulated that arginase 1, expressed by alternatively activated macrophages, can stabilize the atherosclerotic plaque (Morris 2009, Pourcet, Pineda-Torra 2013).

In 2007 Lumeng et al. found that obese mouse had more classically activated macrophages and less alternatively activated macrophages than lean mouse (Lumeng, Bodzin & Saltiel 2007). Also, activation of genes that are known to reduce insulin resistance have been found to polarize adipose tissue macrophages to alternative phenotype (Odegaard et al. 2008, Odegaard et al. 2007). These findings have lead to the hypothesis that alternative activation of macrophages protect lean people from insulin resistance and other obesity-linked metabolic manifestations. (Prieur, Roszer & Ricote 2010, Lumeng, Bodzin & Saltiel 2007). However, the information of macrophage phenotype in human adipose tissue is controversial to that in mice. Both Zeyda et al. and Bourlier et al. showed that the adipose tissue macrophages express alternative phenotype in obese people more than in lean people (Zeyda et al. 2007, Bourlier et al. 2008). Thus, the role of macrophage phenotypes in metabolic diseases is not clear yet.

3 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily. There are three isotypes of PPARs: PPARα, PPARβ/δ and PPARγ. The first isotype, PPARα, was discovered in 1990 when it was found to cause peroxisome proliferation in rodent liver cells (Issemann, Green 1990). Although activation of PPARα does not increase peroxisome proliferation in human cells, the receptor family received its name from this finding (Eldor, DeFronzo & Abdul-Ghani 2013).

Soon after the discovery of the PPARs, it became clear that PPARs are important regulators of cellular metabolic processes. The structure of the PPARs resembles that of many other nuclear receptors (Figure 4) and PPARs regulate the transcription of several genes. When activated, the PPARs interact with retinoid X receptor (RXR) and, as a heterodimer with RXR, bind to a specific DNA sequence motif named

peroxisome proliferator response element (PPRE), which usually is located in promoter regions. Next, coactivators, like the PPARγ coactivator 1α (PGC-1α), histone acetyltransferase p300, CREB binding protein (CBP) and steroid receptor coactivator 1, bind to PPAR and enable the initiation of the transcription of the target gene. Later, PPARs have also been found to regulate gene transcription in other ways, e.g. by binding to other transcription factors and modifying their activity. (Eldor, DeFronzo & Abdul-Ghani 2013, Monsalve et al. 2013)

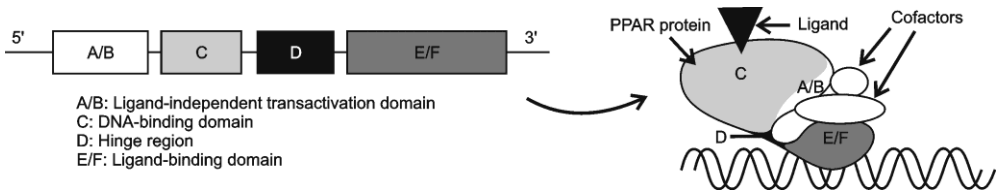

Figure 4. Schematic representation of primary and tertiary structure of PPARs.

PPARα is widely expressed in tissues with high fatty acid catabolic activity e.g. heart, liver, kidney, intestine and brown fat. PPARβ/δ is expressed ubiquitously, but its expression is highest in highly proliferating cells. PPARγ has two isoforms: PPARγ1 and PPARγ2. PPARγ2 is expressed in adipose tissue only, but PPARγ1 is also expressed in other tissues e.g. in skeletal muscle, liver and macrophages at lower levels. (Eldor, DeFronzo & Abdul-Ghani 2013, Monsalve et al. 2013)

3.1 PPAR agonists

According to the current understanding, the main physiological task of PPARs is to link metabolic changes to alterations in gene expression. Both the endogenous and synthetic ligands of PPARs are lipid-soluble and therefore membrane-permeable molecules; hydrophilic compounds could not penetrate the cell membranes effectively and thus could not bind to cytosolic PPAR (Kiss, Czimmerer & Nagy 2013). Due to their effects on energy metabolism, PPARα and PPARγ agonists are used as pharmaceuticals in the treatment of metabolic diseases.

3.1.1 PPARα agonists

Endogenous ligands of the PPARα include saturated and unsaturated fatty acids, 8-hydroxyeicosatetraenoic acid, leukotriene B_4 (LTB_4) and epoxyeicosatrienoic acids.

When bound by these ligands, PPARα promotes the activity of several steps in β- and ω-oxidation. (Monsalve et al. 2013)

Gemfibrozil and fenofibrate, two members of fibrate family, are synthetic PPARα agonists that are in clinical use in the treatment of hyperlipidaemia, especially hypertriglyceridemia. Other synthetic compounds that are not approved for clinical use, but are often used in research purposes to activate PPARα include GW7647, GW9578 and WY14643 (also known as pirinixic acid) (Hamblin et al. 2009). All of these test compounds are selective PPARα agonists (Table 2).

Synthetic ligands of PPARα clearly improve lipid homeostasis by reducing the plasma levels of triglycerides and LDL cholesterol. In the Helsinki Heart study, gemfibrozil reduced the development of coronary heart disease by 34% during a five year follow-up when compared to placebo in middle-aged men suffering from primary dyslipidaemia (Frick et al. 1987). According to the FIELD study, fenofibrate reduced the risk for cardiovascular disease in patients with type II DM and metabolic syndrome. The reduction was highest (27%) in subjects with marked dyslipidaemia (Scott et al. 2009). In a meta-analysis, fibrates generally reduced major cardiovascular events by 10%, coronary events by 13% and the risk of albuminuria progression by 14% (Jun et al. 2010). The benefits were greater in the subjects who had dyslipidaemia characterized by low HDL levels (<1.036 mmol/l) and high triglycerides (>2.26 mmol/l). In those subjects, the risk of cardiovascular events decreased by 29% (Lee et al. 2011). However, the all-cause mortality or cardiovascular mortality was not reduced by fibrate therapy, at least not if the therapy was used in non-targeted dyslipidaemic patients (Jun et al. 2010).

When used as a monotherapy for the treatment of dyslipidaemia, fibrates do not seem to be associated with the risk of serious drug-related adverse events (Jun et al. 2010). Notably, although fibrate therapy decreases the risk of albuminuria progression, the treatment increases creatine levels and reduces calculated glomerular filtrate rate. However, the overall risk for kidney diseases is not increased in fibrate users (Jun et al. 2012).

3.1.2 PPARγ agonists

The ligand pocket of PPARγ is relatively large, and it can allow several different lipid-molecules to bind into it. Thus many molecules with very different chemical structures can act as ligands of PPARγ e.g. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), lysophosphatidic acid, serotonin metabolites, oxidized LDL and oxidized

LDL-derived hydroxyoctadecadienoic acids and hydroxyeicosatetraenoic acids (Kiss, Czimmerer & Nagy 2013, Monsalve et al. 2013, Hamblin et al. 2009).

Thiazolidinediones (TZDs) are synthetic ligands of PPARγ. Three TZD molecules have been used in clinics in the treatment of type II DM: troglitazone, rosiglitazone and pioglitazone. Non-TZD ligands for PPARγ include tyrosine analogues GW1929 and GW7845 (Hamblin et al. 2009) (Table 2).

Table 2. Partial list of synthetic PPARα and PPARγ agonists

PPAR agonist	EC50 selectivity PPARα vs. PPARγ in mice (fold)	Status of clinical development	Reference
PPARγ agonists			
GW1929	< 0.001	Discontinued before entering clinical studies	(Ram 2003)
GW7845	< 0.001	Discontinued before entering clinical studies	(Ram 2003)
Rosiglitazone	< 0.001	Withdrawn from market in Europe	(Ram 2003)
Pioglitazone	< 0.005	In clinical use	(Ram 2003)
Rivoglitazone	< 0.02	In phase III trials (in 2013)	(Kanda et al. 2009, Tao 2013)
Balaglitazone	data not available *	In phase III trials (in 2013)	(Tao 2013)
PPARα/γ dual agonists			
Tesaglitazar	0.01	Discontinued after phase III trials	(Azhar 2010, Ljung et al. 2002)
Chiglitazar	0.1	In phase III trials (in 2014)	(He et al. 2012)
Naveglitazar	0.1	Discontinued after phase II trials	(Azhar 2010, Wood 2006)
Ragaglitazar	0.2	Discontinued after phase II trials	(Azhar 2010, Cai, Xie & Guo 2006)
Muraglitazar	0.3	Discontinued after phase III trials	(Harrity et al. 2006)
Aleglitazar	0.4	Discontinued during phase III trials	(Benardeau et al. 2009)
Saroglitazar	4615	Approved for clinical use in India	(Jani et al. 2014)
Netoglitazone	data not available	In phase II trials (in 2013)	(Tao 2013)
PPARα agonists			
Clofibrate	10	In clinical use	(Ram 2003)
Fenofibrate	14	In clinical use	(Ram 2003)
WY14643	51	Used in basic research only	(Ram 2003, Azhar 2010)
GW9578	300	Used in basic research only	(Ram 2003, Azhar 2010)
GW7647	1300	Used in basic research only	(Azhar 2010, Brown et al. 2001)

*partial agonist of PPARγ

EC50, Median effective concentration

All of the TZDs used in clinics have multiple effects on glucose and lipid metabolism in multiple tissues and the efficacy of TZDs has been evaluated in several clinical studies. They improve insulin sensitivity, β-cell function, glycemic control, endothelial function and lipid distribution in the body (Eldor, DeFronzo & Abdul-Ghani 2013, Monsalve et al. 2013). The mean reduction in HbA1c achieved with TZDs treatment is 0.96 percentage points in subjects with type II DM (Esposito et al. 2012). In terms of the efficacy in improvement of glucose levels in type II DM, it seems that TZDs are one of the best antidiabetic drugs currently on the market. Only insulin, metformin and glucagon-like peptide-1 (GLP-1) agonists have shown better results in diabetic hyperglycaemia (Esposito et al. 2012). The PROactive study showed that pioglitazone could decrease the incidence of all-cause mortality, nonfatal myocardial infarction and nonfatal stroke in patients with type II DM and macrovascular diseases (Dormandy et al. 2005). Similar results were obtained in a meta-analysis which included heterogeneous population of patients with type II DM (Lincoff et al. 2007). TZDs also decrease the severity of proteinuria and hepatic steatosis in diabetic patients (Sarafidis et al. 2010, Zib et al. 2007).

Currently, there is only one TZD, pioglitazone, on the market in Europe. The first TZD, troglitazone, was withdrawn from the market already in 2000 due to liver toxicity (Consoli, Formoso 2013). Another TZD, rosiglitazone, was withdrawn in 2010 due to increased risk for myocardial infarction and cardiovascular death (Nissen, Wolski 2007). In detailed studies, the increased risk of these adverse events seemed to be linked to rosiglitazone specifically, since pioglitazone decreased the incidence of myocardial infarction and deaths (Nagajothi et al. 2008, Lincoff et al. 2007). However, pioglitazone, similarly to other TZDs, is known to increase the risk of fluid retention and heart failure, but the increased mortality was not associated with pioglitazone-induced heart failure (Lincoff et al. 2007). Pioglitazone has also been found to slightly increase the risk of fractures (Loke, Singh & Furberg 2009) and bladder cancer (Ferwana et al. 2013, Zhu et al. 2012). Nevertheless, although the risk of bladder cancer is increased, the overall cancer risk is not higher (Bosetti et al. 2013). This results from decreased risk of lung, colorectal and breast cancers seen after pioglitazone treatment (Colmers, Bowker & Johnson 2012).

3.1.3 PPARα/γ agonists

Recently, also PPARα/γ dual agonists like muraglitazar, naveglitazar, tesaglitazar, aleglitazar, ragaglitazar and netoglitazone have been synthetized to achieve both antidiabetic and cardiovascular protective effects (Hamblin et al. 2009, Rosenson et

al. 2012) (Table 2). Muraglitazar was the first glitazar reaching the US Food and Drug Administration (FDA) for consideration of approval in 2005. Muraglitazar (5 mg) decreased HbA1c levels more effectively than pioglitazone (15 mg or 30 mg) during the 24 week follow-up (-1.18 percentage points in muraglitazar group vs. -0.57 percentage points in pioglitazone 15 mg group and -1.14 percentage points in muraglitazar group vs. -0.85 percentage points in pioglitazone 30 mg group) (Kendall et al. 2006, Rubin, Viraswami-Appanna & Fiedorek 2009). Furthermore, muraglitazar (5 mg) improved lipid parameters significantly better than pioglitazone (30 mg) or placebo (Kendall et al. 2006, Buse et al. 2005). Triglyceride levels were reduced by 27-28% with muraglitazar and 14% and 2% with pioglitazone and placebo, respectively. HDL cholesterol was increased by 16-19% with muraglitazar and 14% and 2% with pioglitazone and placebo.

The FDA advisory committee recommended approval of muraglitazar, but in a meta-analysis of phase 2 and 3 clinical trials, muraglitazar treatment was reported to be associated with an increased incidence of cardiovascular events at least when combined to sulfonylureas or metformin (Nissen, Wolski & Topol 2005). This meta-analysis contained five clinical trials with a total of 3725 subjects. Although the relative risk values for individual end points were not significant if both monotherapies and combination therapies were included, muraglitazar tended to increase the all-cause mortality (RR 3.05), cardiovascular deaths (RR 4.57), fatal or nonfatal myocardial infarction (RR 2.14) and heart failure (RR 7.43) compared to the control group treated with pioglitazone or placebo. In the next year, in May 2006, a clinical trial with 1159 patients revealed similar safety problems (Kendall et al. 2006) and subsequently, muraglitazar's developer, Bristol-Myers Squibb, decided to discontinue further development of the drug.

Tesaglitazar (Goldstein et al. 2006, Fagerberg et al. 2005, Bays et al. 2007, Ratner et al. 2007), ragaglitazar (Skrumsager et al. 2003) and aleglitazar (Henry et al. 2009, Lincoff et al. 2014) have also been documented to improve the levels of HbA1c, triglycerides and HDL when compared to placebo or pioglitazone in type II DM or in non-diabetic patients. The efficacy and safety of these glitazars were comparable to those of muraglitazar. Interestingly, aleglitazar tended to decrease the incidence of myocardial infarction in a 104 week follow-up in a double-blind, placebo-controlled and randomized trial with 7226 patients suffering from acute coronary syndrome and type II DM, but the results did not reach statistical significance (Lincoff et al. 2014).

Saroglitazar is a strong PPARα and moderate PPARγ agonist, which has recently been approved for clinical use in atherogenic diabetic dyslipidaemia in India

(Agrawal 2014). In the reported clinical studies with a total of 411 subjects, saroglitazar significantly reduced the levels of triglycerides and LDL and increased the levels of HDL without causing any adverse events or pathological changes in laboratory parameters (Jani et al. 2014, Pai et al. 2014). However, saroglitazar has only marginal efficacy in improving HbA1c (Jani et al. 2014, Pai et al. 2014). Nonetheless, these promising results obtained with aleglitazar and saroglitazar have encouraged pharmaceutical companies to continue the development of PPARα/γ agonists for the treatment of metabolic diseases.

3.1.4 PPARβ/δ agonists

Endogenous ligands for PPARβ/δ include saturated and unsaturated fatty acids and prostacyclin. In addition, highly selective ligands have been synthesized, but none of them have entered clinical use as yet. Most of the reported studies have been carried out with GW501516, GW0742, MBX-8025 or GFT505. (Mackenzie, Lione 2013, Monsalve et al. 2013)

PPARβ/δ agonists have been reported to exert multiple beneficial effects in several diseases: pulmonary hypertension, septic shock, atherosclerosis, liver diseases, kidney diseases and muscular dystrophy. Thus, drugs are believed to be very promising. For example, PPARβ/δ agonists have demonstrated substantial therapeutic potential in patients with type II DM and metabolic diseases in six phase 2 clinical trials by clearly lowering blood glucose and improving LDL and HDL profiles. However, PPARβ/δ agonists accelerated the tumour development in several animal models, and for this reason GlaxoSmithKline, the drug company that carried out most of the preclinical and clinical trials with PPARβ/δ agonists, decided to discontinue the development of these drugs. In the clinical trials, no increased incidence of tumours was reported, but PPARβ/δ agonists did increase creatinine kinase levels and decreased red blood cell count and haematocrit (Mackenzie, Lione 2013).

Some PPARα/δ dual agonists have also been developed and two clinical investigations with GFT505 have been published. In these studies, GFT505 improved insulin sensitivity as well as levels of triglycerides and HDL in obese, dyslipidaemic and insulin-resistant subjects (Cariou et al. 2013, Cariou et al. 2011).

3.2 Molecular effects of PPARs in metabolism and inflammation

In 1996, the first evidence was published describing the link between PPARs, metabolism and inflammation. In that year, Devchand et al. reported that LTB_4, an endogenous proinflammatory lipid mediator, activated PPARα and the activation of PPARα could shorten the duration of inflammation in mice (Devchand et al. 1996). In 1998, PPARγ was shown to dampen proinflammatory activation of macrophages (Jiang, Ting & Seed 1998, Ricote et al. 1998). Subsequently, the dual action of PPARs has become exceedingly evident. In the previous chapter, the clinical effects of PPAR agonists in pathological conditions were discussed, this chapter will focus on the molecular action of PPARs in metabolism and inflammation.

3.2.1 PPARα

The best known effects of PPARα are linked to fatty acid catabolism and PPARα has been reported to be a critical regulator of fatty acid catabolism during fasting. The phenotype of PPARα deficient mice is normal, but fasting results in elevated plasma free fatty acid levels, accumulation of lipids in the liver, hyperketonaemia, hypoglycaemia and hypothermia (Aoyama et al. 1998, Hashimoto et al. 2000, Leone, Weinheimer & Kelly 1999, Kersten et al. 1999) indicating that normally PPARα shifts the energy source from glucose to fatty acids. An accumulation of lipids in the liver was also seen in PPARα null mice after consuming a high-fat diet (Kersten et al. 1999). In addition, the wide range of genes that are expressed after PPARα activation indicates that PPARα has a central regulatory role in lipid metabolism. The genes that are directly regulated by PPARα include acyl CoA oxidase, carnitine palmitoyl transferase I (CPT I), hydroxymethylglutaryl CoA (HMG-CoA) synthase, cytochrome P450 4A and apolipoprotein A1 and A2 enzymes (Monsalve et al. 2013). Acyl CoA oxidase catalyses β-oxidation, CPT I is essential for the transport of long-chain fatty acids to mitochondria for β-oxidation, HMG-CoA is an intermediate in cholesterol synthesis and ketogenesis and cytochrome P450 4A catalyses the ω-oxidation of fatty acids and thus reduces the synthesis of triglycerides. Apolipoproteins A1 and A2 are the major components of HDL and are involved in the reverse cholesterol transport from peripheral cells. Thus, one can summarize that PPARα is involved in the uptake, binding and oxidation of fatty acids.

There is some evidence showing that PPARα deficient mice might also be sensitive to immunological stress. As mentioned above, PPARα -null mice were shown to display a prolonged inflammatory reaction in response to LTB_4 as

compared to wild-type animals (Devchand et al. 1996). In addition, PPARα agonists reduced the age-related constant NF-κB activation and cytokine production in mice, and this effect of PPARα agonists was abolished in PPARα deficient mice (Poynter, Daynes 1998). Further evidence on the role of PPARα in inflammation was received when Okamoto et al. showed that fenofibrate attenuated signs of arthritis and joint oedema in Freund's adjuvant-induced arthritis in rats (Okamoto et al. 2005). Fenofibrate treatment was also shown to decrease the circulating levels of the proinflammatory cytokines TNF and IFNγ in atherosclerotic patients (Madej et al. 1998) and high-sensitivity CRP and IL-6 in patients with metabolic syndrome (Belfort et al. 2010). PPARα agonists also attenuated leukocyte recruitment and adhesion to endothelial cells (Hamblin et al. 2009). In addition, PPARα agonists have been reported to induce macrophage cholesterol efflux e.g. by increasing the expression of HDL receptor CLA-1 (CD36 and LIMPII analogous 1) (Hamblin et al. 2009). In several studies, PPARα ligands have also been shown to inhibit the proliferation of vascular smooth muscle cells *in vitro* although the *in vivo* evidence is still lacking (Hamblin et al. 2009).

3.2.2 PPARγ

PPARγ knockout mice are not viable, but they die during the embryonic period if PPARγ is missing from the germline (Barak et al. 1999). However, the functions of PPARγ have been studied in several mice with tissue-specific knockout of PPARγ. The critical role of PPARγ in adipocyte differentiation was revealed in mice with non-placental PPARγ deletion. Those mice were not able to generate differentiated adipocytes (Barak et al. 1999). Furthermore, ectopic overexpression of PPARγ in fibroblasts promoted their conversion to adipocytes (Tontonoz, Hu & Spiegelman 1994). On the other hand, a macrophage –specific deficiency of PPARγ resulted in clear signs of insulin resistance, which proofs that PPARγ has an important role in the regulation of insulin sensitivity (Hevener et al. 2007).

The action of PPARγ is dependent not only on the expression of PPARγ and the presence of its ligand, but also on the recruitment of cofactors and the removal of corepressors from the transcription site. There are many cofactors including p300/CBP, p160, PGC-1, thyroid hormone receptor-associated protein 220, PPARγ binding protein, androgen-receptor-associated protein and PPARγ interacting protein, whereas silencing mediator for retinoic and thyroid hormone receptors, nuclear receptor corepressor (N-CoR) and histone deacetylase enzymes can act as corepressors of PPARγ. In addition, as in other nuclear receptors, PPARγ can also

influence gene expression via transrepression, i.e. by physically interfering with the action of another transcription factor (Costa et al. 2010). This complex regulation open up the possibility that distinct PPARγ ligands may be able to induce unique gene expression profiles in a tissue-specific manner (Rogue et al. 2010).

The mechanisms by which TZDs, and PPARγ activation increase the insulin sensitivity are not known. However, adipose tissue is usually considered as the primary target of TZDs. TZDs induce adipogenesis in subcutaneous adipose tissue. In addition, PPARγ activation increases the transcription of genes involved in fatty acid transport and storage (Sears et al. 2007, Sears et al. 2009). It has been speculated that the increased ability to uptake and store lipids would allow lipids to be transferred from skeletal muscle and liver into subcutaneous adipose tissue (Ye et al. 2004). The decreased fat content in skeletal muscle and liver is one way to improve their insulin sensitivity. Furthermore, the fat in visceral adipose tissue is moved to subcutaneous adipose tissue since the lipolytic activity of subcutaneous adipose tissue is lower than that of visceral adipose tissue (Wang 2010). However, TZDs can increase the glucose uptake also through direct mechanisms. Thus, TZDs can increase the expression of glucose transporters types 4 and 1 at the plasma membrane as well as the insulin-induced phosphorylation of IRS-1 and thus improve the insulin-mediated glucose uptake of cells (Monsalve et al. 2013, Eldor, DeFronzo & Abdul-Ghani 2013).

In addition to their effects on energy metabolism, TZDs also suppress inflammation. In adipocytes, TZDs have been reported to inhibit the production of several proinflammatory adipokines like resistin and retinol binding protein 4, and cytokines, like IL-6 and TNF, as well as to increase the secretion of adiponectin (Yamauchi et al. 2001, McTernan et al. 2002, Kolak et al. 2007, Esterson et al. 2013, Westerink, Visseren 2011). Adiponectin has been shown to increase fatty acid oxidation in the liver and skeletal muscle, to improve insulin sensitivity and to decrease glucose production in the liver (Monsalve et al. 2013). On the other hand, PPARγ agonists can modify the function of macrophages directly by shifting the polarization of macrophages towards alternative phenotype (Bouhlel et al. 2007, Odegaard et al. 2007) and PPARγ ligand treatment induced the migration of alternatively activated macrophages to adipose tissue in mice (Stienstra et al. 2008). At the systemic level, PPARγ agonists have been reported to reduce plasma levels of CRP and MCP-1 in patients with type II DM (Mohanty et al. 2004, Pfutzner et al. 2005) as well as those of both CRP and IL-6 in patients with metabolic syndrome without diabetes (Esposito et al. 2006). The extent of the inflammation and subsequent remodelling process in atherosclerotic vessels was also attenuated.

PPARγ agonists reduced vascular smooth muscle cells proliferation and suppressed the production of MMP-9 and MCP-1 in atherotic plaques (Marfella et al. 2006, Tikellis et al. 2008, Yoshimoto et al. 1999). In type II DM patients, the carotid intima-media thickness was also reduced after 26 weeks' pioglitazone treatment (Pfutzner et al. 2005). PPARγ has increased the expression of CD36, a scavenger receptor that mediates the oxidized LDL uptake into macrophages. On the other hand, PPARγ also increased the expression of HDL receptor CLA-1 and liver X receptor thus promoting lipid efflux from the body (Takano, Komuro 2009, Hamblin et al. 2009, Prieur, Roszer & Ricote 2010). In endothelial cells, PPARγ ligands have inhibited the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), two molecules that help leukocytes to adhere endothelial cells at the site of inflammation (Jackson et al. 1999, Pasceri et al. 2000). A PPARγ ligand -induced reduction of levels of VCAM-1 and ICAM-1 was also detected in serum of patients with type II DM and hypertension (Takase et al. 2007).

PPARγ ligands have been demonstrated to possess anti-inflammatory effects also in other types of inflammation. Rosiglitazone, pioglitazone and PPARγ agonist THR0921 have attenuated Freund's adjuvant-induced arthritis in mice (Tomita, Kakiuchi & Tsao 2006, Shiojiri et al. 2002, Cuzzocrea et al. 2003). In mice, troglitazone reduced the inflammation and symptoms of a model of autoimmune encephalomyelitis (Niino et al. 2001) and colitis (Su et al. 1999). Furthermore, ciglitazone has downregulated the symptoms and remodelling in a mouse model of asthma (Honda et al. 2004).

3.2.3 PPARβ/δ

The functions of PPARβ/δ are less well known than those of other PPAR isotypes. Although it is known that PPARβ/δ agonists decrease cholesterol levels and increase insulin sensitivity, the molecular mechanisms behind the effects are unknown. However, it has been proposed that the action of PPARβ/δ in skeletal muscle plays a central role. PPARβ/δ increases fatty acid catabolism, cholesterol efflux and oxidative capacity in the muscle. The loss of PPARβ/δ causes almost complete embryonic lethality in mice (Barak et al. 2002) and surviving PPARβ/δ knockout mice are more prone to obesity than their wild-type counterparts (Wang et al. 2003). In contrast, the specific activation of PPARβ/δ selectively in adipose tissue reduced the adiposity in the mice and the animals were resistant to a high-fat diet (Wang et al. 2003). In macrophages, the removal of PPARβ/δ has been reported to attenuate

expressions of MCP-1 and IL-1β whereas overexpression of PPARβ/δ enhanced inflammatory responses (Hamblin et al. 2009).

AIMS OF THE STUDY

Several findings emerging during the last decade have demonstrated that metabolic and inflammatory pathways share several components and are regulated by mutual factors. Although the functions of PPARs in metabolism have been well characterized there are very few published observations of the potential connection of PPARs to inflammation. The aim of the present study was to investigate the potential therapeutic action of PPAR agonists in inflammatory responses. Macrophages were the main focus of interest since these cells are clearly involved in both metabolic and rheumatoid inflammation.

The detailed aims of the present study were:

I) To investigate whether PPAR agonists could modulate the classical and/or alternative pathways of macrophage activation

II) To characterize the anti-inflammatory potential of PPARα/γ agonist muraglitazar in macrophage activation *in vitro* and in acute inflammation *in vivo*

III) To evaluate if muraglitazar would represent an effective drug for incorporation into a drug-eluting bioabsorbable stent

MATERIALS AND METHODS

1 Materials

GW1929, GW7647 and MG132 were from Tocris Bioscience (Bristol, UK) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) from Calbiochem (San Diego, CA, USA). Rabbit polyclonal iNOS, ac-p65 (Lys310), p-p65 (Ser536), PPARγ, STAT1α p91, STAT6, β-actin and lamin A/C and goat polyclonal arginase 1 as well as goat horseradish peroxidase (HRP)-conjugated anti-rabbit and donkey HRP-conjugated anti-goat polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal NF-κB subunit p65 antibody was from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit polyclonal pSTAT6 (Tyr641) antibody was from Calbiochem (Merck Millipore, Billerica, MA, USA) and rabbit polyclonal PPARα antibody from Alexis Biochemicals (Enzo Life Sciences, Lausen, Switzerland). Polylactic acid (P(L/D)LA 96/4), which is a copolymer of 96% of L-lactide and 4% of D-lactide was from Purac Biochem bv (Gorinchem, The Netherlands). P(L/D)LA 50/50 was from Boehringer Ingelheim (Ingelheim, Germany). Recombinant mouse IL-4 and IL-13 and recombinant human IL-4 were from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (*Escherichia coli* origin) and all other reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA).

Muraglitazar was synthesized in the laboratory of Dr. Paula H. Aulaskari (University of Eastern Finland, Joensuu Campus, Joensuu, Finland) by a five-step procedure according to Devasthale et al (Devasthale et al. 2005). The structure and purity of intermediates and the final product muraglitazar were confirmed by melting point analysis (Gallenkamp melting point apparatus MFB-595), 1H, 13C nuclear magnetic resonance spectroscopy (Bruker Avance 250 MHz and 400 MHz spectrometer) using deuterium chloroform as the solvent and tetramethylsilane as the reference, and by infrared spectroscopy (Nicolet Avatar 320 fourier transform infrared spectrometer) using dry potassium bromide as the salt component of solid mixture. The molecular structure and the purity of muraglitazar were also confirmed by mass spectrometer and elemental analysis. The mass spectrometry measurements were performed on Bruker BioAPEX II 47e Fourier transform ion cyclotron resonance mass spectrometry (Bruker Daltonics, Billerica, MA, USA) equipped with

an InfinityTM cell, 4.7 Tesla 160-mm-bore superconducting magnet (Magnex Scientific Ltd., Abingdon, UK), and an external electron ionization or electrospray ion source (Analytica of Branford Inc., Branford, CT, USA). Elemental analysis was performed on CE Instruments EA 1110 elemental analyser. The results of elemental analysis were within ±0.2% of the theoretical values.

2 Cell culture

Human THP-1 promonocytes (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in 5% CO_2 atmosphere in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza Group Ltd, Basel, Switzerland) adjusted to contain 2 mM L-glutamine (Lonza Group Ltd), 10 mM HEPES (Lonza Group Ltd), 4.5 g/l glucose (Sigma-Aldrich Co.) and 1.5 g/l bicarbonate (Lonza Group Ltd) and supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich Co.). The cells were differentiated by adding phorbol ester 12-O-tetradecanoylphorbol-13-acetate (100 nM) (Sigma-Aldrich Co.) at the time of seeding onto plates. 72 h after the seeding, the cultures were confluent and experiments were started.

Murine J774 macrophages (American Type Culture Collection) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Lonza Group Ltd) supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co.) and harvested with trypsin-ethylenediaminetetraacetic acid (Invitrogen Co.).

J774 cells with luciferase reporter under the control of murine iNOS promoter was created by stable transfection of J774 cells with pGL4(miNOS-prom)neo using Lipofectamine 2000 (Invitrogen Co.) according to the manufacturer's instructions. The pGL-MNOS II-5'-Luc plasmid (Kleinert et al. 1996) containing the 5'-flanking sequence (1171 bp, positions -1570 to +141; promoter and a part of exon 1) of the murine iNOS gene was provided by Professor Harmut Kleinert (Johannes Gutenberg University, Mainz, Germany). This plasmid was digested with KpnI and HindIII, and the restriction fragment, containing murine iNOS promoter and part of exon 1, was then cloned into the KpnI/HindIII site of firefly luciferase reporter plasmid pGL4.17(luc2/neo) (Promega, Madison, WI, USA) generating

pGL4(miNOS-prom)neo, in which the luciferase gene is driven by a murine iNOS promoter. The plasmid was sequenced to confirm the appropriate size, position and orientation of the insert in the plasmid. Transfected cells were selected with G418 disulfate salt (Sigma-Aldrich Co.) treatment (800 μg/ml). After the selection, the surviving clones were pooled to give rise to J774-pGL4(miNOS-prom)neo cell line and further cultured in the presence of 400 μg/ml of G418.

Human HEK293 cells (American Type Culture Collection) were cultured at 37°C in 5% CO_2 atmosphere in Eagle's minimal essential medium (Lonza Group Ltd) supplemented with 0.15% sodium bicarbonate, 100 μM non-essential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co.) and harvested with trypsin-ethylenediaminetetraacetic acid (Invitrogen Co.).

HEK293 cell line with NF-κB reporter plasmid was created by stable transfection of the cells with pGL4.32[luc2P/NF-κB-RE/Hygro] (Promega) using Lipofectamine 2000 (Invitrogen Co.) according to the manufacturer's instructions. Transfected cells were selected with hygromycin B (EMD Biosciences Inc., La Jolla, CA, USA) treatment (200 μg/ml). After the selection, the surviving clones were pooled to give rise to HEK293- pGL4.32[luc2P/NF-κB-RE/Hygro] cell line and further cultured in the presence of 100 μg/ml of hygromycin B.

Cells were seeded on 24-well plates for RNA extraction and nitrite and ELISA measurements, on 24-well plates or 6-well plates for preparation of cell lysates for western blotting, on 10 cm dishes for the preparation of nuclear extracts for western blotting and the NF-κB p65 DNA binding assay and on 96-well plates for the XTT test. Confluent cultures were exposed to fresh culture medium containing the compounds of interest. The drug compounds used were dissolved in dimethylsulfoxide, aliquoted and stored at -20°C before their use. An equal volume of solvent was included in all cell culture incubations and the final concentration of dimethylsulfoxide at cell culture incubation conditions was 1‰. When the effects of PLA fibres were examined, the cells were seeded onto PLA fibres plated in the cell culture wells and the compounds of interest were added to the culture medium 72 h after the seeding of the cells.

Cell viability after treatment with combinations of LPS or cytokine mixture and the tested compounds was assessed by the modified XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) test (Cell proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

3 Preparation of protein extracts

Preparation of cell lysates

At the indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-base, pH 7.4, 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 minutes, lysates were centrifuged (13 400 g, 4°C, 10 min), supernatants were collected and mixed 3:1 with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analysed. The coomassie blue method was used to measure the protein content of the samples (Bradford 1976).

Preparation of nuclear extracts

At the indicated time points, the cells were rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM NaF and 0.1 mM ethylene glycol tetraacetic acid). After incubation on ice for 10 minutes, the cells were vortexed for 30 seconds and the nuclei were separated by centrifugation at 4°C, 21 000 g for 10 seconds. Nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM NaF and 0.1 mM ethylene glycol tetraacetic acid) and incubated on ice for 20 minutes. Nuclei were vortexed for 30 seconds and nuclear extracts were obtained by centrifugation at 4°C, 21 000 g for 2 min. Supernatants were collected and mixed 3:1 with SDS sample buffer. The samples were stored at -70°C until analysed. The coomassie blue method was used to measure the protein content of the samples (Bradford 1976).

4 Western blotting

Prior to western blotting, samples were boiled for 10 minutes and 20 μg of protein was loaded per lane onto 8%, 10% or 12% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After the transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk or 5% bovine serum albumin at room temperature for 1 h and incubated overnight with primary antibody in the blocking solution at 4°C. The membrane was washed with TBS/T, incubated with secondary antibody in the blocking solution at room temperature for 1 hour and washed. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) by FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA) or ImageQuant LAS 4000 mini imaging system (GE Healthcare). The chemiluminescent signal was quantified with FluorChem software version 3.1 or ImageQuant TL 7.0 image analysis software, respectively.

5 RNA extraction and RT-qPCR

At the indicated time points, culture medium on cell line cells was removed and total RNA of the cells was extracted with GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.). In the luciferase mRNA experiments, total RNA was treated with DNase I (Fermentas UAB, Vilnius, Lithuania). 100 ng of total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the reverse transcription reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

The total RNA of the tissue samples was extracted by GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.) with proteinase K digestion. 500 ng of total RNA was reverse-transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit (Fermentas UAB). cDNA obtained from the reverse transcription reaction was diluted 1:10 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes (Table 3) for arginase 1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-6, iNOS and luciferase were designed using Express Software (Applied Biosystems) and purchased from Metabion international AG (Planegg, Germany). The optimization of the primers and probes was performed according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C.

The expression of mouse fizz1, mannose receptor 1, PPARα, PPARγ and TNF and human CCL13 and PDGF mRNA was measured by using TaqMan® Gene Expression Assays (Mm00445109_m1, Mm00485148_m1, Mm00440939_m1, Mm01184322_m1, Mm00443260_g1, Hs01033504_g1, Hs00966522_m1, Applied Biosystems).

The PCR cycling conditions were: incubation at 50°C for 2 min, 95°C for 10 min, thereafter 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Each sample was determined in duplicate. The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2 (arginase 1, iNOS, luciferase) or ΔCt method (TaqMan® Gene Expression Assays).

Table 3. Primer and probe sequences

Gene	Oligonucleotide	Sequence 5' → 3'
Human GAPDH	Forward primer	TCCTACCACCAGCAACCCTGCCA
	Reverse primer	GCAACAATATCCACTTTACCAGAGTTAA
	Probe	CGCCTGGTCACCAGGGCTGC
Luciferase	Forward primer	ACGGCTTCGGCATGTTCA
	Reverse primer	CTCCTCCTCGAAGCGGTACA
	Probe	TTGATCTGCGGCTTTCGGGTCGT
Mouse arginase 1	Forward primer	TCCAAGCCAAAGTCCTTAGAGATTAT
	Reverse primer	CGTCATACTCTGTTTCTTTAAGTTTTTCC
	Probe	CGCCTTTCTCAAAAGGACAGCCTCGA
Mouse GAPDH	Forward primer	GCATGGCCTTCCGTGTTC
	Reverse primer	GATGTCATCATACTTGGCAGGTTT
	Probe	TCGTGGATCTGACGTGCCGCC
Mouse IL-6	Forward primer	TCGGAGGCTTAATTACACATGTTC
	Reverse primer	CAAGTGCATCATCGTTGTTCATAC
	Probe	CAGAATTGCCATTGCACAACTCTTTTCTCA
Mouse iNOS	Forward primer	CCTGGTACGGGCATTGCT
	Reverse primer	GCTCATGCGGCCTCCTT
	Probe	CAGCAGCGGCTCCATGACTCCC

6 Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples were stored at -20°C until assayed. The concentrations of CCL13, MCP-1, TNF and mouse IL-6 in culture medium were determined by ELISA according to the manufacturer's instructions using reagents from R&D Systems Europe (Abingdon, UK). Human IL-6 was analysed using reagents from Sanquin (Amsterdam, The Netherlands; PeliPair ELISA) or eBioscience (San Diego, CA, USA).

7 Nitrite Assay

Nitric oxide production was determined by measuring the accumulation of nitrite, a stable metabolite of nitric oxide in aqueous conditions, into the culture medium by a method developed by Green et al. (Green et al. 1982). Briefly, culture medium samples were collected at indicated time points, the Griess reagent (0.1% napthalethylenediamine dihydrochloridine, 1% sulfanilamine, 2.4% H_3PO_4) was added to the samples and the amount of reaction product was determined by measuring the absorbance at 540 nm. The concentration of nitrite was calculated with sodium nitrate as the standard.

8 NF-κB binding assay

DNA binding activity of NF-κB p65 was evaluated using NF-κB (p65) Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The nuclear extracts for the assay were prepared according to the manufacturer's instructions and 10 μg of nuclear protein per well was used for the experiment. The samples were incubated at 4°C overnight with the dsDNA templates carrying the NF-κB response element. After primary (anti-NF-κB p65) and secondary (goat anti-rabbit HRP) antibody treatments, developing reagents were added and absorbance was read at 450 nm.

9 Carrageenan-induced inflammation in mice

Prior to the experiment investigating the time dependent formation of carrageenan-induced paw oedema, 36 male Charles River mice (weighing 25.0-30.0 g) were housed and cared for according to the guidelines of the institutional animal care and use committee with food and water provided *ad libitum*. The study was approved by Animal Ethic Committee, Universiti Sains Malaysia.

The animals were randomly divided into six groups (six mice per group) and each group was treated orally with the test compound suspended in 2.0% (w/v) carboxymethyl cellulose in distilled water. The test groups were as follows: control (treated with 2.0% carboxymethyl cellulose only), muraglitazar 12.5 mg/kg, muraglitazar 25 mg/kg, muraglitazar 50 mg/kg, L-NIL (N^6-(1-iminoethyl)-L-lysine hydrochloride) 50 mg/kg and dexamethasone 2 mg/kg. An hour after the treatment, 40 µl of carrageenan (1% suspension in normal saline) was injected subcutaneously into the left hind paws of the animals. The thicknesses of the paws were measured with a micrometre one hour before and 1 h, 4 h and 6 h after the carrageenan injection. The results are expressed as percentages of swelling calculated as follows:

Difference = (thickness of the hind paw at indicated time point – thickness of the hind paw before carrageenan) / thickness of the hind paw before carrageenan.

The carrageenan-induced gene expression was investigated in C57BL/6 mice. The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective provincial committee for animal experiments. Animals were housed under standard conditions of light, temperature and humidity (12:12 h light-dark cycle, 22±1°C, 50-60%) with food and water provided *ad libitum*.

Mice were randomly divided into two study groups with six mice in both groups and muraglitazar 50 mg/kg was injected intraperitoneally into one of the groups. Two hours after the treatment, the mice were anesthetized with an intraperitoneal injection of 0.5 mg/kg of medetomidine (Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Pfizer Oy Animal Health, Helsinki, Finland), and 30 µl of λ-carrageenan (1.5% suspension in normal saline) was injected subcutaneously into a hind paw. As a control, 30 µl of saline was injected into a contralateral paw. After six hours of carrageenan injection, the mice were sacrificed by cervical dislocation. Carrageenan-treated and control paws were skinned and the soft tissues of the paws were collected for RNA extraction.

10 Processing fibre and stents

The self-expandable biodegradable vascular stents were designed and manufactured in the Institute of Biomaterials, Tampere University of Technology, Tampere, Finland. The stent polylactic acid (PLA) (96/4) fibre was manufactured by extrusion with a single screw extruder (Extrudex, Mühlacker, Germany). The extrusion was followed by a drawing process to create a fibrillated monofilament. The final diameter of the fibre was 0.2 mm. Next, 16 fibers were braided into tubular form onto a mandrel (diameter of 6.0 mm) using one-over-one –type of braid with 110° braiding angle (Pick Master, J.B.Hyde & Code. Ltd., Cheshire, England). After braiding, the stents were heat-treated for 10 min at 110°C to stabilize the braided structure. The heat-treated braid was manually cut into 15 mm long stents.

Stents were coated using the solvent coating method as described earlier (Mikkonen et al. 2009). Briefly, coating polymer (PLA 50/50) and the drug (44.13 wt-%) were dissolved in acetone, and the stents were supported with a metallic mandrel and immersed into the polymer-drug solution. Polymers were coated twice to achieve desired amount of the drug in the coating. Control stents were coated with the coating polymer only. Finally, stents were sterilized using gamma irradiation of >25 kGy (BBF Sterilisationsservice GmbH, Kernen, Germany).

11 Measurement of chemical and physical properties of stents

Drug content of the stents was calculated by weighing the stent before and after coating. Drug release from the stents was studied by incubating the stents in PBS at 37°C under gentle agitation or in RPMI 1640 medium (Lonza Group Ltd) at 37°C in humidified 5% CO_2 atmosphere. PBS or cell culture medium was renewed at desired time-points, and the amount of the drug in the collected PBS or cell culture medium was measured by high-performance liquid chromatography (HPLC) at wavelength of 280 nm (Agilent 1100 series HPLC system containing pump, autosampler and adjustable wavelength UV detector, Agilent Technologies, Germany) as described by Li et al. (Li et al. 2006). Samples were injected into Interchrom C18 column (Interchim SA, Montluçon, France) preceded by Chromolith Guard Column (Merck KGaA, Darmstadt, Germany) and run through mobile phase consisting of acetonitrile (55%) and trifluoroacetic acid (0.06%) in water. Peak for muraglitazar appeared at the retention time of 9.1 min, and the

amount of the drug in the sample was calculated against standard curve prepared for each run.

The effects of hydrolysis on the mechanical properties of the stents were examined in PBS with pH 7.40±0.2. The radial pressure stiffness of the stents was measured by a loop test using Instron 4411 Materials tester (Instron Ltd., High Wycombe, UK) with a cell of 500N. Stents were placed inside the plastic collar, which radially compressed the stent. Distance of the grips was 100 mm, and the collar was pulled with a crosshead speed of 50 mm/min. Radial pressure stiffness was calculated from the load/displacement curve as described earlier (Nuutinen et al. 2003).

12 Statistics

Results are expressed as mean +/± standard error of mean (SEM). When indicated, statistical significance was calculated by analysis of variance followed by Dunnett's multiple comparisons test or unpaired t test with Welch correction. All the statistical analyses were performed using GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p<0.05$.

SUMMARY OF THE RESULTS

1 J774 and THP-1 macrophages express PPARs and are able to represent classical and alternative activation phenotypes

Murine J774 macrophages were able to express both classical and alternative phenotypes when stimulated with LPS or the combination of IL-4 and IL-13, respectively. The activation of the macrophages with LPS increased the levels of markers linked to classical activation, including nitric oxide, iNOS (Figure 5), TNF and IL-6 whereas costimulation with IL-4 and IL-13 induced the production of alternative activation markers arginase 1, fizz1 and mannose receptor 1 (Figure 6).

Similarly to J774 cells, human THP-1 macrophages also expressed the classical phenotype when exposed to LPS as shown by the increased production of TNF and IL-6. In addition, stimulation with IL-4 increased the mRNA levels of markers linked to alternative activation in human macrophages, CCL13 and PDGF (Figure 7).

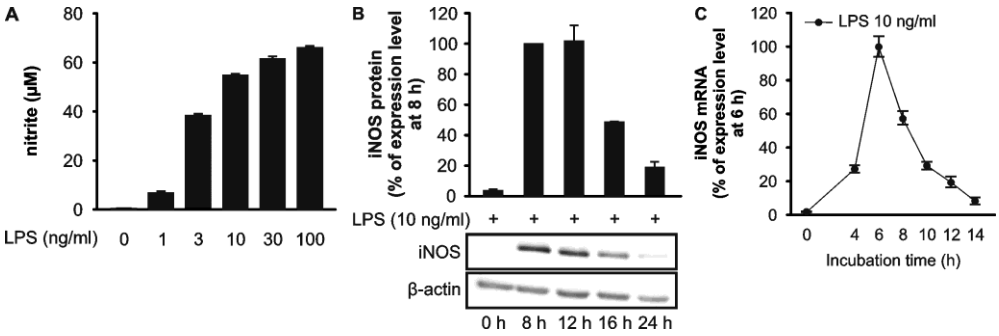

Figure 5. LPS induces nitric oxide production and iNOS expression in J774 macrophages. A. Cells were stimulated with LPS for 24 hours and nitrite, a stable metabolite of nitric oxide, concentrations in culture medium was analysed by Griess reaction. B. Cells were stimulated with LPS for the indicated periods of time and the levels of iNOS protein were analysed by western blotting. β-actin was used as a loading control. C. Cells were stimulated with LPS for the indicated periods of time and the levels of iNOS mRNA were analysed by RT-qPCR. The results were normalized against GAPDH mRNA. Values represent the mean +/± SEM (n=3-4).

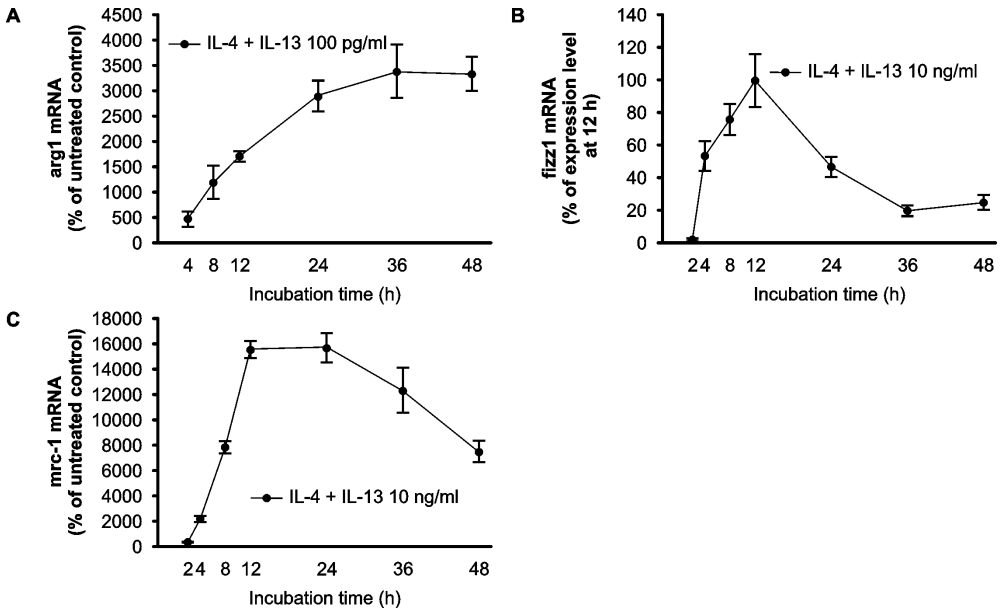

Figure 6. Time-dependent expression of alternative activation markers in J774 macrophages. The cells were stimulated with IL-4 and IL-13 for the indicated periods of time and the levels of arginase 1 (arg1) (A), fizz1 (B) and mannose receptor 1 (mrc-1) (C) mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean ± SEM (n=4). (Paukkeri et al. 2015, Immunometabolism 2:1-11)

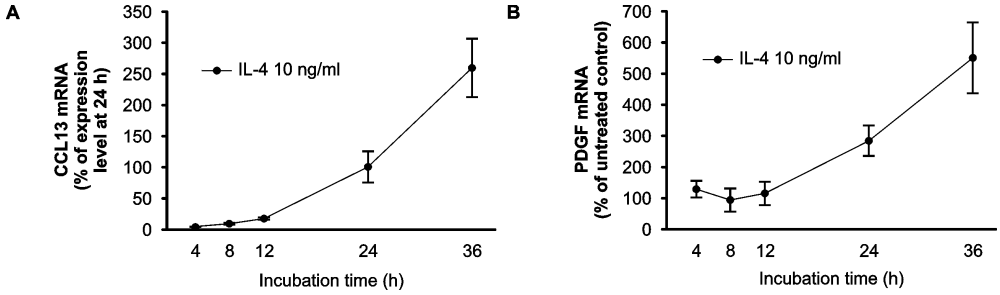

Figure 7. Time-dependent expression of alternative activation markers in THP-1 macrophages. Cells were stimulated with IL-4 for indicated periods of time and the levels of CCL13 (A) and PDGF (B) mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean ± SEM (n=4). (Paukkeri et al. 2015, Immunometabolism 2:1-11)

Both murine J774 macrophages and human THP-1 macrophages expressed PPARα and PPARγ (Figure 8).

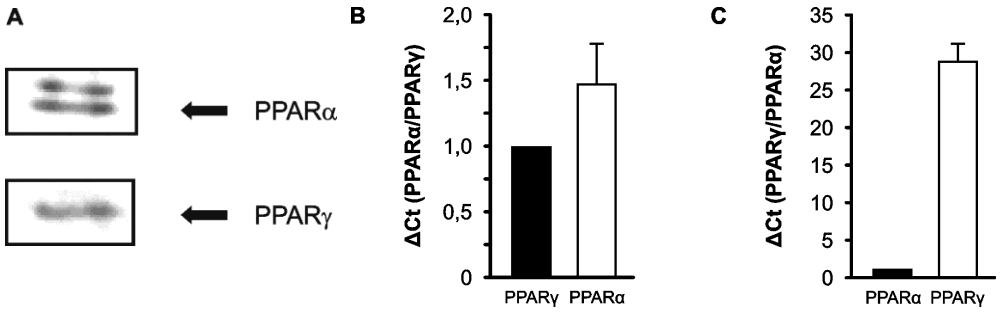

Figure 8. The expression of PPARα and PPARγ in J774 and THP-1 macrophages. A. Proteins of J774 cells were extracted and PPARα and PPARγ protein expression was determined by western blotting. The gels shown are representatives of three other experiments with similar results. B-C. Total RNA of J774 cells (B) and THP-1 cells (C) was extracted and the levels of PPARα and PPARγ mRNA were analysed by RT-qPCR. The results were normalized against GAPDH mRNA. Values represent the mean + SEM (n=4-6). (Paukkeri et al. 2015, Immunometabolism 2:1-11, modified)

2 PPAR agonists modify the activation of macrophages

In the present study, GW7647, WY14643 and fenofibrate were used as selective PPARα agonists and 15d-PGJ_2 and GW1929 as selective PPARγ agonists to evaluate the effects of PPARs on macrophage activation. The effects of a dual PPARα/PPARγ agonist muraglitazar were also investigated. The toxicity of these agonists was evaluated in the XTT test and they were found to be non-toxic.

2.1 The effects of PPAR agonists on classical activation of macrophages

The PPARα agonists GW7647, WY14643 and fenofibrate decreased nitric oxide production and iNOS expression (Figure 9a-d) in J774 macrophages, which were stimulated with LPS for 24 hours. In addition, although LPS-induced IL-6 production was attenuated (Figure 9e), TNF production was not reduced (Figure 9f) by PPARα agonists. The effects of PPARα agonists were compared to those of PPARγ agonists and similarly to PPARα agonists, also the PPARγ agonists 15d-PGJ_2 and GW1929 and the PPARα/γ agonist muraglitazar decreased LPS-induced iNOS expression and nitric oxide and IL-6 production (Figure 10a-e). Contrary to PPARα agonists, also TNF production was reduced (Figure 10f).

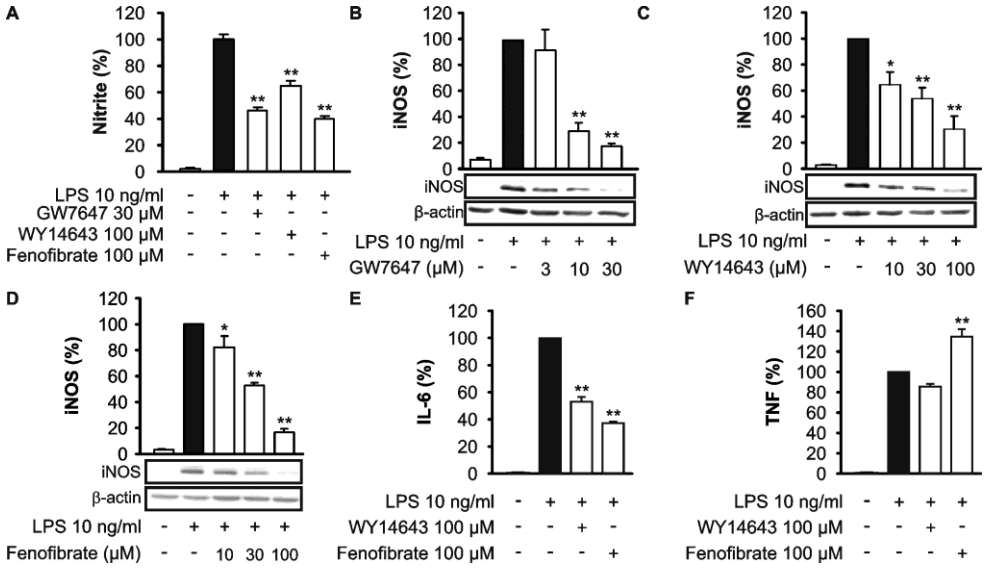

Figure 9. The effects of PPARα agonists on markers of classical activation in J774 macrophages. Cells were stimulated with LPS and treated with the PPARα agonists for 24 hours. Nitrite, a stable metabolite of nitric oxide (A), IL-6 (E) and TNF (F) concentrations in culture medium were analysed by Griess reaction and ELISA, respectively. B-D The levels of iNOS protein in cell lysates were analysed by western blotting. β-actin was used as a loading control. Results represent the mean + SEM (n=4). * $p<0.05$ and ** $p<0.01$ as compared to cells treated with LPS alone. (Reprinted with permission from Paukkeri et al. 2007, Br J Pharmacol 152: 1081-1091 © John Wiley Sons Inc., modified; Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

Most of the known regulators of iNOS expression modify the expression at the transcriptional level (Korhonen et al. 2005, Pautz et al. 2010). Thus, the effects of PPAR agonists on iNOS mRNA levels were tested next. PPARγ agonists and muraglitazar attenuated the levels of LPS-induced iNOS mRNA (Figure 11b). Interestingly, PPARα agonists did not reduce iNOS mRNA expression (Figure 11a).

The different effects of PPARα and PPARγ agonists on iNOS mRNA levels might result either from reduced transcription of iNOS gene or increased decay of iNOS mRNA by PPARγ agonists. However, the stability of iNOS mRNA was not changed by PPAR agonists (Figure 12a). Thus, PPARα agonists and PPARγ agonists seemed to exert different effects on the transcription of the iNOS gene. This was further demonstrated by studying the effect of PPAR agonists on J774 cells that were stably transfected with the luciferase reporter gene under the control of an iNOS promoter. As shown in Figure 12b, the PPARγ agonist GW1929 and the PPARα/γ agonist muraglitazar both reduced the levels of luciferase mRNA, while the PPARα agonist fenofibrate had no effect indicating that PPARγ but not PPARα activation

could decrease the iNOS promoter-mediated transcription. NF-κB inhibitor MG132 was used as a positive control.

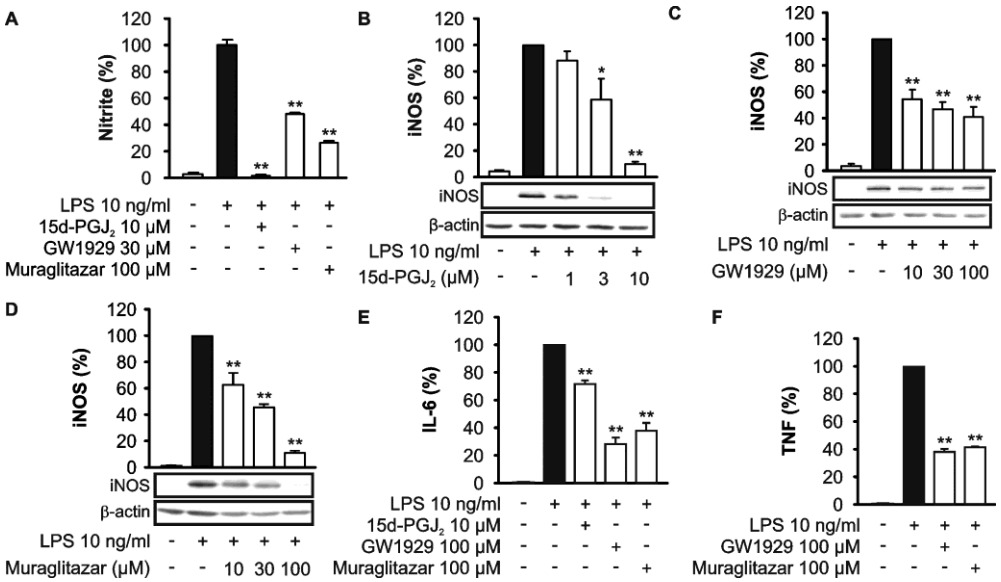

Figure 10. PPARγ agonists and PPARα/γ agonist muraglitazar reduce the expression of classical activation markers in J774 macrophages. Cells were stimulated with LPS and treated with PPARγ agonists or muraglitazar for 24 hours. Nitrite, a stable metabolite of nitric oxide (A), IL-6 (E) and TNF (F) concentrations in the culture medium were analysed by the Griess reaction and ELISA, respectively. B-D. The levels of iNOS protein in cell lysates were analysed by western blotting and β-actin was used as a loading control. Results represent the mean + SEM (n=4). * $p<0.05$ and ** $p<0.01$ as compared to cells treated with LPS alone. (Reprinted with permission from Paukkeri et al. 2007, Br J Pharmacol 152: 1081-1091 © John Wiley Sons Inc., modified; Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

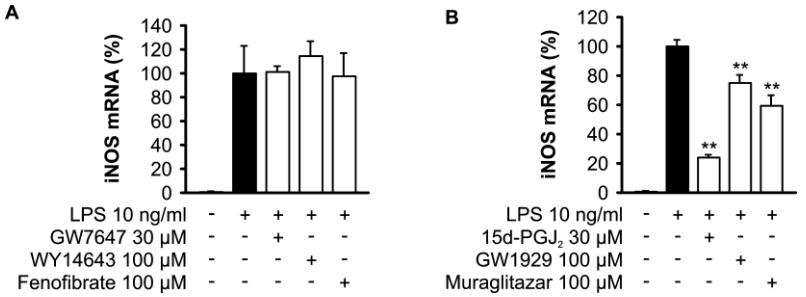

Figure 11. PPARα and PPARγ agonists have different effects on iNOS mRNA levels. J774 cells were stimulated with LPS and treated with PPAR agonists for 6 hours and the levels of iNOS mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). ** $p<0.01$ as compared to cells treated with LPS alone.

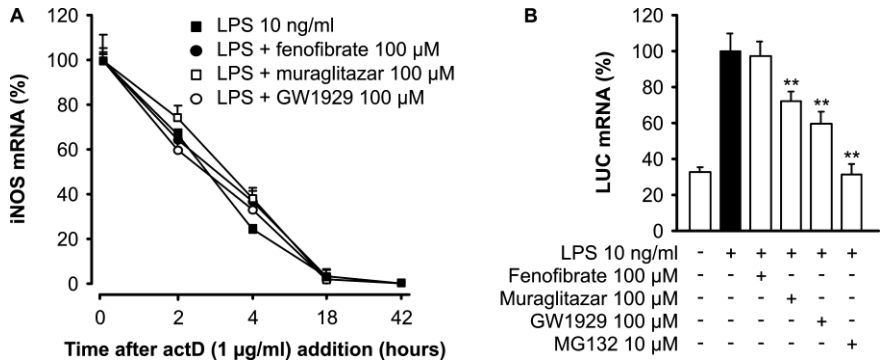

Figure 12. Effects of PPAR agonists on iNOS mRNA degradation and iNOS promoter activity. A. J774 cells were stimulated with LPS and treated with PPAR agonists. After 6 h incubation, actinomycin D was added to the cell culture to stop transcription and the incubations were terminated at the indicated time points. B. J774 cells that were stably transfected with the luciferase reporter gene under the control of iNOS promoter were incubated with LPS and pharmacological compounds for 6 hours. iNOS and LUC mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=3-4). ** $p<0.01$ as compared to cells treated with LPS alone. (Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

2.1.1 The effects of PPAR agonists on transcription factors

To understand the mechanism for the inhibitory action of PPARγ agonists, the effects of PPAR agonists on transcription factors were investigated. The NF-κB pathway is the best known transcription factor regulating both iNOS and IL-6 and thus the activation of this pathway was studied first. In J774 macrophages, the nuclear translocation of NF-κB starts during the first hour after the onset of LPS-stimulation and peaks at the 4 hour time point (Figure 13).

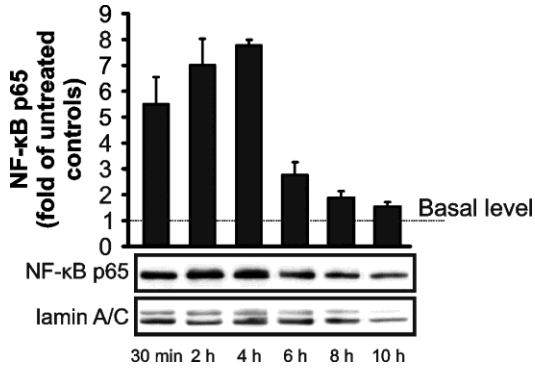

Figure 13. LPS-induced nuclear translocation of NF-κB in J774 macrophages. Cells were stimulated with LPS for the indicated periods of time and the nuclear levels of p65 were analysed by western blotting. The band density of LPS (10 ng/ml) -induced sample was compared to that of untreated sample at each time point. Lamin A/C was used as a loading control. The results represent the mean + SEM (n=3).

PPAR agonists did not reduce the nuclear translocation of NF-κB as indicated by the levels of p65 in nuclear extracts after both 30 min (Figure 14a) and 4 hour incubation with LPS. However, the transcriptional activity of p65 can also be modified by dephosphorylation or deacetylation (Huang, Hung 2013), and hence it was dedicated to examine the effects of PPAR agonists on the activation status of p65. As seen in Figure 14b-c, PPAR agonists did not reduce the levels of either phosphorylation or acetylation of p65.

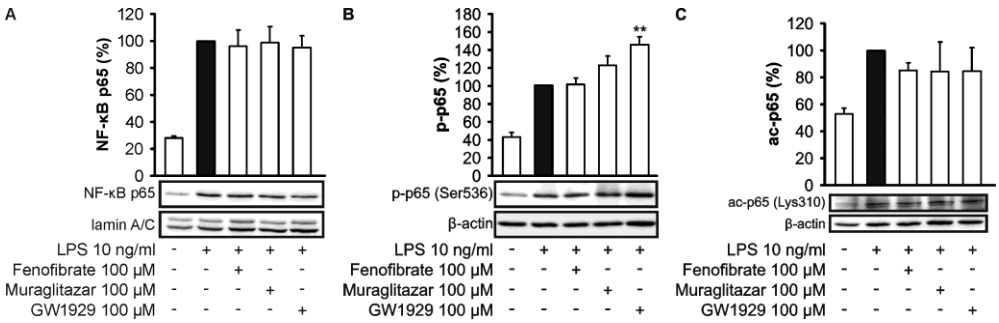

Figure 14. PPAR agonists do not inhibit NF-κB translocation or activation in J774 macrophages. A. Cells were preincubated with PPAR agonists for 2 h before addition of LPS for 30 min. The nuclear levels of p65 were analysed by western blotting. B. Cells were preincubated with PPAR agonists for 2 h before addition of LPS for 1 h. The levels of p-p65 in cell lysates were analysed by western blotting. C. Cells were stimulated with LPS and treated with PPAR agonists for 6 h and the levels of ac-p65 in cell lysates were analysed by western blotting. Lamin A/C or β-actin was used as a loading control. The results represent the mean + SEM (n=3-6). ** $p<0.01$ as compared to cells treated with LPS alone. (Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

Further, the effects of PPAR agonists on binding and transcription activity of NF-κB were studied. As anticipated, PDTC, an inhibitor of NF-κB, clearly reduced the DNA binding of NF-κB, but PPAR agonists did not exert any effects (Figure 15a).

The transcription activity of NF-κB was investigated in HEK293 cells that were stably transfected with a reporter gene carrying the luciferase reporter under an NF-κB-responsive promoter. Again, PPAR agonists did not reduce the levels of luciferase mRNA, in contrast to the positive control PDTC (Figure 15b).

Since STAT1 and MAPK transcription factors are also important regulators of iNOS (Pautz et al. 2010, Korhonen et al. 2005), the effects of PPAR agonists on STAT1 and MAPK activation were also investigated. However, PPAR agonists did not reduce either the translocation of STAT1α to nucleus or the phosphorylations of p38 or JNK (Figure 16).

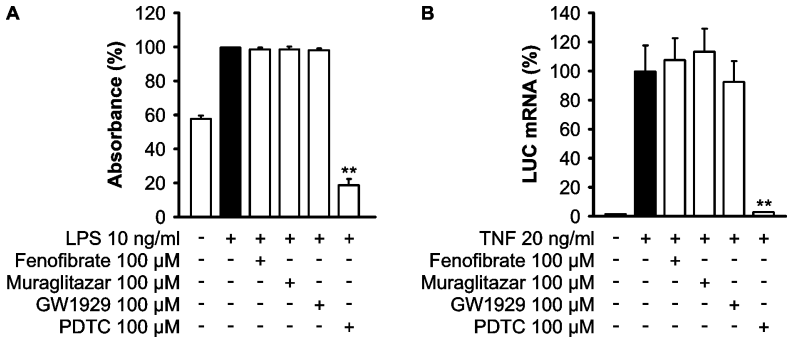

Figure 15. PPAR agonists do not reduce DNA binding or transcription activity of NF-κB. J774 cells (A) or HEK293 cells that were stably transfected with a reporter gene carrying the luciferase reporter under NF-κB-responsive promoter (B) were preincubated with pharmacological compounds for 2 h before addition of LPS or TNF. A. After 1 h incubation with LPS the binding activity of NF-κB p65 to DNA was analysed as described in the methods. B. After 6 hour incubation with TNF, the levels of LUC mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=3-4). **p<0.01 as compared to cells treated with LPS or TNF alone. (Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

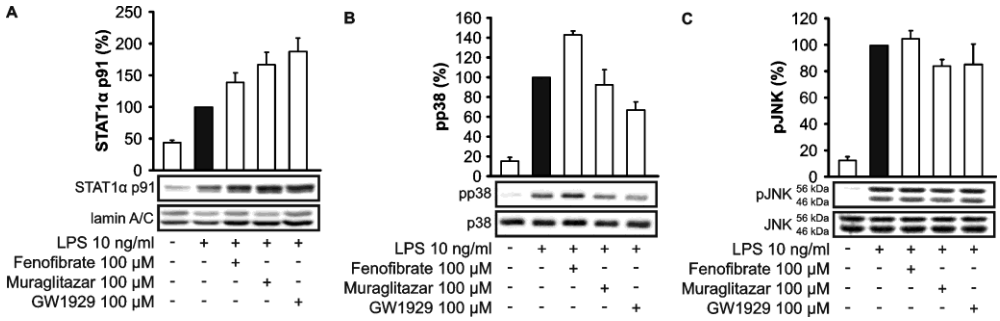

Figure 16. PPAR agonists do not reduce the activation of STAT1, p38 or JNK. A. J774 cells were incubated with LPS and PPAR agonists for 6 h and the nuclear levels of STAT1α were analysed by western blotting. B-C. J774 cells were preincubated with PPAR agonists for 2 h and LPS was added for 30 min. The levels of pp38 and pJNK in cell lysates were analysed by western blotting. Lamin A/C, p38 or JNK were used as a loading control. The results represent the mean + SEM (n=3).

2.1.2 The effects of PPAR agonists on proteasome

Since PPARα agonists reduced the levels of iNOS protein, but had no effect on iNOS mRNA levels, it was hypothesized that PPARα could be able to increase iNOS protein degradation. To evaluate this possibility, first the effects of PPARα on LPS-induced iNOS protein expression were studied after different incubation times. PPARα agonists reduced the protein expression in a time-dependent manner: the

reduction in protein levels obtained with PPARα agonists was much greater after 24 h incubation than after 8 h incubation (Figure 17). Furthermore fenofibrate and muraglitazar, in contrast to GW1929, were able to reduce iNOS protein levels when added to culture medium after a 10 h stimulus with LPS (Figure 18).

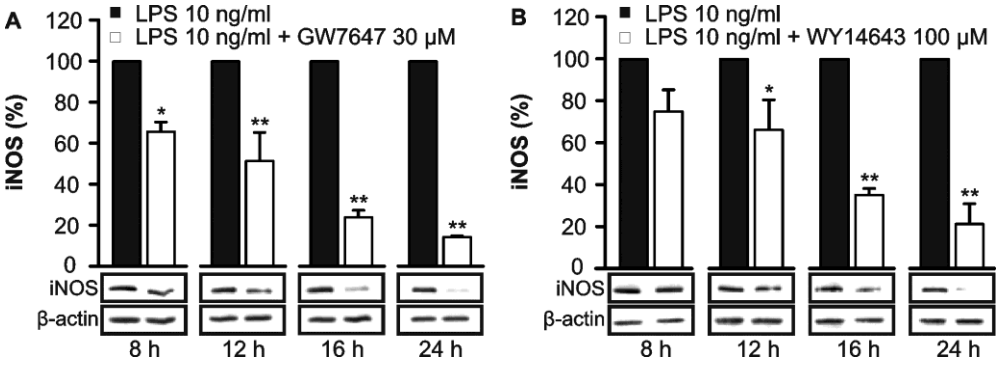

Figure 17. PPARα agonists reduce iNOS protein levels in a time-dependent manner. J774 cells were incubated with LPS and PPARα agonists for the indicated periods of time. The levels of iNOS protein in cell lysates were analysed by western blotting. At each time point, the band density of LPS–stimulated sample was set as 100% and the band with LPS+PPARα agonist treatment was compared to that. β-actin was used as a loading control. Results are expressed as mean + SEM (n=3). * p<0.05 and ** p<0.01 as compared to cells treated with LPS alone. (Reprinted with permission from Paukkeri et al. 2007, Br J Pharmacol 152: 1081-1091 © John Wiley Sons Inc., modified)

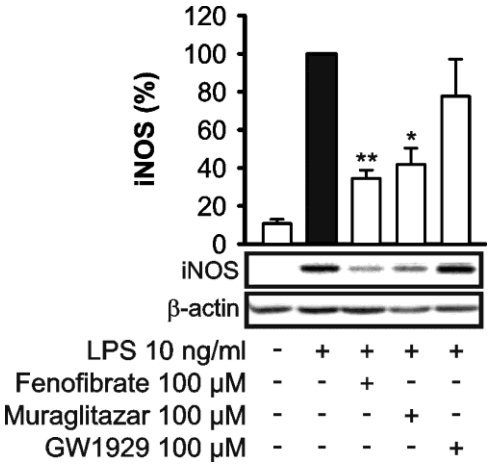

Figure 18. Posttranslational effects of PPAR agonists on iNOS protein expression. J774 cells were stimulated with LPS for 10 hours. After the stimulation, the culture medium was changed and the cells were further incubated with PPAR agonists or vehicle (dimethyl sulfoxide) without LPS for an additional 14 hours. The levels of iNOS protein in cell lysates were analysed by western blotting. β-actin was used as a loading control. Results are expressed as mean + SEM (n=3). * p<0.05 and ** p<0.01 as compared to cells treated with LPS alone. (Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

iNOS protein is known to be degraded by proteasome (Korhonen et al. 2005). To study if PPARα agonists could modify the action of the proteasome, a proteasome inhibitor, lactacystin, was used. Since proteasome is required for IκB degradation and NF-κB activation, lactacystin was added to cell culture 8 hours after the onset of LPS-stimulation and PPARα agonist treatment. The cells were harvested

after 24 h incubation. As anticipated, lactacystin increased iNOS protein levels in comparison to cells treated with LPS alone. However, the suppressing effect of PPARα agonists on iNOS levels virtually disappeared in the samples that were also treated with lactacystin (Figure 19). The results support the concept that PPARα agonists can reduce iNOS expression by accelerating iNOS protein degradation in the proteasome.

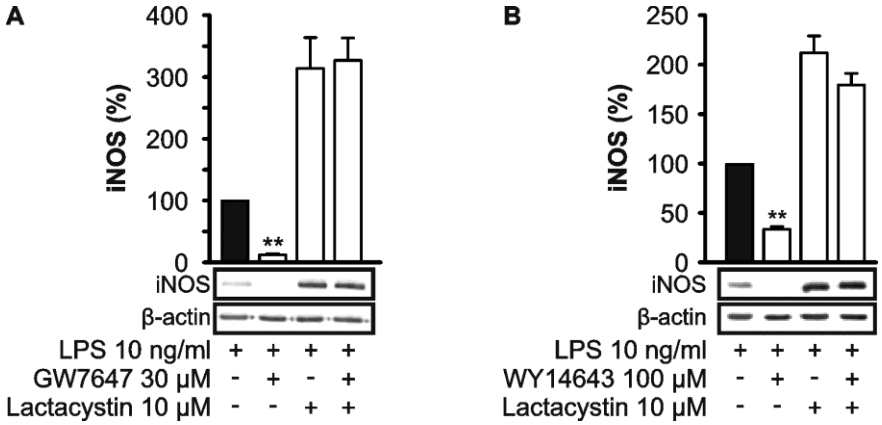

Figure 19. A proteasome inhibitor can abolish the effects of PPARα agonists on iNOS protein expression in J774 macrophages. Cells were incubated with PPARα agonists and LPS for 8 h. Lactacystin was added into the culture medium and the incubation was continued for an additional 16 h. The levels of iNOS protein in cell lysates were analysed by western blotting. The band density of LPS-stimulated sample was set as 100% and the bands with other treatments were compared to that. β-actin was used as a loading control. Results represent the mean + SEM (n=3). ** $p<0.01$ as compared to cells treated with LPS alone. (Reprinted with permission from Paukkeri et al. 2007, Br J Pharmacol 152: 1081-1091 © John Wiley Sons Inc., modified)

2.2 The effects of PPAR agonists on alternative activation of macrophages

In the previous chapter, both PPARα and PPARγ agonists were shown to reduce the classical activation of macrophages. Subsequently, the effects of PPAR agonists on mannose receptor 1, fizz1 and arginase 1 expression in IL-4 and IL-13 – stimulated J774 macrophages were studied to investigate the role of PPARs on alternative macrophage activation. The PPARγ agonist tended to induce the mRNA levels of all three markers (Figure 20), but, in contrast to the PPARγ agonist, PPARα agonists attenuated their mRNA levels (Figure 21).

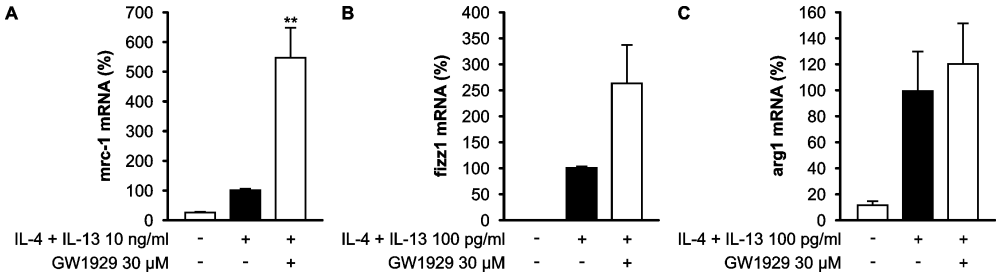

Figure 20. PPARγ agonist tends to increase the alternative activation markers in J774 macrophages. Cells were incubated with GW1929 and a combination of IL-4 and IL-13 for 24 h and the levels of mannose receptor 1 (mrc-1) (A), fizz1 (B) and arginase 1 (arg1) (C) mRNA were determined by RT-qPCR. The results were normalized against GAPDH. Results represent the mean + SEM (n=4). **p<0.01 as compared to cells treated with IL-4+IL-13 only. (Paukkeri et al. 2015, Immunometabolism 2:1-11)

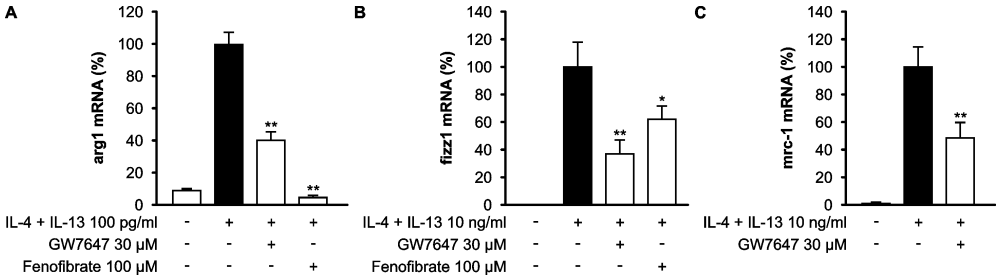

Figure 21. PPARα agonists attenuate the expression of alternative activation markers in J774 macrophages. Cells were incubated with PPARα agonists and a combination of IL-4 and IL-13 for 24 h and the levels of arginase 1 (arg1) (A), fizz1 (B) and mannose receptor 1 (mrc-1) (C) mRNA were determined by RT-qPCR. The results were normalized against GAPDH. Results represent the mean + SEM (n=4). *p<0.05 and ** p<0.01 as compared to cells treated with IL-4+IL-13 only. (Paukkeri et al. 2015, Immunometabolism 2:1-11)

The effects of PPARα agonists on alternative activation were confirmed in THP-1 cells and PPARα agonists were able to reduce the IL-4-induced levels of CCL13 and PDGF mRNA (Figure 22). Both CCL13 and PDGF are linked to alternative activation phenotype in human macrophages (Martinez et al. 2006, Murray, Wynn 2011).

Since STAT6 is an essential transcription factor for the alternative activation of macrophages, next the effects of PPAR agonists of STAT6 activation were studied. In J774 cells, STAT6 became maximally phosphorylated during the first hour after the onset of IL-4+IL-13 stimulus (Figure 23a). Neither the PPARα nor the PPARγ agonists were able to modify the extent of phosphorylation (Figure 23b-c) or nuclear translocation of STAT6 in J774 or THP-1 cells.

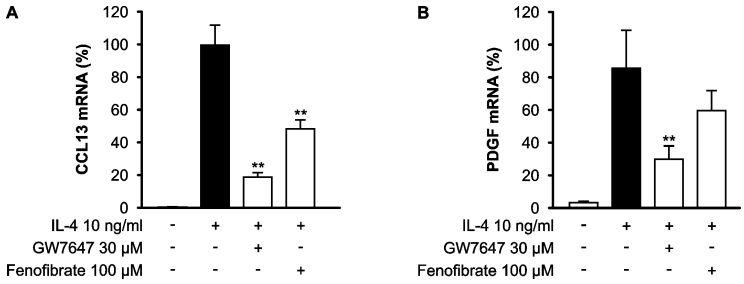

Figure 22. PPARα agonists attenuate the expression of alternative activation markers in THP-1 macrophages. Cells were incubated with PPARα agonists and IL-4 for 24 h and the levels of CCL13 (A) and PDGF (B) mRNA were determined by RT-qPCR. The results were normalized against GAPDH. Results represent the mean + SEM (n=4). ** p<0.01 as compared to cells treated with IL-4 alone. (Paukkeri et al. 2015, Immunometabolism 2:1-11)

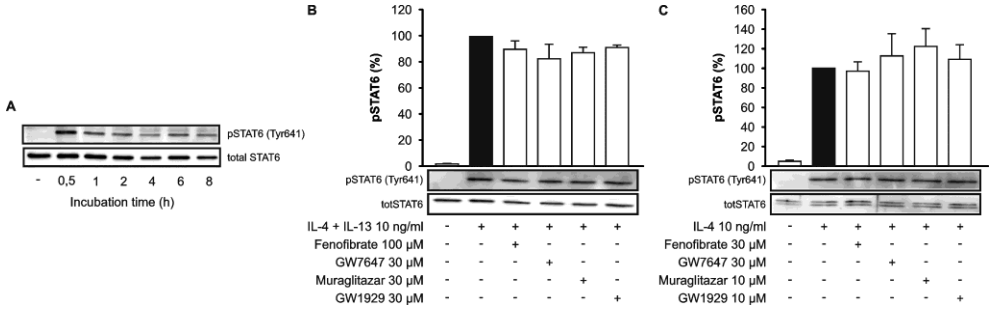

Figure 23. PPAR agonists do not modify the activation of STAT6. A. J774 cells were stimulated with IL-4 and IL-13 for the indicated periods of time and the levels of pSTAT6 in cell lysates were analysed by western blotting. Total STAT6 was used as a loading control. The gel shown is representative of three others with similar results. B-C. J774 (B) and THP-1 (C) macrophages were preincubated with PPAR agonists for one hour and IL-4 with or without IL-13 was added for an additional 30 min. The levels of pSTAT6 in cell lysates were analysed by western blotting. Total STAT6 was used as a loading control. Results represent the mean + SEM (n=4). (Paukkeri et al. 2015, Immunometabolism 2:1-11, modified)

3 PPARα/γ agonist muraglitazar attenuates acute inflammation *in vivo*

As shown above, muraglitazar has several anti-inflammatory effects in macrophages. To determine if muraglitazar would be able to suppress inflammation also *in vivo*, the effect of muraglitazar on carrageenan-induced paw oedema was investigated. Muraglitazar attenuated the development of oedema in a dose-dependent manner (Figure 24). The golden standard of anti-inflammatory agents, dexamethasone, and the iNOS inhibitor L-NIL were used as control compounds. In addition,

muraglitazar decreased the proinflammatory gene expression in carrageenan-induced inflammation as shown by the reduced mRNA levels in the inflammatory tissue (Figure 25).

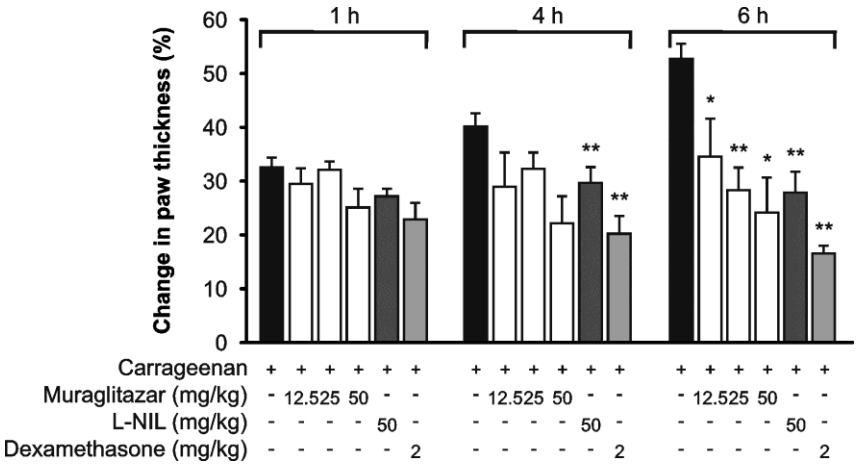

Figure 24. Muraglitazar attenuates carrageenan-induced paw oedema in the mouse. Muraglitazar, the iNOS inhibitor L-NIL, dexamethasone or vehicle (2.0% carboxymethyl cellulose) were administered to mice per os 1 hour prior to carrageenan (1%) injection. Paw oedema was measured before and 1 h, 4 h and 6 h after carrageenan injection by micrometer. Oedema is expressed as increase in paw thickness at indicated time points compared to thickness before carrageenan. Values represent the mean + SEM (n=6). *p<0.05 and **p<0.01 as compared to mice treated with carrageenan alone. (Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

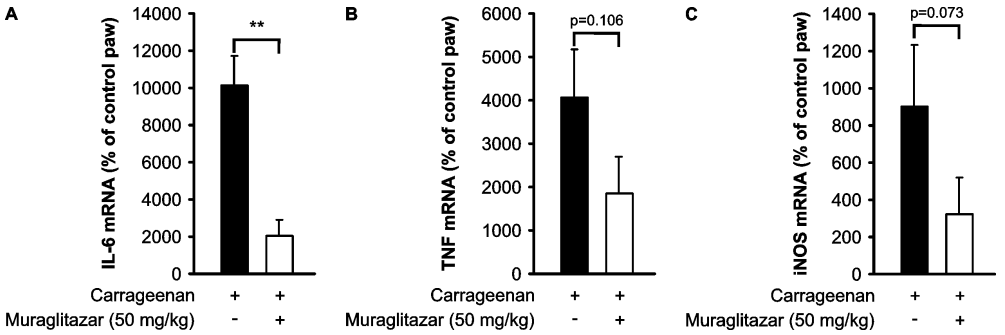

Figure 25. Muraglitazar reduces carrageenan-induced expression of inflammatory mediators in the mouse paw. Muraglitazar was administered to animals intraperitoneally 2 hours prior to carrageenan (1.5%) injection. Six hours after carrageenan injection, the animals were sacrificed and total RNA was extracted from subcutaneous connective tissue of carrageenan–injected and control paws. The levels of IL-6, TNF and iNOS mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA and mRNA levels in carrageenan-treated paw were compared to the levels in control paw. Values represent the mean + SEM (n=5-7). **p<0.01 as compared to mice treated with carrageenan alone. (Paukkeri et al. 2013, Arthritis Res Ther 15: R51, modified)

4 Novel coronary stent with muraglitazar coating expresses promising effects in *in vitro* studies

As described earlier, it was found that muraglitazar exhibited anti-inflammatory effects both *in vitro* and *in vivo*. Furthermore, there are reports showing that muraglitazar also has several beneficial metabolic effects (Buse et al. 2005, Rubin, Viraswami-Appanna & Fiedorek 2009, Kendall et al. 2006). To investigate whether it would be possible to combine the benefits of both of these effects, a biodegradable vascular stent with muraglitazar coating was manufactured. The rate of muraglitazar release from the muraglitazar-coated stent was evaluated in PBS and in cell culture medium at 37°C until > 95% of the drug content had been released. The rate of drug release into PBS was highest during the first two weeks, but the elution continued at an almost constant rate for at least the remaining five weeks of follow-up (Figure 26). In subsequent studies, the muraglitazar coating did not impair the mechanical properties of the stent. Also, the manufacturing process did not impair the biological efficacy of muraglitazar as compared to native muraglitazar. This was shown by the effects of muraglitazar stent on LPS-induced IL-6, TNF and MCP-1 production in THP-1 cells (Figure 27).

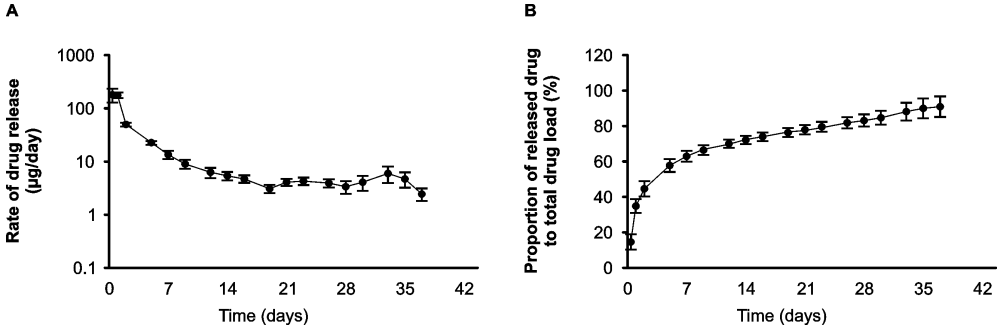

Figure 26. The elution kinetics of muraglitazar *in vitro*. Muraglitazar-eluting stents were incubated in PBS for 7 weeks and the concentrations of muraglitazar released in PBS were measured by HPLC. The results represent the mean ± SEM (n=4).

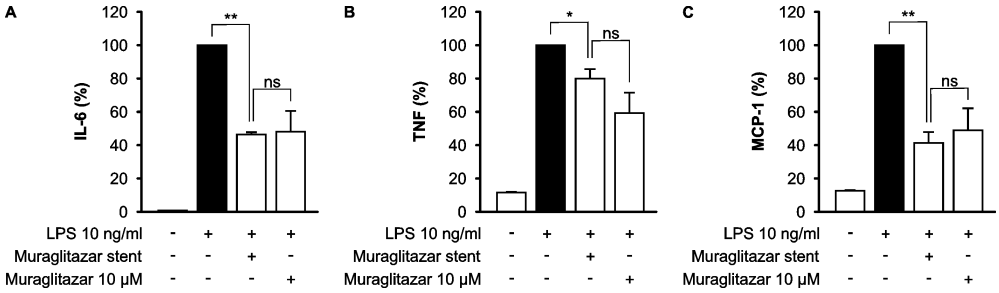

Figure 27. The effects of muraglitazar-coated stent on cytokine production in THP-1 cells. Cells were incubated with LPS and vehicle (non-coated stent), muraglitazar with a non-coated stent and a muraglitazar-coated stent for 24 h. The levels of IL-6, TNF and MCP-1 in culture medium were measured by ELISA. The concentration of native muraglitazar was comparable to the amount of muraglitazar released from muraglitazar-coated stent during the incubation time. The results represent the mean + SEM (n=4). *p<0.05 and **p<0.01 as compared to the cells treated with LPS only.

DISCUSSION

1 Methodology

In the present study, PPAR agonists were used to evaluate the effects of PPARs in cells. Apart from the PPARα/γ dual agonist, muraglitazar, all of the agonists used are selective with at least 10 times higher affinity to one particular PPAR subtype (Ram 2003, Azhar 2010). However, 15d-PGJ_2 has also PPAR-independent effects in cells (Lu et al. 2013, Giri et al. 2004, Han, Sidell 2002) and therefore 15d-PGJ_2 was used only in a few experiments here and its effects were confirmed by comparing them with another PPARγ agonist GW1929. The effects of PPARα were examined with at least two, molecularly distinct PPARα agonists. Most of the PPAR agonists require micromolar concentrations to activate PPAR (Yamazaki et al. 2004), and therefore micromolar concentrations were also used in this study. The possibility of PPAR-independent effects cannot be completely ruled out with these methods even though several PPAR agonists were used, the activities of the PPAR agonists did seem to be selective and PPARs were being expressed in the cells used.

Most of the experiments in the present study were carried out with immortalized macrophages growing in cell culture conditions. Non-modified primary macrophages change their phenotype immediately after the initiation of culture and the phenotype continues to develop rapidly during culturing. However, immortalized cells maintain their basal phenotype unchanged for several months and the possible discrepancies in results can therefore be explained by treatments or methodological reasons (Maqsood et al. 2013). Thus, immortalized cells are a good model with which to study complex intracellular molecular pathways. In addition, cell culture methods do not have similar ethical problems than encountered with the use of animals or primary cells. On the other hand, immortalized cells have undergone mutations in their path to becoming immortal and those mutations may affect the cellular responses. For this reason the results obtained in immortal cells should be considered when interpreting the results.

J774 cells are monocyte-macrophages that were originally harvested from ascites of female BALB/c mice with reticulum cell sarcoma in 1968 (Ralph, Prichard & Cohn 1975). These cells express several characteristics of primary macrophages: they

synthesize and secrete lysozymes, express Fc receptors, phagocytose carbon particles and immunoglobulin G -labeled host cells (Muschel, Rosen & Bloom 1977). In this study, J774 cells were shown to express both classically activated and alternatively activated macrophage phenotypes according to the environmental stimuli provided.

THP-1 cells were the other macrophage cell line used in this study. These cells are monocytes that have been derived from the peripheral blood of a 1 year old boy with acute monocytic leukaemia. THP-1 cells express Fc and C3b receptors and are able to phagocytose (Tsuchiya et al. 1980). The gene expression profile of LPS-stimulated THP-1 monocytes has been shown to be very similar to that of LPS-stimulated peripheral blood mononuclear cells (PBMCs) (Sharif et al. 2007). Unlike PBMCs, THP-1 cells require special treatments to differentiate into macrophages and some treatments may change the responses of the cells (Takashiba et al. 1999). In this study, the macrophage differentiation was induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, which is the most commonly used differentiation stimulus according to the literature.

In the experiments focusing on iNOS promoter activity and NF-κB mediated transcription, cells were transfected with transfection vectors expressing either iNOS promoter or repeated NF-κB binding sites prior to the luciferase reporter. Transfection is a powerful method to investigate a certain step of the gene regulation. The transfection of macrophages is generally difficult, since these cells express several potent degrading enzymes that can disrupt nucleic acid integrity (Zhang, Edwards & Mosser 2009). On the other hand, HEK293 cells are practical and one of the most commonly used cell types to study transcription (Thomas, Smart 2005) and HEK293 cells were used to investigate NF-κB-mediated transcription also in this study.

This is the first study to demonstrate that muraglitazar has anti-inflammatory potential *in vitro*. The possibility that muraglitazar acts as an anti-inflammatory compound *in vivo* was investigated by examining its protective effects in carrageenan-induced paw oedema in mice. This model is representative of acute inflammation and it has been widely used in pharmacological studies (Vogel 2002). Carrageenan-induced inflammation has been described to occur in two phases. The local first phase starts almost immediately after the injection of carrageenan and lasts for 1-2 hours. During this phase, the most abundant mediators are histamine, serotonin and the kinins. The second phase starts during the first hours after carrageenan injection and has been reported to involve the activation of inflammatory cells including macrophages. In this phase, the expressions of iNOS and cyclooxygenase 2, and the syntheses of prostaglandins, oxygen-derived free radicals, TNF, IL-1 and IL-6 are

increased in the inflamed tissue (Salvemini et al. 1996, Morris 2003, Loram et al. 2007).

The intracellular mRNA and protein levels of the genes interested were studied by standard cell biological methods, RT-qPCR and western blotting, respectively. Although RT-qPCR is a highly specific and reproducible method for detecting the mRNA levels of a gene, the specificity of western blotting can be impaired for several reasons. Most importantly, the protein is usually detected with a polyclonal antibody, which may recognize several different, even slightly mismatched, amino acid sequences. The size, responses to treatment and negative and positive controls can be used to estimate whether a certain band represents the target protein. However, the running behaviour of proteins in SDS-polyacrylamide gel electrophoresis gels is not only dependent on size but also on charges in the structure of the proteins. Thus, the size of a detected protein estimated by protein standards on a gel quite often differs from the calculated size of the protein. Furthermore, the total amount of protein may differ in different lines of a gel. These aspects were noted in the present work and all results were confirmed in at least another independent experiment. The loading controls were used to ensure that the amount of protein was similar between the bands that were being compared to each other. In addition, in RT-qPCR, the levels of target genes were normalized to those of GAPDH, a house-keeping gene known to be constantly expressed in several, although not all (Kozera, Rapacz 2013), cellular conditions.

2 Regulation of classical macrophage activation by PPAR agonists

In the present study, both PPARα and PPARγ agonists were found to attenuate the classical activation of macrophages. The regulation of iNOS expression was the main focus in this part of the study, but also the effects of PPAR agonists on IL-6 and TNF production were investigated. One novel finding was that PPARα agonists were found to reduce iNOS expression by increasing the degradation of iNOS protein. The identification of the anti-inflammatory action of muraglitazar was also an original observation.

The regulation of iNOS expression is an intensively studied area, but the main focus has been on the regulation of iNOS gene transcription and iNOS mRNA stability. For long, very little was known about the regulation of iNOS protein stability, although the degradation of iNOS protein in the proteasome had been

shown to be the main mechanism to terminate iNOS activity. However, after the publication of the present results, several reports of both the molecular mechanisms of flagging iNOS for degradation and compounds that modify the rate of iNOS degradation have been published.

The very first reports of the involvement of the ubiquitin-proteasome pathway in the degradation of iNOS protein were published by Felley-Bosco et al., Musial and Eissa and Kolodiejski et al. (Felley-Bosco et al. 2000, Musial, Eissa 2001, Kolodziejski et al. 2002). These studies demonstrated that ubiquitinylation of iNOS was required for its degradation and that the proteasome pathway was the main, if not the only, pathway for the degradation of iNOS. Later, it became evident that the COOH terminus of heat shock protein 70-interacting protein (CHIP) (Chen et al. 2009) and the SPRY domain-containing suppressor of cytokine signaling box protein 1 (SPSB1), SPSB2 and SPSB4 (Kuang et al. 2010, Nishiya et al. 2011) were required for effective ubiquitinylation and subsequent proteasomal degradation of iNOS in murine macrophages. In addition, it was reported that regulatory particle non-ATPase subunit 13 increased iNOS protein degradation by recruiting iNOS and deubiquitination enzyme UCH37 to the 19S proteasome cap (Mazumdar et al. 2010). In the present study, the proteasome inhibitor, lactacystin, abolished the effect of PPARα agonists on iNOS protein. This might be evidence that PPARα agonists can promote the action of the proteasome. However, on the basis of current knowledge, it is also possible that PPARα agonists increase the ubiquitinylation of iNOS e.g. by inducing the action of CHIP or SPSB proteins.

Before the publication of the present study, only a few molecules had been found to regulate the stability of iNOS protein. In 1996 Kunz et al. showed that dexamethasone could increase the degradation of IL-1β-induced iNOS in mesangial cells (Kunz et al. 1996). In addition, scaffold protein calveolin-1 and TGF-β had been demonstrated to increase the degradation of iNOS protein (Vodovotz et al. 1993, Vuolteenaho et al. 2005). However, during recent years, several pharmacological or test compounds have been reported to stimulate the degradation of iNOS. Lin et al. reported that the prostaglandin E_2 receptor 4 inhibitor, AH23848, induced iNOS degradation in mesangial cells (Lin et al. 2008) and there are claims that rapamycin and the polyphenolic compound curcumin could promote iNOS degradation and the effects on degradation could be abolished by treatment with proteasome inhibitors (Ben et al. 2011, Jin et al. 2009). In addition, the dibenzylbutyrolactone lignan arctigenin stimulated iNOS degradation in a CHIP-dependent manner (Yao et al. 2012). Finally, the antidiabetic drug, the GLP-1

receptor agonist exendin-4, increased iNOS degradation in macrophages with this effect being possibly mediated by induction of cAMP levels (Chang et al. 2013).

Studies carried out in retinal pigment epithelial cells, mesangial cells and astrocytes suggest that the anti-inflammatory effects of PPARα agonists are mediated through the inhibition of NF-κB activation (Shen et al. 2014, Kono et al. 2009, Xu et al. 2006). A similar result was also achieved in mouse peritoneal macrophages in a single study (Crisafulli, Cuzzocrea 2009). Although PPARα agonists did not reduce NF-κB activation in the cells used in this study, the results achieved by other groups are not necessarily in conflict with the present results describing the effects of PPARα agonists on proteasome. One reason is that proteasome-mediated degradation of IκB, the critical inhibitor of NF-κB in resting state, is an essential step in NF-κB signalling. In some cell lines, PPARα agonists may inhibit the degradation of IκB and further decrease the transcriptional activity of NF-κB in addition to altering the degradation of iNOS protein. Alternatively, the effects of PPARα agonists may be cell type specific and PPARα agonists may be able to regulate protein expression in different mechanisms in different tissues.

In the present study, the detailed mechanisms for the suppressing effect of PPARγ agonists on iNOS protein and mRNA levels in J774 cells could not be elucidated. However, the PPARγ agonist GW1929 and PPARα/γ dual agonist muraglitazar were shown to decrease the transcription activity of iNOS without affecting the DNA binding of NF-κB or the activation of STAT-1 or MAPKs. Since the NF-κB, STAT-1 and MAPK pathways are the most important mediators of LPS-stimulation of iNOS transcription, these results suggest that GW1929 and muraglitazar most probably affect the transcription process in a direct manner. One possible molecular mechanism could be N-CoR -mediated inhibition. In resting cells, the transcription machinery of the iNOS gene is inhibited by N-CoR. Upon LPS-stimulation, N-CoR is degraded by the proteasome, which makes the activation of the transcriptional machinery possible. Pascual et al. reported that rosiglitazone-activated PPARγ was able to prevent the degradation of N-CoR, which prevented subsequent iNOS transcription in mouse macrophages (Pascual et al. 2005).

There are no published reports where the effect of PPARγ agonists on NF-κB activation in macrophages would be clearly established. The PPARγ agonist, CLX-090717, reduced LPS-induced IκBα degradation in human peripheral blood-derived monocyte-macrophages (Sumariwalla et al. 2009). However, Guyton et al. reported that 15d-PGJ_2 could reduce IκBα degradation in LPS-stimulated rat peritoneal macrophages, but this effect could not be replicated with the selective PPARγ agonist BRL49653 (Guyton et al. 2001). In Kuppfer cells, pioglitazone has been

reported to reduce LPS-induced nitric oxide production, but it could not suppress nuclear translocation or DNA binding of NF-κB (Uchimura et al. 2001). Thus, further studies are needed to clarify the role of PPARγ in the regulation of NF-κB signalling.

In the present study, PPARγ agonists decreased both IL-6 and TNF production, while PPARα agonists decreased only IL-6 production. This might be explained by the different mechanisms of anti-inflammatory action of PPARα and PPARγ in the tested cells. NF-κB is an important transcription factor for both of these cytokines, but both of the cytokines are also regulated at the posttranscriptional level. However, only IL-6 has been reported to be regulated by the proteasome (LaPensee, Hugo & Ben-Jonathan 2008). Thus, the effect of PPARγ agonists on IL-6 and TNF production might be mediated by decreased transcription of IL-6 and TNF genes, while PPARα might affect the expression of IL-6 at the posttranslational level.

As described, the effects of PPARγ and PPARα agonists in inflammation are partly different, but muraglitazar exhibited both PPARγ- and PPARα-linked effects. Muraglitazar was able not only to inhibit iNOS transcription but it could also promote iNOS protein degradation. In addition, muraglitazar was able to decrease TNF production and in this respect it differed from the PPARα agonists. The results suggest that combining the effects of PPARα and PPARγ by using a dual PPARα/γ agonist can achieve an improved anti-inflammatory action in comparison with either PPARα or PPARγ agonists on their own.

In addition to its anti-inflammatory effects *in vitro*, muraglitazar also reduced carrageenan-induced paw oedema in mice. The decrease was seen only at the later time points, suggesting that muraglitazar specifically was inhibiting the second phase of inflammation, i.e. the activation of macrophages and other immune cells. This result is in line with the results *in vitro* where muraglitazar reduced the production of nitric oxide, IL-6 and TNF and the expression of iNOS. Similarly, muraglitazar suppressed IL-6, TNF and iNOS mRNA levels in the carrageenan-inflamed paw tissue.

3 Regulation of alternative macrophage activation by PPAR agonists

PPARγ agonists have been previously reported to promote the alternative activation of macrophages and this effect was also shown in this study. In contrast to the

PPARγ agonists, PPARα agonists were, for the first time, found to decrease the alternative activation.

In several studies, the alternative activation of macrophages has been linked to fibrosis. Alternatively activated macrophages secrete TGF-β and PDGF that increase the proliferation and collagen synthesis of human fibroblasts (Song et al. 2000). A homodimer of PDGF isoform PDGF-C, PDGF-CC, derived from alternatively activated macrophages also promotes the differentiation of fibroblasts to myofibroblast (Glim et al. 2013). Bellon et al. found that the proportion of CD163-expressing alternatively activated macrophages of all macrophages increased during infectious peritonitis in patients with peritoneal dialysis and these $CD163^+$ macrophages stimulated fibroblast proliferation to a larger extent than $CD163^-$ macrophages (Bellon et al. 2011). In addition, patients with neuromuscular sarcoidosis expressed higher levels of alternatively activated macrophages, IL-4, IL-4R, IL-13 and more fibrosis in their muscle tissue than control subjects (Prokop et al. 2011).

At present, only a few factors have been shown to modify alternative activation of macrophages. Yang et al. reported that oestrogen could repress the alternative activation of macrophages by decreasing the phosphorylation of STAT6 (Yang et al. 2012). In addition, serum amyloid P has reduced alternative activation and collagen deposition of chronic fungus-induced allergic airway disease in mice (Moreira et al. 2010). Furthermore, the mineralocorticoid receptor antagonist spironolactone attenuated the infiltration of alternatively activated macrophages and collagen deposition in a bleomycin-induced pulmonary reaction in mice (Ji et al. 2013). In contrast, glucocorticoids increased the activity of alternatively activated macrophages (Stein et al. 1992). IL-5 has also been shown to increase the alternative activation of macrophages and schistosomiasis-induced liver fibrosis in mice (Reiman et al. 2006). There is one report that galectin-3 expression is needed for effective IL-4-induced alternative activation in mice (MacKinnon et al. 2008).

The hypothesis to account for the antifibrotic effects of PPARα agonists is supported by recent findings in animal models of fibrosis. In the study by Li et al., fenofibrate reduced fibrosis in kidney in a rat model of diabetes as evaluated by collagen accumulation and increased levels of α-smooth muscle actin (Li et al. 2010). The amounts of collagen and α-smooth muscle actin were also attenuated by fenofibrate in concanavalin A -induced liver fibrosis in mice (Mohamed et al. 2013). Fenofibrate treatment was also able to ameliorate the extent of fibrosis in bleomycin-induced pulmonary fibrosis in rats (Samah et al. 2012). Finally, fenofibrate decreased the amounts of fibrosis as assessed histologically as well as by mRNA levels of

collagen type I and type III in left ventricle of pressure-overloaded rat heart (Ogata et al. 2002). These results suggest that PPARα agonists have potent antifibrotic effects but further confirmation will be needed especially in human subjects. The present study supports previous findings by providing a potential mechanism for the antifibrotic action of PPARα agonists.

4 Potential of PPAR agonists in novel applications

At present, PPAR agonists have been used as oral treatments for metabolic disorders. However, there is increasing evidence to suggest that PPAR agonists may confer additional benefits in novel indications. New indications or novel applications with local dosage may also help to limit the range of side effects linked to PPAR agonists.

A novel muraglitazar -eluting bioabsorbable stent was investigated in the present study. The *in vitro* data revealed that muraglitazar did not lose its biological properties during the manufacturing process.

The primary treatment of ischemic myocardial infarct is to open the coronary artery with percutaneous coronary intervention (PCI) and to support the artery with a stent (De Luca et al. 2008). However, although stenting clearly improves the results of PCI, the risk for restenosis of stents remains a significant problem (Spaulding et al. 2006, Laarman et al. 2006). In-stent restenosis is linked to chronic vascular inflammation and hypertrophic wound healing and macrophages are key players in this pathogenic process (Farb et al. 2002, Moreno et al. 1996). In the present study, muraglitazar was shown to decrease the production of proinflammatory and chemotactic proteins by human macrophages, which suggests that a muraglitazar-eluting stent might be able to reduce the risk of in-stent restenosis. This suggestion can be further supported by protective effect of orally administrated pioglitazone on the restenosis risk in clinical settings. In two meta-analyses, oral pioglitazone treatment clearly reduced the risk of in-stent restenosis after bare metal stenting (Nishio, Kobayashi 2010, Patel et al. 2011). In addition, in a prospective, multicentre, randomized trial pioglitazone treatment decreased need for revascularization and neointimal thickness in stented area during 6-month follow-up after bare metal stenting in patients with type II DM (Takagi et al. 2009). The local drug delivery of muraglitazar-eluting stent would offer the additional benefit over oral administration, since the drug would affect only the target tissue. In addition, when compared to metal stents, the bioabsorbable characteristic of the stent would help

to provide the mechanical support, but avoid triggering any long term tissue reactions in the vessel walls. The degradation of PLA occurs by hydrolysis, which means that the elimination is not dependent on the function of kidney or liver. The degradation residues of PLA are non-toxic, which further improves its safety (Shikanov, Kumar & Domb 2005).

In line with the anti-inflammatory effects of PPAR agonists seen in this research project, several studies about the effectiveness of PPAR agonists in autoimmune diseases have been published recently. Shahin et al. reported that 12-week treatment with pioglitazone improved the symptoms and CRP levels in patients suffering from both RA and type II DM (Shahin et al. 2011). All the enrolled patients used methotrexate 15 mg/wk and oral corticosteroids ≤7.5 mg/day. The provision of pioglitazone treatment reduced the mean swollen joint count from 4.7 to 2.7, the tender joint count from 6 to 3.1 and CRP levels from 20.4 mg/l to 8.1 mg/l. However, there was no appropriate control group in that study and it was conducted single-blinded only. In another randomized, double-blinded and placebo-controlled crossover study by Marder et al., a 3-month treatment of patients suffering from RA with pioglitazone decreased CRP-based disease activity score 28 (DAS28) and CRP levels in RA patients without type II DM (Marder et al. 2013). All the patients had earlier started disease-modifying antirheumatic drugs (DMARDs) or corticosteroid treatment, and then pioglitazone was added to the previous treatment regimen. The improvement in the CRP-based DAS28 value was statistically significant (0.31±1.2 in the pioglitazone group vs. 0.15±1.2 in the placebo group, p=0.02), although the basal disease activity of the patients was low (CRP-based DAS28 = 2.8±1.98).

In addition, fenofibrate has been shown to reduce RA activity in patients with both RA and dyslipidaemia (Goto 2010). In that study, 44 patients were randomly assigned to fenofibrate 200 mg/day or statin (either atorvastatin, pravastatin or pitavastatin, dosages were not described) group while the patients' other antirheumatic drug treatment remained unchanged. After 6 months of treatment, visual analogue scale (VAS), a questionnaire used to estimate pain, values were improved in both fenofibrate and statin groups with a tendency to a greater improvement in the fenofibrate group (49.1±24.7 to 14.7±11.2 vs. 47.4±29.7 to 20.2±16.5, respectively), but the difference was not statistically significant. In addition, CRP levels tended to decline in both groups (from 10.3±2.05 to 7.0±11.5 mg/l in the fenofibrate group and from 11.4±13.6 to 7.4±12.9 mg/l in the statin group), but again the differences were not statistically significant.

In two double-blind, randomized, placebo-controlled clinical trials of the effects of pioglitazone in plaque psoriasis, pioglitazone treatment improved the psoriasis

area and severity index (PASI) score by 12.5 (Mittal et al. 2009) and 25.9 (Shafiq et al. 2005) percentage points more than control group. The differences in the results between the two trials might be explained by study designs. In the former study, the dose of pioglitazone was smaller (15 mg/day) than in the latter study (30 mg/day). Furthermore, in the study by Mittal et al. acitretin, a second-generation retinoid, was started concurrently with pioglitazone and placebo for all study patients (Mittal et al. 2009).

Pioglitazone has also been reported to improve non-alcoholic steatohepatitis. In two meta-analyses, pioglitazone significantly improved lobular inflammation, steatosis, ballooning degeneration and fibrosis of the liver (Boettcher et al. 2012, Mahady et al. 2011). This is an important finding, since there is no established medication for non-alcoholic steatohepatitis currently available (Del Ben et al. 2014).

As previously discussed, PPARα agonists were shown to reduce the alternative activation of macrophages in the present study. This and previous studies seem to suggest that PPARα agonists would also possess antifibrotic effects. Thus, PPARα agonists may be beneficial in the treatment of diseases in which fibrosis plays a central role, such as SSc. Also, the findings discussed above support the idea that the non-metabolic effects of both PPARα and PPARγ agonists are clinically relevant and PPAR agonists appear to be beneficial also in other than metabolic indications. The results encourage to continue the investigation of PPAR agonists in novel indications, especially in inflammatory diseases and fibrosis.

5 The safety of PPAR agonists

In recent years, concerns have arisen in the public media about the adverse effects of lipid-lowering drugs, in particular the risk of myopathy. However, in a recent meta-analysis with 15 313 participants, fibrate therapy did not increase the risk of rhabdomyolysis or muscle abnormalities (Jun et al. 2010). It has also been suggested that the risk of myopathy may be increased if fibrates are combined with statin-type drugs (Enger et al. 2010), but a recent randomized, double-blind clinical trial involving 474 patients stated that none of the patients treated with a combination of rosuvastatin and fenofibrate suffered from serious adverse events or drug-related myopathy (Roth et al. 2010).

The beneficial effects of fibrates are the clearest in patients with hypertriglyceridemia or combined hypertriglyceridemia and low HDL values (Lee et al. 2011, Bruckert et al. 2011). In patients suffering from hypertriglyceridemia, fibrate

therapy has reduced the risk of vascular events by 25-28 %, while the reduction was 29-30 % in patients with both hypertriglyceridemia and low HDL (Lee et al. 2011, Bruckert et al. 2011). In fact, in two meta-analyses fibrate therapy did not significantly reduce the vascular events in patients with dyslipidaemia without hypertriglyceridemia or low HDL (Lee et al. 2011, Bruckert et al. 2011). Thus, targeting of fibrate therapy to the right patients is an important factor in the estimation of safety-efficacy relation.

The safety of PPARγ agonists also have evoked discussion during the past years. Rosiglitazone was found to increase risk of myocardial infarction and cardiovascular deaths (Nissen, Wolski 2007). Muraglitazar has been reported to cause similar cardiovascular adverse effects as rosiglitazone (Nissen, Wolski & Topol 2005). Although pioglitazone does not seem to increase the risk of macrovascular outcomes, it has been linked to a higher risk of heart failure, bone fractures and bladder cancer in diabetic patients (Dormandy et al. 2009). However, the difference in the spectrum of adverse effects linked to different TZDs indicates that the adverse effects are not consequence of PPARγ activation. Thus, the development of new PPARγ or PPARα/γ agonists may help to avoid at least some of the adverse effects. The previous cardioprotective results with aleglitazar support this hypothesis (Lincoff et al. 2014). It is also worth considering that most of the safety studies of TZDs and muraglitazar have been carried out with type II DM patients, who already have an increased risk for cardiovascular events. It is likely that the profile of adverse effects would not be the same in other groups of patients, e.g. in patients with RA or other autoimmune disease.

One way to avoid the systemic adverse effects linked to PPARγ agonists would be to utilize local drug delivery. One example examined in this thesis is the PPAR agonist-eluting vascular stents. In addition, the PPAR agonist might be dosed directly to an inflamed joint in a form where the drug is released slowly. This might help to attenuate joint inflammation in RA and at the same time avoid the systemic adverse effects linked with both PPAR agonists.

6 Hypotheses of the links between inflammation and metabolism

If immunity is primarily considered as a protection system against pathogens, then the mind of obesity-induced inflammation and subsequent changes in cellular function is hard to understand. As earlier discussed, invaded pathogen promotes inflammation, which further promotes changes in cellular metabolism. These

changes in metabolism help to eradicate the pathogen effectively. For example, during infection, insulin resistance in muscle and adipose cells increases the availability of glucose for immune cells, and the production of leptin supports the proinflammatory activity of immune cells. Thus, from this point of few, the inflammation linked to metabolic disruption is a protective phenomenon. On the other hand, the obesity-linked inflammation would be a purely pathological phenomenon and some kind of mistake of evolution not conferring any reasonable benefit to the individual. However, another possibility is that immunity and metabolism would be tightly connected to each other also in normal physiology and immunity may regulate normal metabolic homeostasis in the body. If the latter is true, it is tempting to hypothesize that metabolic changes could also contribute to the inflammation in autoimmune diseases, even without obesity. Proof for this hypothesis could be obtained if a link between inflammation and metabolism could be established.

It is well known that inflammation modifies energy consumption. The intracellular molecular mechanisms contributing to this phenomenon have been extensively investigated in macrophages. In several studies, classically activated macrophages have been reported to utilize anaerobic glycolysis and pentose phosphate pathway as their primary energy source (Haschemi et al. 2012), while alternatively activated macrophages primarily adopt aerobic fatty acid oxidation. This is understandable since anaerobic energy metabolism increases and aerobic metabolism decreases NAD+/NADH ratio and NAD+ is needed for synthesis of ROS necessary to kill pathogenic microbes. In addition, glycolysis produces energy rapidly, whereas aerobic oxidative phosphorylation requires more time. Proinflammatory NF-κB signalling seems to have a central role in the regulation of aerobic-anaerobic switching and thus on the energy source of cells. However, several mediators have been reported to be involved in this regulation. As already discussed in this thesis, PPARγ suppresses the production of several NF-κB-mediated cytokines. Additionally, PPARγ has also been shown to promote aerobic metabolism in macrophages possibly through induction of genes controlling fatty acid oxidation (Luo et al. 2010). Secondly, hypoxia sensing protein hypoxia-induced factor 1α (HIF-1α) is needed for efficient glycolysis (Weidemann, Johnson 2008). In addition to hypoxia, HIF-1α expression is clearly increased by NF-κB activation, linking HIF-1α to inflammation (Weidemann, Johnson 2008). Thirdly, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) promotes the function of mitochondria and thus fatty acid and glucose oxidation in macrophages. On the other hand, NF-κB signalling has been reported to suppress PGC-1α activation,

which decreases fatty acid oxidation (Kauppinen et al. 2013). Fourthly, sirtuin 1 is needed for adequate fatty acid oxidation (Liu et al. 2012). Sirtuin 1 also increases the expression of PGC-1α and suppress the signalling of NF-κB (Kauppinen et al. 2013). In summary, inflammatory signalling and the availability of oxygen regulate the switch between energy sources and several factors modify this regulation.

There is rather little evidence supporting the hypothesis that glucose or fatty acids would promote inflammation. However, Shi et al. demonstrated that both glucose uptake and glycolysis are needed for the differentiation of T_h17 cells (Shi et al. 2011). On the other hand, blockade of glycolysis promoted T_{reg} differentiation. They also indicated that HIF-1α is the key enzyme in glycolytic activity and T_h17-T_{reg} switching (Shi et al. 2011). Interesting results were obtained by Haschemi et al. who showed that suppression of pentose phosphate pathway increased the production of proinflammatory cytokines in macrophages, even without an inflammatory stimulus (Haschemi et al. 2012). Further evidence has been provided by the finding earlier discussed in this thesis that saturated free fatty acids activate TLR4 (Nguyen et al. 2007, Shi et al. 2006).

It is also possible that there exists "a master regulator" of both metabolism and inflammation and it exerts its effects extracellularly. For example, this kind of "master regulator" could be leptin or the sympathetic nervous system. Leptin is mainly secreted from adipose tissue and the produced levels correlate to the mass of adipose tissue. However, inflammatory stimuli also increase leptin production. On the other hand, leptin can modify both inflammatory and metabolic reactions. It is known that leptin induces macrophages to proliferate and produce ROS, leukotrienes, nitric oxide, TNF, IL-6, IL-8 and IFNγ, and it increases the proliferation of naïve T cells while decreasing the proliferation of T_{reg} cells. In adipocytes, leptin increases glucose and fatty acid oxidation and reduces adipocyte proliferation (Stofkova 2009, Harris 2014). Nonetheless, although the link between leptin, metabolism and inflammation is clear, the detailed effects and causality need to be clarified.

In several studies, catecholamines that are produced from the sympathetic neurons in response to activation of the sympathetic nervous system can modify the activation of macrophages and adaptive immune cells, leukocyte trafficking and the proliferation of fibroblasts (Koopman et al. 2011). Cytokines can also influence the activity of the sympathetic nervous system (Koopman et al. 2011, Canale et al. 2013). On the other hand, it is known that several factors related to metabolic homeostasis such as leptin, certain fatty acids and insulin can modify the action of the sympathetic

nervous system (Canale et al. 2013). However, the effects of the sympathetic nervous system are highly complex and proper evidence of the connection is still lacking.

The action of PPARs to regulate inflammation and metabolism seem to be different at the molecular level. In the present study, the effects of PPARs on inflammation were mainly mediated by either reducing the transcription of proinflammatory genes or by affecting the posttranscriptional processing of the gene, while there are reports in the literature that the effects of PPARs on metabolism are mediated by PPAR-induced transcription of certain genes. If the hypothesis of inflammation as a regulator of metabolic homeostasis were true, PPARs might mediate the regulation in the following manner. Lipid compounds activate PPARs that first promote the transcription of several genes involved in the β-oxidation of lipids. Subsequently, activated PPARs prevent the inflammatory pathways from shifting the metabolism towards glycolysis. Nevertheless, new research will surely improve our understanding of the directions of these signalling pathways.

In summary, PPARs act as interesting links between metabolism and inflammation. This makes PPARs an attractive target for novel drug discoveries. The current study provides encouragement to continue research of the PPAR agonists in inflammatory diseases.

SUMMARY

The objectives of the present study were to clarify the effects of PPAR agonists on classical and alternative pathways of macrophage activation and to investigate the potential of PPARα/γ dual agonist muraglitazar as an anti-inflammatory drug.

The major findings are as follows:

I) a) PPARα agonists attenuate both classical and alternative activation of macrophages, while PPARγ agonists reduced classical but enhanced alternative activation of macrophages.

b) Both PPARα and PPARγ agonists reduced the protein levels of iNOS, which is one of the most important markers of classical macrophage activation, but the mechanisms controlling the downregulation were different. PPARα agonists increased iNOS degradation by activating the proteasome pathway, but PPARγ agonists reduced iNOS transcription.

II) Muraglitazar downregulated the production of proinflammatory cytokines in macrophages *in vitro* and carrageenan-induced inflammation *in vivo*.

III) The biological efficacy of muraglitazar was retained during the manufacturing process of drug-coated stent. In addition, the drug-elution kinetics of the muraglitazar-coated stent proved to be promising.

The present study has provided new information about the immunomodulating effects of PPAR agonists. Since alternative activation of macrophages is linked to fibrosing diseases, such as systemic sclerosis, PPARα agonism might be a beneficial feature of novel anti-fibrotic drugs. On the other hand, PPARγ agonists and muraglitazar hold potential as anti-inflammatory therapeutics in inflammatory diseases, such as rheumatoid arthritis. In the literature, the metabolic effects of PPARs have been described in detail and in that respect, the present study further highlights the role of PPARs as players linking inflammation and metabolism.

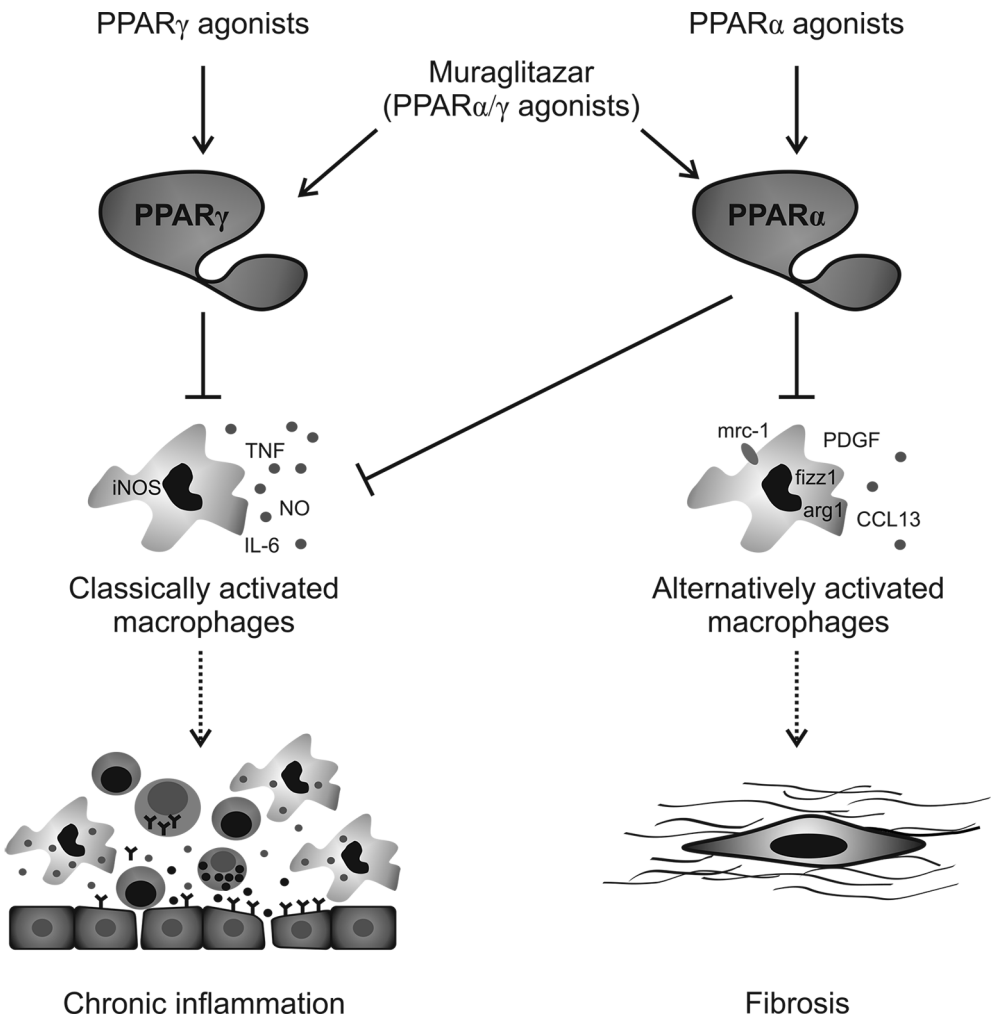

Figure 28. Summary of the effects of PPAR agonists on macrophage activation. Arg1, arginase 1; CCL13, (C-C motif) ligand 13; fizz1, found in inflammatory zone 1; IL, interleukin; iNOS, inducible nitric oxide synthase; mrc-1, mannose receptor 1; NO, nitric oxide; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; TNF, tumour necrosis factor; ⋯⋯→ Hypothetical connection

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RESEARCH PAPER

PPARα agonists inhibit nitric oxide production by enhancing iNOS degradation in LPS-treated macrophages

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Background and purpose: Nitric oxide (NO) production through the inducible nitric oxide synthase (iNOS) pathway is increased in response to pro-inflammatory cytokines and bacterial products. In inflammation, NO has pro-inflammatory and regulatory effects. Peroxisome proliferator-activated receptors (PPARs), members of the nuclear steroid receptor superfamily, regulate not only metabolic but also inflammatory processes. The aim of the present study was to investigate the role of PPARα in the regulation of NO production and iNOS expression in activated macrophages.
Experimental approach: The effects of PPARα agonists were investigated on iNOS mRNA and protein expression, on NO production and on the activation of transcription factors NF-κB and STAT1 in J774 murine macrophages exposed to bacterial lipopolysaccharide (LPS).
Key results: PPARα agonists GW7647 and WY14643 reduced LPS-induced NO production in a dose-dependent manner as measured by the accumulation of nitrite into the culture medium. However, PPARα agonists did not alter LPS-induced iNOS mRNA expression or activation of NF-κB or STAT1 which are important transcription factors for iNOS. Nevertheless, iNOS protein levels were reduced by PPARα agonists in a time-dependent manner. The reduction was markedly greater after 24 h incubation than after 8 h incubation. Treatment with the proteasome inhibitors, lactacystin or MG132, reversed the decrease in iNOS protein levels caused by PPARα agonists.
Conclusions and implications: The results suggest that PPARα agonists reduce LPS-induced iNOS expression and NO production in macrophages by enhancing iNOS protein degradation through the proteasome pathway. The results offer an additional mechanism underlying the anti-inflammatory effects of PPARα agonists.
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Keywords: iNOS; macrophages; nitric oxide; PPAR; protein degradation; proteasome

Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; CRP, C-reactive protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor κB; NO, nitric oxide; PPAR, peroxisome proliferator-activated receptor; STAT1, signal transducer and activator of transcription 1; TNF-α, tumour necrosis factor-α

Introduction

Nitric oxide (NO) is an important modulator of immune response in human tissues. It has cytotoxic and cytostatic effects, which are beneficial in host defence against pathogenic microbes. In inflammatory diseases, the regulatory, pro-inflammatory and destructive effects of NO modulate the responses also in host tissues (Moilanen *et al*., 1999; Abramson *et al*., 2001; Korhonen *et al*., 2005) and inhibitors of iNOS have been found to be beneficial in various models of inflammatory diseases (Vallance and Leiper, 2002). High amounts of NO are produced through the inducible nitric oxide synthase (iNOS) pathway in response to proinflammatory cytokines and bacterial products. Expression of iNOS has been shown to be regulated both at transcriptional and post-translational levels in activated macrophages, but many of the mechanisms are still unknown (MacMicking *et al*., 1997; Alderton *et al*., 2001; Kleinert *et al*., 2003; Aktan, 2004; Korhonen *et al*., 2005).

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear steroid receptor superfamily. Three members of the family have been identified: PPARα, PPARβ/δ and

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PPARγ. Originally, the receptors were found to be involved in the regulation of the oxidation of fatty acids, but recently other functions of PPARs have been described (Berger and Moller, 2002; Kota *et al*., 2005). For example, they regulate the transcription of genes that are involved in lipid and glucose metabolism and play a role in adipocyte differentiation and apoptosis (Delerive *et al*., 2001; Moore *et al*., 2001a; Kota *et al*., 2005). Furthermore, recent observations suggest that the PPARs, especially PPARα and PPARγ, are involved in the regulation of the immune and inflammatory responses. Although both anti-inflammatory and pro-inflammatory effects of PPARs have been reported (Delerive *et al*., 2001; Moore *et al*., 2001a; Cabrero *et al*., 2002; Clark, 2002; Zhang and Young, 2002; Genolet *et al*., 2004) the role of PPARs in inflammation is not clear.

PPARγ agonists have been shown to decrease interferon γ- or lipopolysaccharide (LPS)-induced NO production (Ricote *et al*., 1998; Alleva *et al*., 2002; Chen *et al*., 2003) and iNOS expression (Castrillo *et al*., 2000; Chen *et al*., 2003). iNOS expression was shown to be modulated at the transcriptional level. PPARγ agonists were proposed to inhibit the action of inflammatory transcription factors nuclear factor κappa B (NF-κB), activator protein 1 and signal transducer and activator of transcription 1 (STAT1) (Ricote *et al*., 1998; Chen *et al*., 2003). The effects of PPARα agonists on NO production and iNOS expression in macrophages have been less studied (Colville-Nash *et al*., 1998; Cernuda-Morollón *et al*., 2002). The results of the two studies were contradictory and the mechanisms of action were not investigated in detail.

The aim of the present study was to investigate the effects of PPARα agonists on LPS-induced NO production and iNOS expression in macrophages. The results suggest that PPARα agonists suppress LPS-induced NO production and iNOS expression by enhancing the degradation of iNOS protein through the proteasome pathway.

Methods

Cell culture

J774 macrophages (American Type Culture Collection) were cultured at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Cambrex BioScience, Verviers, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (Cambrex BioScience), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 250 ng ml^{-1} amphotericin B (Gibco, Paisley, UK) and harvested with trypsin-EDTA (Gibco). Cells were seeded on 24-well plates (0.2×10^6 cells per well) for nitrite measurements and real-time PCR assays, on six-well plates (0.9×10^6 cells per well) for preparation of cell lysates for iNOS and PPARα Western blot analysis, on 10 cm dishes (4×10^6 cells per dish) for preparation of nuclear extracts and cell lysates for ubiquitin western blotting, and on 96-well plates (4×10^4 cells per well) for cell viability assays. Confluent cells were exposed to fresh culture medium containing the compounds of interest. PPAR agonists were added together with LPS (10 ng ml^{-1}) in all experiments.

Nitrite determination

Measurement of nitrite accumulation into the culture medium was used to determine NO production. The culture medium was collected at indicated time points and nitrite was measured by the Griess reaction (Green *et al*., 1982). The concentration of nitrite was calculated by using sodium nitrite added to the culture medium (including supplements) as a standard. A selective iNOS inhibitor 1400W was used to differentiate nitrite derived from other biochemical pathways and cellular sources.

Cell viability assays

Cell viability was tested using Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN, USA). Cells were incubated with the tested compounds for 20 h before addition of sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate (final concentration 0.3 mg ml^{-1}) and *N*-methyl dibenzopyrazine methyl sulphate (final concentration 1.25 mM). Then the cells were further incubated for 3 h and the amount of formazan accumulated into the growth medium was assessed spectrophotometrically. Triton X-100-treated cells were used as a positive control. A direct cytotoxicity of the tested compounds was evaluated by Trypan blue staining. Triton X-100-treatment was used as a positive control in the cytotoxicity tests.

Preparation of cell lysates for iNOS, PPARα and ubiquitin western blotting

At indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in cold lysis buffer containing 10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 μg ml^{-1} leupeptin, 50 μg ml^{-1} aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. When preparing cell lysates for ubiquitin western blotting, lysis buffer contained also 20 μg ml^{-1} ubiquitin aldehyde and 25 μM MG132. After incubation on ice for 15 min, lysates were centrifuged (13 400 *g*, 4 °C, 10 min), supernatants were collected and mixed 3:1 with SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford, 1976).

Preparation of nuclear extracts for STAT1α, NF-κB and PPARγ western blotting

At indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 1 mM NaF and 0.1 mM EGTA). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 °C, 21 000 *g* for 10 s. Nuclei were resuspended in

buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 1 mM NaF and 0.1 mM EGTA) and incubated for 20 min on ice. Nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4 °C, 21 000 *g* for 2 min. Supernatants were collected and mixed 3:1 with SDS sample buffer. Coomassie blue was used to measure the protein content of the samples (Bradford, 1976).

Western blotting
Prior to western blotting, samples were boiled for 10 min and 20 μg (240 μg in ubiquitin western blotting) of protein was loaded per lane on 5% (ubiquitin), 8% (iNOS, STAT1α), 10% (PPARα, PPARγ) or 12% (NF-κB p65) SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK). The membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 °C overnight. Thereafter, the membrane was washed with TBS/T, incubated with secondary antibody in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using SuperSignal West Pico, Dura or Femto chemiluminescent substrate (Pierce, Rockford, IL, USA) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). Actin or lamin A was used as a loading control.

RNA extraction and quantitative real-time PCR
Cell homogenization, RNA extraction, reverse transcription of RNA to cDNA and PCR of iNOS were performed as described previously (Lahti *et al*., 2003). Glyceraldehyde-3-phosphate dehydrogenase was used as a control gene.

Statistics
Results are expressed as mean ± s.e.m. When indicated, statistical significance was calculated by analysis of variance followed by Dunnett's multiple comparisons test. Differences were considered significant at $P<0.05$.

Materials

Reagents were obtained as follows: GW7647 and MG132 from Tocris Cookson Ltd. (Bristol, UK), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) from Calbiochem (San Diego, CA, USA), ubiquitin aldehyde from Boston Biochem (Cambridge, MA, USA), LPS (*Escherichia coli* 0111:B4, product no. L-4391) from Sigma Chemical Co. (St Louis, MO, USA), rabbit polyclonal actin, lamin A/C, iNOS, NF-κB subunit p65, PPARγ and STAT1α p91 antibodies and goat anti-rabbit polyclonal HRP-conjugated antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), rabbit polyclonal PPARα antibody from Alexis Biochemicals (Lausen, Switzerland), mouse monoclonal ubiquitin antibody from Zymed (San Fransisco, CA, USA) and anti-mouse polyclonal HRP-conjugated antibody from Pierce (Cheshire, UK). 1400W was a kind gift from Dr Richard Knowles (GlaxoSmithKline, Stevenage, UK). All other reagents were from Sigma Chemical Co.

Results

Effects of PPARα agonists on LPS-induced NO production
J774 macrophages were found to express PPARα and PPARγ as detected by western blot and LPS treatment for 24 h did not alter their expression levels (data not shown). Resting cells did not produce detectable amounts of NO (measured as nitrite accumulated in the culture medium), but LPS induced NO production and iNOS expression in J774 macrophages. To test the effect of PPARα activation on LPS-induced NO production, we measured NO production in the presence of a selective PPARα agonist GW7647 or WY14643. GW7647 and WY14643 inhibited LPS-induced NO production in a dose-dependent manner, GW7647 being more potent than WY14643 (Figures 1a and b). GW7647 and WY14643 did not affect cell viability when determined by sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate test or Trypan blue staining.

Effects of PPARα agonists on iNOS mRNA levels and activation of transcription factors NF-κB and STAT1
To measure the effects of PPARα agonists on iNOS mRNA expression, the LPS-induced iNOS mRNA levels in the presence and absence of PPARα agonists were determined by quantitative RT-PCR. Neither GW7647 nor WY14643 had any effect on iNOS mRNA expression when measured 6h or 10 h after addition of LPS (Figures 2a and b).

We tested also the effect of WY14643 on the activation of NF-κB and STAT1, which are important transcription factors for iNOS expression. The activation was examined by measuring the translocation of NF-κB (as measured by an antibody against p65 subunit) or STAT1α to the nuclei by western blot. LPS increased the translocation of NF-κB, which peaked at 30 min and decreased thereafter, and that of STAT1, which increased up to 6 h after LPS. WY14643 did not alter LPS-induced NF-κB or STAT1 translocation (Figures 3a and b).

Since PPARγ agonists have been previously reported to inhibit LPS-induced iNOS mRNA expression in macrophages (Ricote *et al*., 1998; Castrillo *et al*., 2000; Chen *et al*., 2003), we wanted to compare the effects of PPARα agonists to those of PPARγ agonists. Although PPARα agonists had no effect on iNOS mRNA expression, we saw a marked reduction in LPS-induced iNOS mRNA levels after treatment with 15d-PGJ_2, a natural ligand of PPARγ (Figure 4a). 15d-PGJ_2 reduced also LPS-induced iNOS protein expression and NO production (Figures 4b and c) as reported previously (Ricote *et al*., 1998; Petrova *et al*., 1999). These results suggest that the mechan-

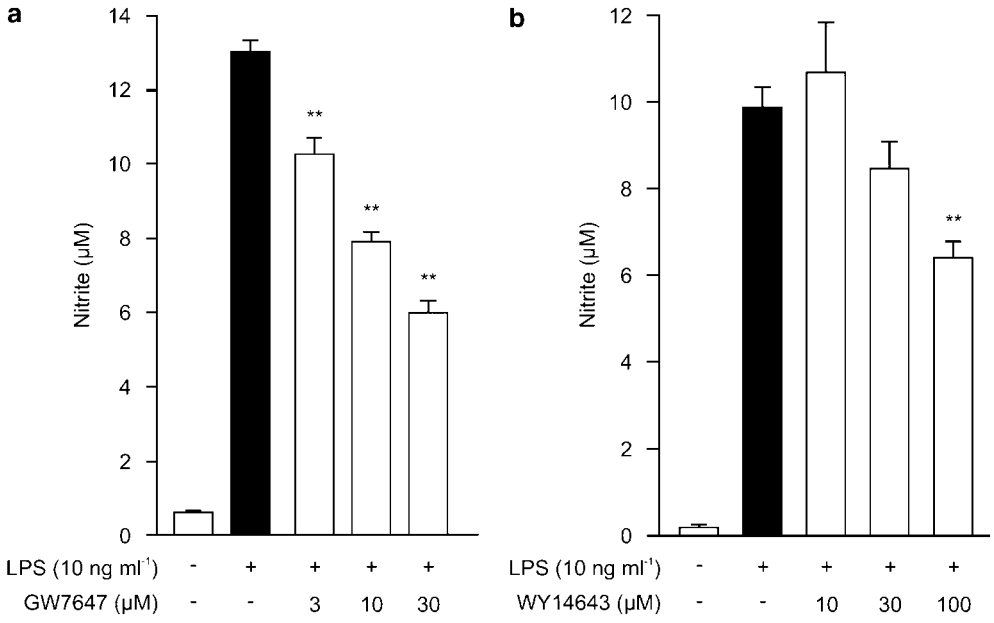

Figure 1 Effects of PPARα agonists on NO production in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of GW7647 (**a**) or WY14643 (**b**). After 24 h incubation nitrite accumulated in the culture medium was measured by Griess reaction, as a marker of NO production. Results are expressed as mean ± s.e.m. ($n = 6$). **$P < 0.01$ as compared to cells treated with LPS alone.

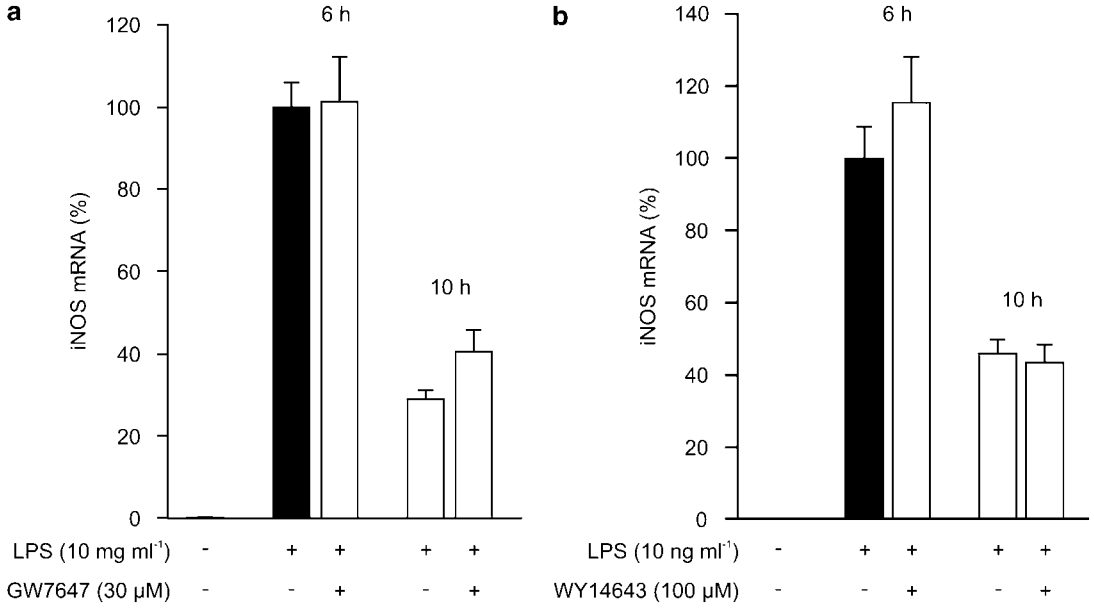

Figure 2 Effects of PPARα agonists on iNOS mRNA expression in J774 macrophages. Cells were incubated with LPS (10 ng ml^{-1}) and GW7647 (30 μM) (**a**) or WY14643 (100 μM) (**b**). Total RNA was extracted at the indicated time points and iNOS mRNA was measured by RT-PCR. The results were normalized against GAPDH mRNA. Levels of iNOS mRNA are expressed relative to that induced by LPS at 6 h (set to 100%). Results are expressed as mean ± s.e.m. ($n = 3$).

ism of the inhibitory effect of PPARα agonists on NO production is different from that of PPARγ agonists.

Effects of PPARα agonists on iNOS protein levels

In further studies, we determined the effects of PPARα agonists on iNOS protein expression by western blot analysis. LPS-induced iNOS expression was reduced by PPARα agonists in a dose-dependent manner (Figures 5a and b). After 24 h incubation, the reduction of iNOS expression was about 70% (WY14643) and 80% (GW7647) at the highest agonist concentrations used, thus showing a greater reduction on iNOS protein levels than on NO production (Figure 1).

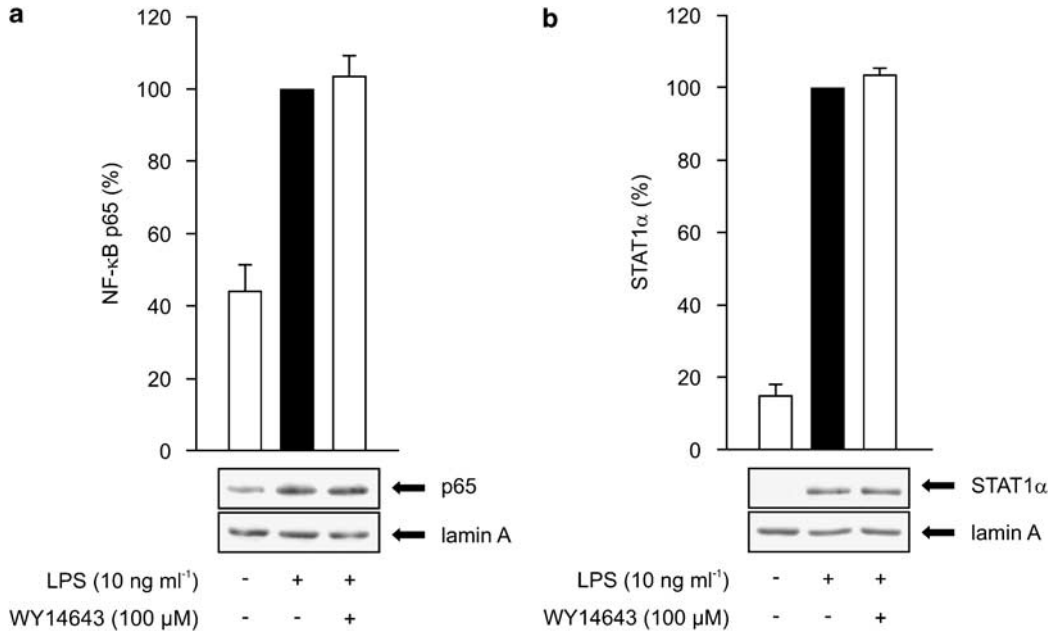

Figure 3 (**a**) Effects of PPARα agonists on NF-κB activity in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with WY14643 (100 μM) for 30 min. Nuclear extracts were prepared and the p65 subunit of NF-κB was measured by western blot. (**b**) Effects of PPARα agonists on STAT1α activity in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with WY14643 (100 μM) for 6 h. Nuclear extracts were prepared and STAT1α was measured by western blot. Protein levels are expressed relative to that in LPS-treated cells (set to 100%). Lamin A was used as a loading control. Results are expressed as mean ± s.e.m. ($n = 3$).

Since PPARα agonists reduced the expression of iNOS protein, but had no effect on iNOS mRNA levels, we hypothesized that PPARα agonists could enhance iNOS degradation. Therefore, we measured the effects of PPARα agonists on LPS-induced iNOS protein levels by western blot after different incubation times (Figures 6a and b). After 8 h incubation, the level of iNOS protein expression was 20–30% lower in cells treated with combinations of LPS and GW7647 or LPS and WY14643 than in cells treated with LPS alone. In contrast, when measured after 12, 16 and 24 h incubations, iNOS protein levels were 50, 75 and 85% lower, respectively in (LPS + GW7647)-treated cells than in cells treated with LPS alone (Figure 6a). A similar pattern of reduction was seen in cells treated with LPS + WY14643 as compared to cells treated with LPS only (Figure 6b).

iNOS protein has been reported to be degraded through the proteasome pathway (Felley-Bosco *et al*., 2000; Musial and Eissa, 2001). Therefore we investigated the role of proteasomes in the suppressive effect of GW7647 and WY14643 on iNOS protein levels. For this purpose, we used two proteasome inhibitors, lactacystin and MG132. To ensure that the proteasome pathway was blocked by these proteasome inhibitors, we first assessed the effect of lactacystin on ubiquitinated protein levels. As detected by western blot, lactacystin increased the ubiquitinated protein levels both in cells incubated with and without LPS (Figure 7).

In subsequent studies, lactacystin (10 μM) or MG132 (10 μM) was added to the cells 8 hours after the commencement of the incubation with LPS or LPS and a PPARα agonist, and the cells were harvested after 24 h incubation. As a response to LPS, J774 macrophages expressed iNOS protein reaching maximum between 8 and 12 h after stimulation and decreasing thereafter (Figure 8). In the (LPS + lactacystin)-treated cells, iNOS protein levels were higher after 24 h incubation than in LPS-treated cells (Figures 9a and b) supporting the idea that lactacystin inhibits iNOS degradation. In addition, GW7647 and WY14643 had practically no effect on iNOS levels in the presence of lactacystin while they reduced iNOS protein levels by more than 65% in the absence of lactacystin (Figures 9a and b). Another proteasome inhibitor, MG132, also reduced the inhibitory effect of WY14643 on LPS-induced iNOS protein expression (Figure 9c). Similarly, proteasome inhibitors reversed the inhibitory effects of WY14643 on NO production as measured by nitrite accumulation in the culture medium (data not shown). These results suggest that treatment with proteasome inhibitors reversed the degradation of iNOS protein induced by PPARα agonists GW7647 and WY14643.

Discussion

In the present study, we have shown that PPARα agonists GW7647 and WY14643 reduce LPS-induced iNOS expression and NO production in macrophages. Our results suggest that this effect is mediated through enhanced degradation of iNOS protein via the proteasome pathway. Because the proteasome pathway is involved in the degradation of several inflammatory factors, the present findings may well provide an explanation for the anti-inflammatory effects of PPARα agonists.

In several studies, activation of PPARα has been reported to have anti-inflammatory effects *in vivo*. A clear evidence of

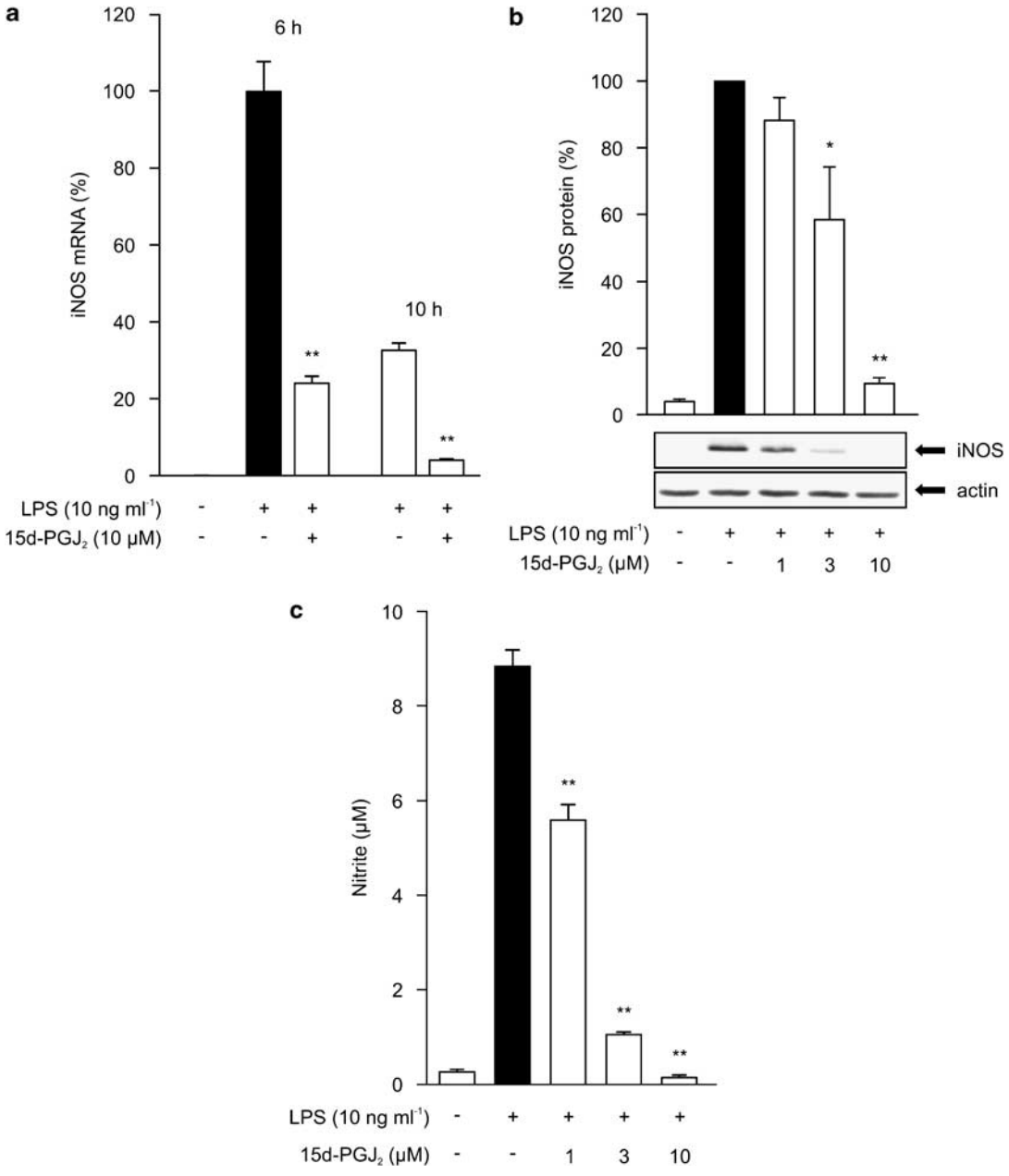

Figure 4 (**a**) Effect of PPARγ agonist 15d-PGJ_2 on iNOS mRNA expression in J774 macrophages. Cells were incubated with LPS (10 ng ml^{-1}) and 15d-PGJ_2 (10 μM). Total RNA was extracted at the indicated time points and iNOS mRNA was measured by real-time PCR. The results were normalized against GAPDH mRNA. Levels of iNOS mRNA are expressed relative to that induced by LPS at 6 h (set to 100%). Results are expressed as mean ± s.e.m. ($n = 3$). **$P < 0.01$ as compared to cells treated with LPS alone. (**b**) Effect of PPARγ agonist 15d-PGJ_2 on iNOS protein expression in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of 15d-PGJ_2. After 24 h incubations, proteins were extracted and iNOS protein was measured by western blot. Protein levels are expressed relative to that in LPS-treated cells (set to 100%). Actin was used as a loading control. Results are expressed as mean ± s.e.m. ($n = 3$). *$P < 0.05$ and **$P < 0.01$ as compared to cells treated with LPS alone. (**c**) Effect of PPARγ agonist 15d-PGJ_2 on NO production in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of 15d-PGJ_2. After 24 h incubation, nitrite accumulated into the culture medium was measured by Griess reaction as a marker of NO production. Results are expressed as mean ± s.e.m. ($n = 6$). **$P < 0.01$ as compared to cells treated with LPS alone.

the immunomodulating effects of PPARα was unveiled in 1996, when PPARα-null mice were shown to present a prolonged inflammatory reaction in response to leukotriene B_4 as compared to wild-type animals (Devchand *et al*., 1996). Later, fibrates, which act as PPARα ligands, have been shown to decrease plasma levels of interleukin-6 (IL-6), interferon-γ, tumour necrosis factor-α, fibrinogen and C-reactive protein in hyperlipidemic patients (Madej *et al*., 1998; Staels *et al*., 1998). In addition, numerous studies have clarified the role of PPARγ agonists on inflammatory responses. For example, members of antidiabetic thiazolidinediones, which are synthetic PPARγ ligands, have been shown to reduce

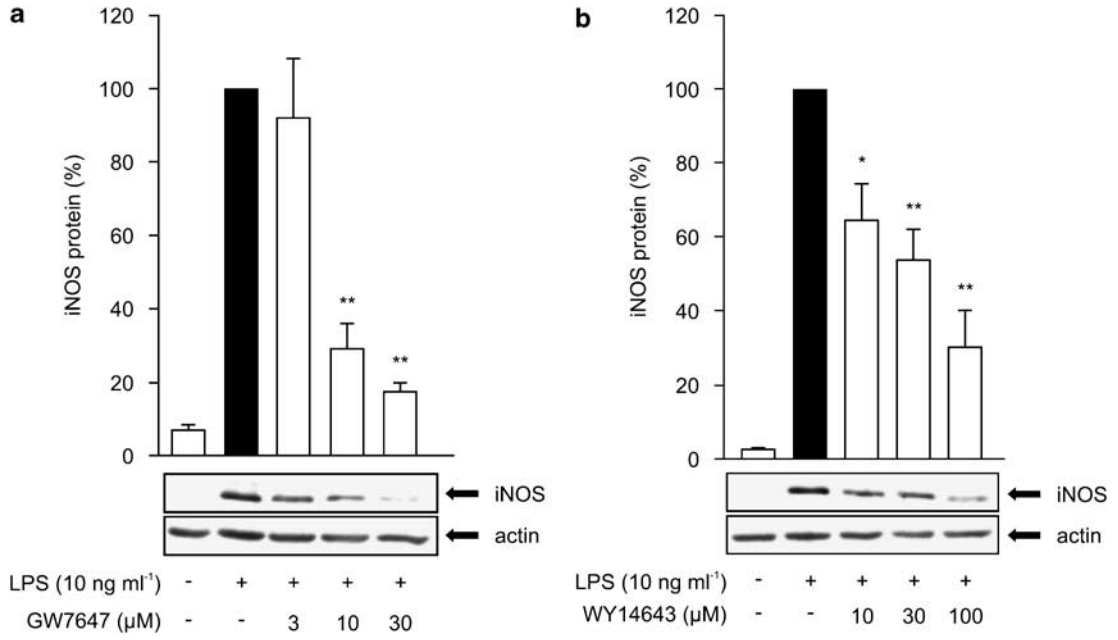

Figure 5 Dose-dependent effects of PPARα agonists on iNOS protein expression in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of GW7647 (**a**) or WY14643 (**b**). After 24 h incubations, proteins were extracted and iNOS protein was measured by Western blot. iNOS protein levels are expressed relative to that in LPS-treated cells (set to 100%). Actin was used as a loading control. Results are expressed as mean ± s.e.m. ($n = 3$). *$P < 0.05$ and **$P < 0.01$ as compared to cells treated with LPS alone.

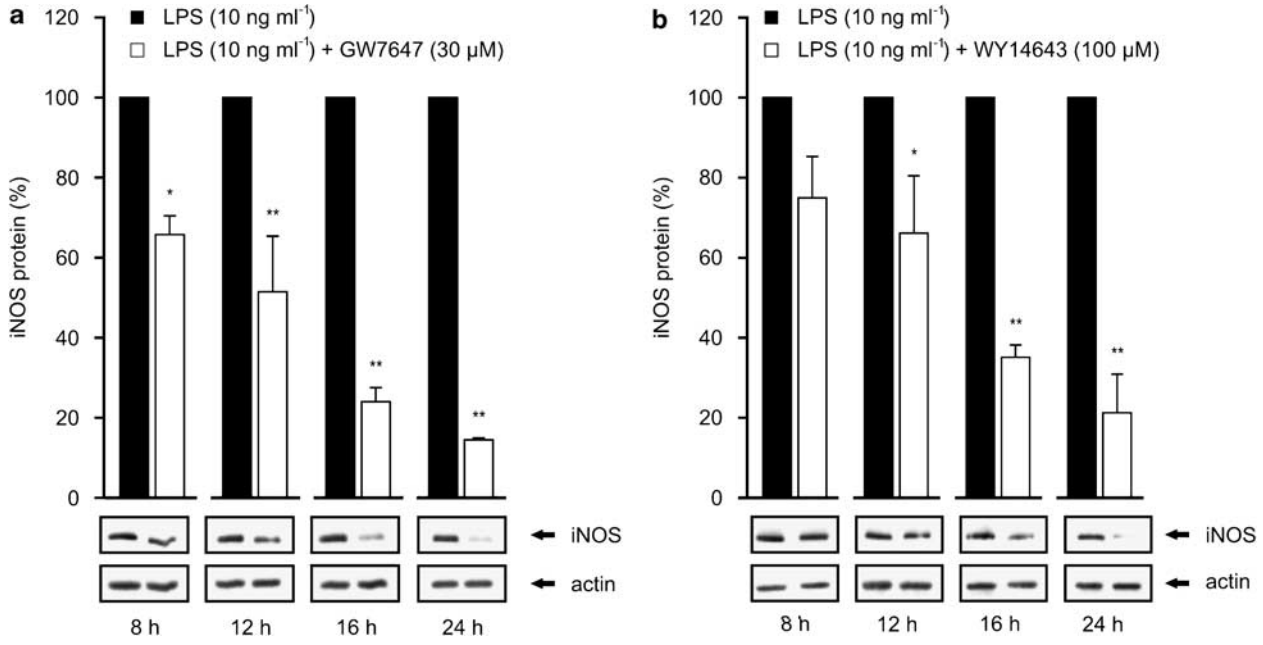

Figure 6 Time-dependent effects of PPARα agonists on iNOS protein expression in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) and treated with GW7647 (30 μM) (**a**) or WY14643 (100 μM) (**b**). Proteins were extracted at indicated time points and iNOS protein was measured by western blot. At each time point, iNOS protein levels are expressed relative to that in LPS-treated cells (set to 100%). Actin was used as a loading control. Results are expressed as mean ± s.e.m. ($n = 3$). *$P < 0.05$ and **$P < 0.01$ as compared to cells treated with LPS alone.

inflammation in a mouse model of inflammatory bowel disease (Su *et al.*, 1999) and in adjuvant-induced arthritis in rats (Kawahito *et al.*, 2000). Rosiglitazone has also been reported to decrease plasma concentrations of C-reactive protein and matrix metalloproteinase-9 (Haffner *et al.*, 2002), and inhibit the development of atherosclerosis in low-density lipoprotein receptor-deficient mice (Li *et al.*, 2000). However, although there are a large number of studies reporting anti-inflammatory actions of PPAR ligands, some observations suggest that PPAR agonists may also have pro-

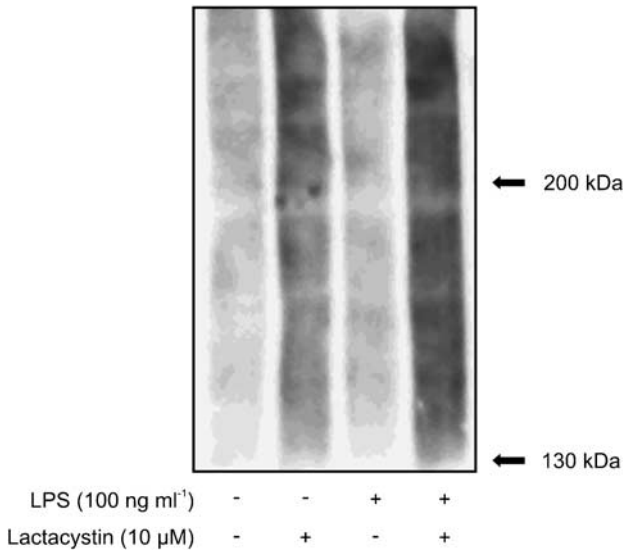

Figure 7 Effect of lactacystin on ubiquitinated protein levels in J774 macrophages. When indicated, LPS 10 ng ml^{-1} was added 8 h prior to lactacystin (10 μM). Proteins were extracted 16 h after the addition of lactacystin and ubiquitinated protein levels were analysed by Western blot. A representative gel is shown, from three experiments with similar results.

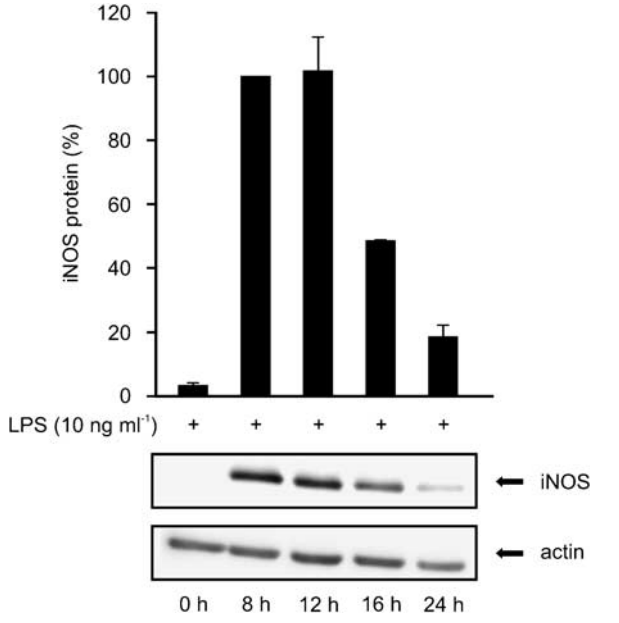

Figure 8 The effect of LPS on iNOS protein expression in J774 macrophages. J774 macrophages were stimulated by LPS (10 ng ml^{-1}). Proteins were extracted at indicated time points and iNOS protein was measured by western blot. iNOS protein levels are expressed relative to that in LPS-treated cells at 8 h (set to 100%). Actin was used as a loading control. Values expressed are mean ± s.e.m. ($n = 3$).

inflammatory effects (Thieringer *et al*., 2000; Guyton *et al*., 2001; Moore *et al*., 2001b; Fu *et al*., 2002). Thus, PPARs have been shown to have *in vivo* relevance with inflammatory processes, and that is the reason why the mechanisms underlying these effects are highly interesting to clarify.

In the present study, PPARα agonists suppressed LPS-induced NO production and iNOS protein expression in a dose-dependent manner, but they had no effect on iNOS mRNA levels or on activation of NF-κB or STAT1, which are important transcription factors for iNOS. These results together suggest that the suppressive effects of PPARα agonists on iNOS expression and NO production are mediated through post-transcriptional mechanisms.

When we investigated the effects of PPARα agonists on iNOS protein expression at different time points, we found that PPARα agonists reduced LPS-induced iNOS protein expression significantly more when measured 24 h after addition of LPS than at the 8 h time point. These findings suggest that PPARα agonists GW7647 and WY14643 enhance the degradation of iNOS protein in macrophages and this is the mechanism for the inhibition of NO production by PPARα agonists. This idea is also supported by the fact that the suppressing effect of PPARα agonists was greater on iNOS protein than on NO levels at the equal time point. There is evidence showing that iNOS protein is degraded by the proteasome pathway (Felley-Bosco *et al*., 2000; Musial and Eissa, 2001). In the present study, we found that two proteasome inhibitors lactacystin and MG132 reversed the effects of PPARα agonists on iNOS protein expression. Therefore, we proposed that PPARα agonists reduced NO production through iNOS pathway by enhancing the degradation of iNOS protein by proteasomal enzymes. This assumption is supported by the recent data from mRNA microarrays showing that PPARα agonists enhance expression of proteasomal genes in cynomolgus monkey liver (Cariello *et al*., 2005) and in murine hepatocytes (Anderson *et al*., 2004).

The regulation of iNOS protein degradation is poorly known. However, there are data that support the importance of the proteasome pathway in this degradation process (Felley-Bosco *et al*., 2000; Musial and Eissa, 2001). In those reports, the proteasome inhibitor lactacystin was shown to enhance iNOS protein levels in murine RAW 264.7 macrophages and in human cell lines (Felley-Bosco *et al*., 2000; Musial and Eissa, 2001). The present findings support the earlier data by showing that two proteasome inhibitors, lactacystin and MG132, inhibited iNOS protein degradation in LPS-treated J774 macrophages supporting the significant role of proteasomes in the degradation of iNOS protein.

In the literature, only a few factors have been described to regulate iNOS protein stability. TGF-β, in addition to its effects on iNOS mRNA stability and translation, has been found to increase degradation of iNOS protein in macrophages (Vodovotz *et al*., 1993; Mitani *et al*., 2005) and in chondrocytes (Vuolteenaho *et al*., 2005). In addition, dexamethasone has been reported to decrease iNOS protein stability in IL-1-stimulated mesangial cells (Kunz *et al*., 1996).

There are some data on the role of PPARs in the regulation of iNOS expression and NO production, but most of the interest has been focused on PPARγ. PPARγ agonists have been shown to decrease NO production and iNOS expression in macrophages (Ricote *et al*., 1998; Castrillo *et al*., 2000; Alleva *et al*., 2002; Chen *et al*., 2003), and this inhibitory effect seems to take place at a transcriptional level by

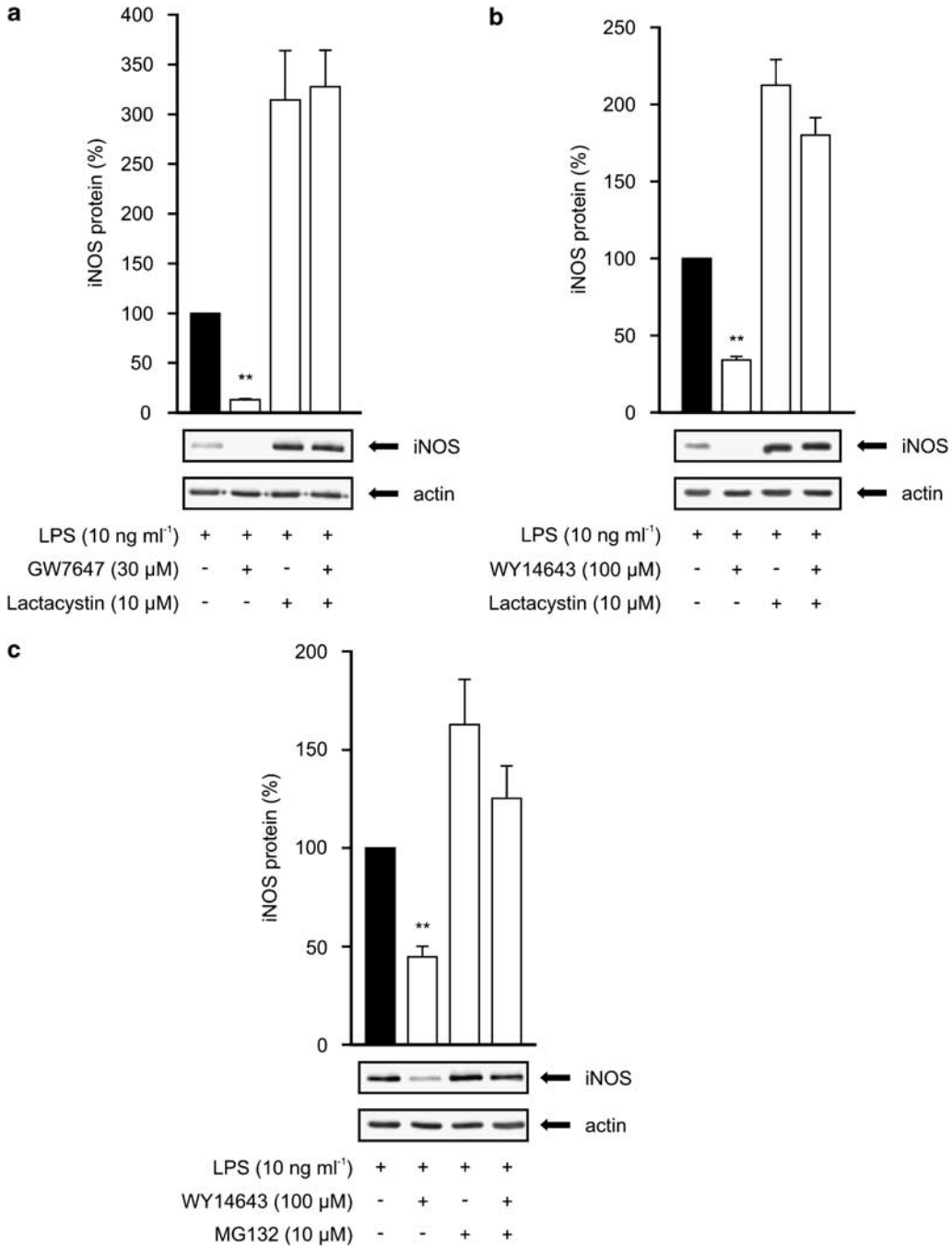

Figure 9 Effects of proteasome inhibitors on iNOS protein expression in J774 macrophages. Cells were stimulated by LPS (10 ng ml^{-1}) with or without GW7647 (**a**) or WY14643 (**b**, **c**). After 8 h incubation a proteasome inhibitor lactacystin (**a**, **b**) or MG132 (**c**) was added into the culture medium. Proteins were extracted after 24 h incubation and iNOS protein was analysed by western blot. iNOS protein levels are expressed relative to that in LPS-treated cells (set to 100%). Actin was used as a loading control. Results are expressed as mean ± s.e.m. ($n = 3$). **$P < 0.01$ as compared to cells treated with LPS alone.

inhibiting the action of transcription factors NF-κB and STAT1 (Ricote *et al*., 1998; Chen *et al*., 2003). There are, however, only two previous reports on the effects of PPARα agonists on iNOS expression and NO production in macrophages showing contradictory results (Colville-Nash *et al*., 1998; Cernuda-Morollón *et al*., 2002). Colville-Nash *et al*. (1998) found that a selective PPARα ligand WY14643 reduced interferon-γ and LPS-induced NO production in RAW 264.7 macrophages. Cernuda-Morollón *et al*. (2002) reported that WY14643 amplified LPS- or LPS and interferon-γ-stimulated iNOS protein expression in RAW 264.7 macrophages. The present results are in line with those reported by Colville-Nash *et al*. (1998) and they extend the earlier data by showing a cellular mechanism that could, at least in part, explain the inhibitory effect of PPARα agonists on LPS-induced iNOS protein expression and NO production in

activated macrophages. As the proteasome pathway is involved in the degradation of several inflammatory proteins (Ben-Neriah, 2002; Colmegna *et al*., 2005), PPARα agonists may well regulate the levels of an array of inflammatory factors by the same mechanism.

In conclusion, the present data show that PPARα agonists GW7647 and WY14643 suppress LPS-induced iNOS protein expression and NO production in macrophages, and this effect is likely to be mediated by enhanced iNOS protein degradation through the proteasome pathway. These results offer an additional mechanism for the anti-inflammatory effects of PPARα agonists and point to the significance of proteasomes in the degradation of iNOS protein and as a target of anti-inflammatory drugs.

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Conflict of interest

The authors state no conflict of interest.

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RESEARCH ARTICLE Open Access

Anti-inflammatory properties of a dual PPARgamma/alpha agonist muraglitazar in *in vitro* and *in vivo* models

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Abstract

Introduction: Peroxisome proliferator-activated receptor (PPAR) agonists are widely used drugs in the treatment of diabetes and dyslipidemia. In addition to their metabolic effects, PPAR isoforms PPARα and PPARγ are also involved in the regulation of immune responses and inflammation. In the present study, we investigated the effects of a dual PPARγ/α agonist muraglitazar on inflammatory gene expression in activated macrophages and on carrageenan-induced inflammation in the mouse.

Methods: J774 murine macrophages were activated by lipopolysaccharide (LPS) and treated with dual PPARγ/α agonist muraglitazar, PPARγ agonist GW1929 or PPARα agonist fenofibrate. The effects of PPAR agonists on cytokine production and the activation of inducible nitric oxide synthase (iNOS) pathway were investigated by ELISA, Griess method, Western blotting and quantitative RT-PCR. Nuclear translocation, DNA-binding activity and reporter gene assays were used to assess the activity of nuclear factor kappa B (NF-kB) transcription factor. Carrageenan-induced paw oedema was used as an *in vivo* model of acute inflammation.

Results: Muraglitazar as well as PPARγ agonist GW1929 and PPARα agonist fenofibrate inhibited LPS-induced iNOS expression and NO production in activated macrophages in a dose-dependent manner. Inhibition of iNOS expression by muraglitazar included both transcriptional and post-transcriptional components; the former being shared by GW1929 and the latter by fenofibrate. All tested PPAR agonists also inhibited IL-6 production, while TNFα production was reduced by muraglitazar and GW1929, but not by fenofibrate. Interestingly, the anti-inflammatory properties of muraglitazar were also translated *in vivo*. This was evidenced by the finding that muraglitazar inhibited carrageenan-induced paw inflammation in a dose-dependent manner in mice as did iNOS inhibitor L-NIL and anti-inflammatory steroid dexamethasone.

Conclusions: These results show that muraglitazar has anti-inflammatory properties both *in vitro* and *in vivo* and these effects reflect the agonistic action through both PPARα and PPARγ.

Introduction

It has long been known that macrophages are pivotal cells in the pathogenesis of autoimmune diseases, including rheumatoid arthritis [1,2]. During the last decades macrophages have also been found to affect metabolism in various tissues and today it is widely agreed that macrophages are also associated with the pathophysiology of many obesity-linked diseases [3-5]. The mechanisms connecting macrophage activation and metabolism are not fully known but some hypotheses have been proposed. Macrophages are present in adipose tissue and when aberrantly activated in relation to obesity they produce inflammatory factors by themselves and also activate surrounding adipocytes to release signalling proteins called adipokines. Adipokines are known to regulate energy metabolism and appetite but also inflammatory responses, arthritis and catabolic processes in articular cartilage [6]. It is also obvious that obesity-related changes in energy

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metabolism directly regulate macrophage responses [7]. From the pharmacological point of view, an interesting question exists of if or how the macrophage phenotype and secretory profile differ between chronic inflammation typical, for example, in rheumatoid arthritis and obesity-related systemic inflammation, and if the same known and future anti-inflammatory compounds would have therapeutic value in only one or both of those inflammatory conditions.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and they are strongly linked to the regulation of energy homeostasis in cells. PPARs are expressed in three isoforms: PPARα, PPARγ and PPARβ/δ. PPARγ agonists thiazolidinediones (TZDs) and PPARα agonist fibrates are widely used as pharmacological agents in the treatment of diabetes and dyslipidemia, respectively. Although the main interest in PPAR-related studies has been focused on the role of PPARs in energy homeostasis, PPARs, especially PPARα and PPARγ, are also shown to be involved in the regulation of the immune and inflammatory responses in obesity-linked diseases [8-10]. During the last few years some reports have been published showing that PPARγ agonists reduce inflammatory responses in animal models of rheumatoid arthritis [11]. Nevertheless, we found only a single study suggesting that also a PPARα agonist fenofibrate would have anti-inflammatory effects in experimentally induced arthritis [12].

Muraglitazar is a dual PPARγ/α-agonist that has strong PPARγ and moderate PPARα effects [13]. Originally, it was developed for the treatment of type II diabetes with a view to combining both insulin sensitizing and antihyperlipidemic effects of the PPAR agonists. Muraglitazar has been shown to decrease the levels of HbA_{1c}, FFA and triglycerides and to increase the levels of HDL in humans when compared to placebo, and it appeared to be more potent than pioglitazone [14,15]. However, despite the fact that there are several reports on the antidiabetic effects of muraglitazar, there are no previous studies on the effects of muraglitazar in inflammatory processes.

In the present study, we aimed to investigate the anti-inflammatory effects of muraglitazar. Since the immunoregulatory effects of PPARα and PPARγ are somewhat different, we wanted to study whether muraglitazar has more potent anti-inflammatory effects than PPARγ or PPARα agonists alone. To examine this we investigated the effects of muraglitazar on the activation of the inducible nitric oxide synthase (iNOS) pathway and on the production of inflammatory cytokines in activated macrophages. We extended our study by testing the effect of muraglitazar on carrageenan-induced paw inflammation in the mouse.

Materials and methods

Materials

Reagents were obtained as follows: GW1929 and MG132 from Tocris Bioscience (Bristol, UK), N^6-(1-iminoethyl)-L-lysine (L-NIL) from Enzo Life Sciences Ltd. (Exeter, UK), rabbit polyclonal β-actin, lamin A/C and iNOS antibodies and goat HRP-conjugated anti-rabbit polyclonal antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Nuclear factor kappa B (NF-κB) subunit p65 antibody was from Cell Signaling Technology Inc. (Danvers, MA, USA). Muraglitazar was synthesised in the laboratory of Dr. Paula H. Aulaskari (University of Eastern Finland, Joensuu Campus, Joensuu, Finland), see below. Other reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA).

Muraglitazar was synthesised by a five-step procedure according to Devasthale *et al.* [13]. The structure and purity of intermediates and the final product muraglitazar were confirmed by melting point analysis (Gallenkamp melting point apparatus MFB-595, Gallenkamp, Loughborough, UK), ^1H, ^{13}C NMR spectroscopy (Bruker Avance 250 MHz and 400 MHz spectrometer, Bruker BioSpin AG, Fällanden, Switzerland) using deuteriumchloroform as a solvent and tetramethylsilane as a reference, and by IR spectroscopy (Nicolet Avatar 320 FT-IR spectrometer, Thermo Electron Scientific Instruments, LLC, Madison, WI, USA) using dry potassium bromide as a salt component of solid mixture. The molecular structure and the purity of muraglitazar were also confirmed by mass spectrometer and elemental analysis. The mass spectrometer measurements were performed on Bruker BioAPEX II 47e Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an InfinityTM cell, 4.7 Tesla 160-mm-bore superconducting magnet (Magnex Scientific Ltd., Abingdon, UK), and an external electron ionization (EI) or electrospray ion source (ESI) (Analytica of Branford Inc., Branford, CT, USA). Elemental analysis was performed on CE Instruments EA 1110 elemental analyser (CE Instruments Ltd., Rodano, MI, Italy). The results of elemental analysis were within ± 0.2% of the theoretical values.

Cell culture

Murine J774 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Lonza Group Ltd., Basel, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were seeded on 24-well plates for RNA extraction and

nitrite and ELISA measurements, on 24-well plates or 6-well plates for preparation of cell lysates for Western blotting, on 10 cm dishes for preparation of nuclear extracts for Western blotting and NF-κB p65 DNA binding assay and on 96-well plates for an XTT test. Confluent cultures were exposed to fresh culture medium containing the compounds of interest.

Human HEK293 cells (American Type Culture Collection) were cultured at 37°C in 5% CO_2 atmosphere in Eagle's minimal essential medium (Lonza Group Ltd.) supplemented with 0.15% sodium bicarbonate, 100 μM non-essential aminoacids, 1 mM sodium pyruvate, 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co.) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were seeded on 24-well plates for RNA extraction. Confluent cultures were exposed to fresh culture medium containing the compounds of interest.

Cell viability after treatment with combinations of LPS or cytokine mixture and the tested compounds was assessed by modified XTT test (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Preparation of stable J774-pGL4(miNOS-prom)neo and HEK293-pNF-κB(luc)neo reporter cell lines

The pGL-MNOS II-5'-Luc plasmid [16] containing 5'-flanking sequence (1,171 bp, positions -1,570 to +141; promoter and a part of exon 1) of the murine iNOS gene was provided by Professor Harmut Kleinert (Johannes Gutenberg University, Mainz, Germany). This plasmid was digested with *Kpn*I and *Hind*III, and the restriction fragment, containing murine iNOS promoter and part of exon 1, was then cloned into the *Kpn*I/*Hind*III site of firefly luciferase reporter plasmid pGL4.17(luc2/neo) (Promega, Madison, WI, USA) generating pGL4(miNOS-prom)neo, in which the luciferase gene is driven by murine iNOS promoter. The plasmid was sequenced to confirm the appropriate size, position and orientation of the insert in the plasmid. To create a stable transfection with murine iNOS promoter reporter plasmid, J774 cells were transfected with pGL4(miNOS-prom)neo using Lipofectamine 2000 (Invitrogen Co.) according to the manufacturer's instructions. Transfected cells were selected with G418 disulfate salt (Sigma-Aldrich Co.) treatment (800 μg/ml). After the selection, the survived clones were pooled to give rise to J774-pGL4(miNOS-prom)neo cell line and further cultured in the presence of 400 μg/ml of G418.

To create a stable transfection with NF-κB reporter plasmid, HEK293 cells were transfected with pGL4.32 [luc2P/NF-κB-RE/Hygro] (Promega) using Lipofectamine 2000 (Invitrogen Co.) according to the manufacturer's instructions. Transfected cells were selected with hygromycin B (EMD Biosciences Inc., La Jolla, CA, USA) treatment (200 μg/ml). After the selection, the survived clones were pooled to give rise to HEK293- pGL4.32[luc2P/NF-κB-RE/Hygro] cell line and further cultured in the presence of 100 μg/ml of hygromycin B.

Nitrite determination

Nitrous oxide (NO) production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous condition, into the culture medium. The culture medium was collected at indicated time points and nitrite was measured by the Griess reaction [17].

Preparation of cell lysates for Western blotting

At indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in cold lysis buffer containing 10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 minutes, lysates were centrifuged (13,400 *g*, 4°C, 10 minutes), supernatants were collected and mixed 3:1 with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analysed. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [18].

Preparation of nuclear extracts for Western blotting

At indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA). After incubation on ice for 10 minutes, the cells were vortexed for 30 seconds and the nuclei were separated by centrifugation at 4°C, 21,000*g* for 10 seconds. Nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA) and incubated on ice for 20 minutes. Nuclei were vortexed for 30 seconds and nuclear extracts were obtained by centrifugation at 4°C, 21,000 *g* for 2 minutes. Supernatants were collected and mixed 3:1 with SDS sample buffer. The samples were stored at -70°C until analysed. Coomassie blue method was used to measure the protein content of the samples [18].

Western blotting

Prior to Western blotting, samples were boiled for 10 minutes and 20 μg of protein was loaded per lane on 10% or 12% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After the transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk or 5% bovine serum albumin at room temperature for one hour and incubated with primary antibody in the blocking solution at 4°C overnight. The membrane was washed with TBS/T, incubated with secondary antibody in the blocking solution at room temperature for one hour and washed. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and ImageQuant LAS 4000 mini imaging system (GE Healthcare). The chemiluminescent signal was quantified with ImageQuant TL 7.0 image analysis software.

RNA extraction and quantitative real-time PCR

At the indicated time points, culture medium was removed and total RNA of the cultured cells was extracted using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.). For luciferase mRNA experiments, total RNA was treated with DNase I (Fermentas UAB, Vilnius, Lithuania). Total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the reverse transcription reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Total RNA of the tissue samples was extracted by GenElute™ Mammalian Total RNA Miniprep kit with proteinase K digestion (Sigma-Aldrich Co.). Total RNA (500 ng) was reverse-transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit (Fermentas UAB). cDNA obtained from the reverse transcription reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes for luciferase, IL-6, iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. The expression of mouse TNFα mRNA was measured by using TaqMan® Gene Expression Assay (Mm00443260_g1, Applied Biosystems, Foster City, CA, USA).

Table 1 Primer and probe sequences.

Gene	Oligonucleotide	Sequence 5′ → 3′
Human GAPDH	Forward primer	TCCTACCACCAGCAACCCTGCCA
	Reverse primer	GCAACAATATCCACTTTACCAGAGTTAA
	Probe	CGCCTGGTCACCAGGGCTGC
Luciferase	Forward primer	ACGGCTTCGGCATGTTCA
	Reverse primer	CTCCTCCTCGAAGCGGTACA
	Probe	TTGATCTGCGGCTTTCGGGTCGT
Mouse GAPDH	Forward primer	GCATGGCCTTCCGTGTTC
	Reverse primer	GATGTCATCATACTTGGCAGGTTT
Mouse IL-6	Probe	TCGTGGATCTGACGTGCCGCC
	Forward primer	TCGGAGGCTTAATTACACATGTTC
	Reverse primer	CAAGTGCATCATCGTTGTTCATAC
	Probe	CAGAATTGCCATTGCACAACTCTTTTCTCA
Mouse iNOS	Forward primer	CCTGGTACGGGCATTGCT
	Reverse primer	GCTCATGCGGCCTCCTT
	Probe	CAGCAGCGGCTCCATGACTCCC

PCR reaction parameters were as follows: incubation at 50°C for 2 minutes, incubation at 95°C for 10 minutes, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 minute. Each sample was determined in duplicate.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2.

NF-κB p65 DNA binding assay

DNA binding activity of NF-κB p65 was evaluated using NF-κB (p65) Transcription Factor Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The nuclear extracts for the assay were prepared according to the manufacturer's instructions and 10 μg of nuclear protein per well was used for the experiment. The samples were incubated at +4°C overnight with the dsDNA templates carrying NF-κB response element. After primary (anti-NF-κB p65) and secondary (goat anti-rabbit HRP) antibody treatments, developing reagents were added and absorbance was read at 450 nm.

Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples were kept at -20°C until assayed. The concentrations of IL-6 and TNFα in culture medium were determined by ELISA according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK).

Carrageenan-induced paw oedema in the mouse

Prior to the experiment investigating the time dependent formation of carrageenan-induced paw oedema, 36 male Charles River mice (weighing 25.0 to 30.0 g) were housed

and cared for under the guidelines of the institutional animal care and use committee with food and water provided *ad libitum*. The study was approved by the Animal Ethic Committee, Universiti Sains Malaysia.

The animals were randomly divided into six groups (six mice per group) and each group was treated orally with the test compound suspended in 2.0% (w/v) carboxymethyl cellulose (CMC) in distilled water. The test groups were as follows: control (treated with 2.0% CMC only), muraglitazar 12.5 mg/kg, muraglitazar 25 mg/kg, muraglitazar 50 mg/kg, L-NIL 50 mg/kg and dexamethasone 2 mg/kg. An hour after the treatment, 40 μl of carrageenan (1% suspension in normal saline) was injected subcutaneously into the left hind paws of the animals. Thicknesses of the paws were measured by micrometer one hour before and one hour, four hours and six hours after the carrageenan injection. The results are expressed as percentages of swelling calculated as follows:

Difference = (thickness of the hind paw at indicated time point − thickness of the hind paw before carrageenan)/thickness of the hind paw before carrageenan.

The carrageenan-induced gene expression was investigated in C57BL/6 mice. The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective provincial committee for animal experiments. Animals were housed under standard conditions of light, temperature and humidity (12:12 h light-dark cycle, 22 ± 1°C, 50 to 60%) with food and water provided *ad libitum*.

Mice were randomly divided into two study groups with five and seven mice in the groups and muraglitazar 50 mg/kg was injected intraperitoneally into the group of five mice. Two hours after the treatment, the mice were anesthetized with an intraperitoneal injection of 0.5 mg/kg of medetomidine (Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Pfizer Oy Animal Health, Helsinki, Finland), and 30 μl of λ-carrageenan (1.5% suspension in normal saline) was injected subcutaneously into a hind paw of the animals. As a control, 30 μl of saline was injected into the contralateral paws. The paw volumes were measured before and six hours after carrageenan injection by plethysmometer (Ugo Basile Srl, Comerio, Italy). Oedema is expressed as the difference between the change in carrageenan-treated paw volume and the control paw volume in μl. After six hours of carrageenan injection, the mice were sacrificed by cervical dislocation. Carrageenan-treated and control paws were skinned and the soft tissues of the paws were collected and RNA was extracted as described above.

Statistics

Results are expressed as mean + standard error of mean (SEM). When indicated, statistical significance was calculated by analysis of variance followed by Dunnett's multiple comparisons test or Mann-Whitney test. Differences were considered significant at $P < 0.05$.

Results

Muraglitazar *and PPARα and PPARγ agonists decreased NO production and iNOS expression*

Resting J774 macrophages did not produce detectable amounts of NO, but when the cells were activated through TLR4 pathway by bacterial endotoxin LPS, NO production and iNOS expression were increased. Muraglitazar, PPARα agonist fenofibrate and PPARγ agonist GW1929 decreased LPS-induced iNOS expression (Figure 1) and NO production (Figure 2) in a dose-dependent manner. Muraglitazar, fenofibrate or GW1929 did not affect cell viability at the concentration used as determined by XTT test.

Muraglitazar and a PPARγ agonist, but not a PPARα agonist, reduced iNOS mRNA expression

Since all three PPAR agonists decreased iNOS expression and NO production, we went further and determined the effects of PPAR agonists on iNOS mRNA expression. Muraglitazar and GW1929 reduced the levels of iNOS mRNA when determined six hours after the addition of LPS. However, fenofibrate had no effect on iNOS mRNA levels as compared to the cells treated with LPS only. NF-κB inhibitor MG132 was used as a control compound and it reduced LPS-induced iNOS mRNA levels as expected (Figure 3a).

Since the reduction of iNOS mRNA levels may be a sign of either transcriptional inhibition or increase in iNOS mRNA degradation, we studied the effect of PPAR agonists on the half-life of iNOS mRNA. After six-hour incubation with LPS alone or together with PPAR agonists, actinomycin D was added to the cells to stop mRNA synthesis. Incubations were terminated at different time points after the addition of actinomycin D. None of the PPAR agonists did affect the stability of iNOS mRNA when compared to the cells treated with LPS only (Figure 3b). These results suggest that the suppressive effect of muraglitazar and GW1929 on LPS-induced iNOS mRNA levels is mediated at the level of iNOS transcription.

We further investigated the effect of muraglitazar on the activity of iNOS promoter in J774 macrophages stably transfected to express luciferase reporter gene under the control of full length murine iNOS promoter. Similar to the effects on iNOS mRNA levels in wild-type J774 cells, muraglitazar and GW1929, but not fenofibrate, reduced LPS-induced iNOS promoter activity (Figure 3c).

The different effects of fenofibrate and GW1929 on iNOS mRNA expression may be explained by our previous findings that PPARα agonists regulate iNOS expression at the post-transcriptional level [19]. To

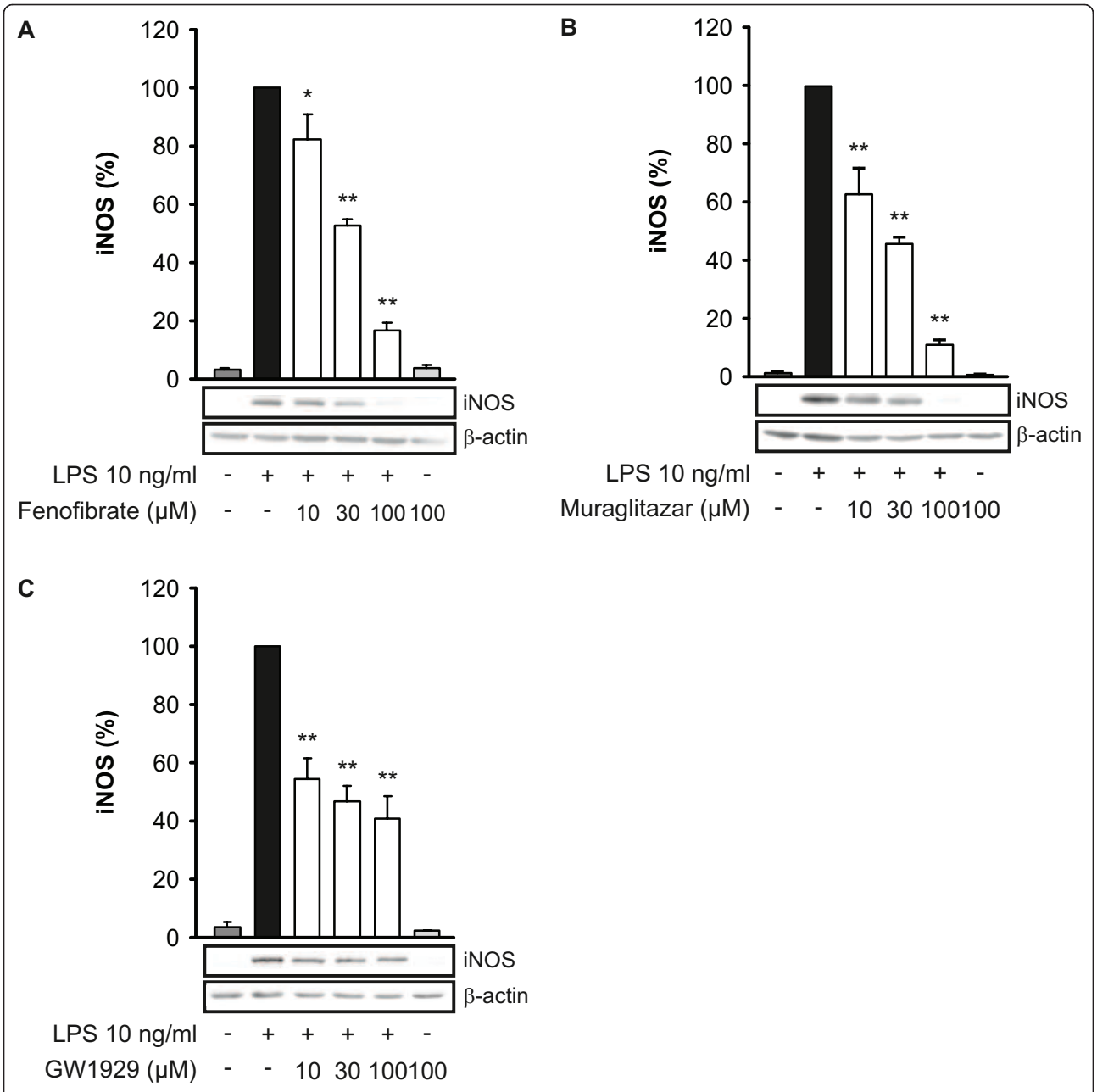

Figure 1 Effects of PPAR agonists on iNOS protein expression in J774 macrophages. Cells were stimulated with lipopolysaccharide (LPS) and treated with increasing concentrations of fenofibrate (**a**), muraglitazar (**b**) or GW1929 (**c**). After 24 h incubation, proteins were extracted and the levels of inducible nitric oxide synthase (iNOS) protein were analysed by Western blotting. β-actin was used as a loading control. Results represent the mean + SEM of three experiments, from which one representative Western blot is shown. *P <0.05 and **P <0.01 as compared to cells treated with LPS alone.

study the post-transcriptional effects of muraglitazar and the other two PPAR agonists on iNOS protein levels, we first stimulated J774 macrophages with LPS for 10 hours, and PPAR agonists were added thereafter for 14 hours. Muraglitazar and fenofibrate, but not GW1929, were able to reduce iNOS protein levels in these post-transcriptional time points (Figure 3d).

Effects of muraglitazar and PPARα and PPARγ agonists on TNFα and IL-6 production

In order to investigate if PPAR agonists regulate the expression of other genes related to innate immunity, we measured their effects on IL-6 and TNFα production. LPS induced TNFα and IL-6 production in J774 cells when measured with ELISA in the culture medium

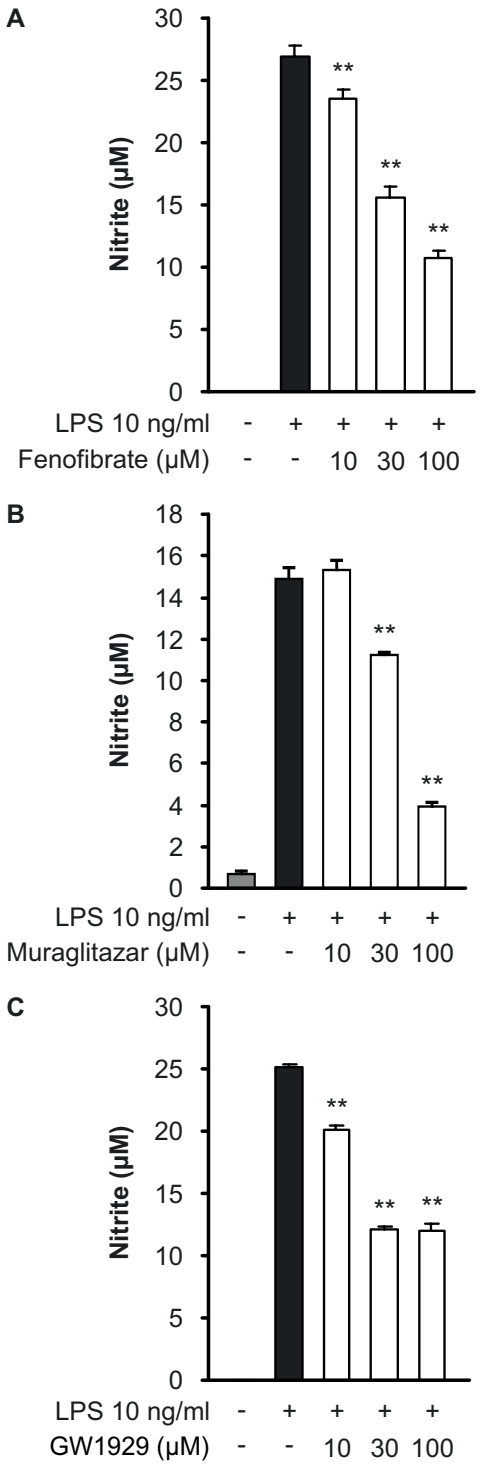

Figure 2 Effects of PPAR agonists on NO production in J774 macrophages. Cells were stimulated with lipopolysaccharide (LPS) and treated with increasing concentrations of fenofibrate (**a**), muraglitazar (**b**) or GW1929 (**c**). After 24 h incubation, nitrite accumulated into the culture medium was measured by Griess reaction as a marker of nitric oxide (NO) production. Results represent the mean + SEM (n = 6). **P <0.01 as compared to cells treated with LPS alone.

after 24 hours incubation. All the agonists reduced IL-6 production in a dose-dependent manner (Figure 4). However, LPS-induced TNFα production was reduced only by GW1929 and muraglitazar at the highest concentration used (Figure 5c, b). Fenofibrate, on the other hand, tended to increase TNFα production at higher concentrations (Figure 5a).

Muraglitazar or PPARα or PPARγ agonists had no effect on NF-κB activation

As we have reported in this paper, muraglitazar reduces the synthesis of iNOS, IL-6 and TNFα. The syntheses of all these inflammatory markers are regulated by transcription factor NF-κB [20,21]. Therefore, we hypothesized that muraglitazar might affect the activity of NF-κB.

To test this, we first investigated the effect of muraglitazar on the nuclear translocation of NF-κB. LPS enhanced the nuclear translocation of NF-κB peaking at 30 minutes of stimulation. Muraglitazar, fenofibrate or GW1929 did not affect the nuclear levels of NF-κB p65 when compared to cells treated with LPS only (Figure 6a).

We continued by studying whether PPAR agonists affect the DNA binding activity of NF-κB. As illustrated in Figure 6b, muraglitazar, fenofibrate or GW1929 did not reduce the ability of NF-κB to bind to dsDNA fragments containing NF-κB response element. NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used as a positive control compound and it reduced LPS-induced NF-κB binding as expected.

Further, we evaluated the effect of PPAR agonists on NF-κB -mediated transcription using HEK293 cells stably transfected with a luciferase reporter gene under the control of NF-κB-responsive promoter. PPAR agonists did not modulate NF-κB-mediated transcription while NF-κB inhibitor PDTC had an effect as expected (Figure 6c).

Muraglitazar reduced carrageenan-induced inflammatory responses in the mouse

To determine if the anti-inflammatory properties of muraglitazar are also translated to *in vivo* situations, we examined the effect of muraglitazar on carrageenan-induced inflammatory paw oedema in mice. Muraglitazar prevented the development of oedema in a dose-dependent manner (Figure 7). With the highest dose of muraglitazar (50 mg/kg) used, carrageenan-induced oedema was reduced by 54% at the six-hour time point. Dexamethasone 2 mg/kg and iNOS inhibitor L-NIL 50 mg/kg were used as control compounds and they reduced the oedema by 69% and 48%, respectively.

In the following series of experiments, we analysed the effect of muraglitazar on inflammatory gene expression

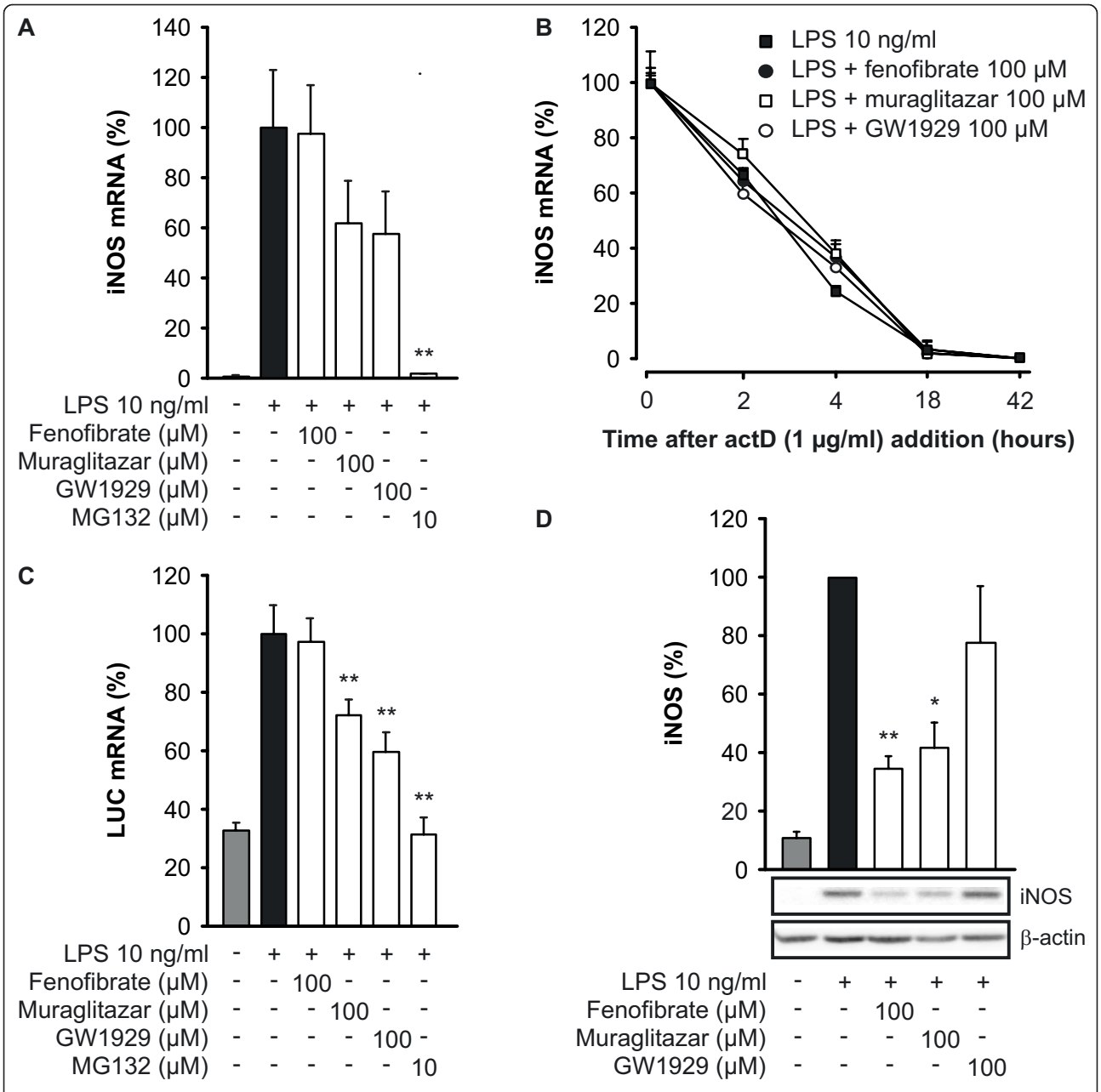

Figure 3 Effects of PPAR agonists on iNOS expression in activated J774 macrophages. (**a**) Effects of peroxisome proliferator-activated receptor (PPAR) agonists on inducible nitric oxide synthase (iNOS) mRNA expression. Cells were treated with PPAR agonists, NF-κB inhibitor MG132 or vehicle (dimethyl sulfoxide, DMSO) and stimulated with LPS for six hours. Total RNA was extracted and iNOS mRNA was determined by RT-PCR. The results were normalized against GAPDH mRNA. (**b**) Effects of PPAR agonists on iNOS mRNA degradation. Cells were treated with PPAR agonists or vehicle (DMSO) and stimulated with lipopolysaccharide (LPS). After six hours incubation, actinomycin D (1 μg/ml) was added to the cell culture to stop transcription. Total RNA was extracted at indicated time points after actinomycin D addition, and iNOS mRNA was determined by RT-PCR. The results were normalized against GAPDH mRNA. (**c**) Effects of PPAR agonists on iNOS promoter activity in J774 macrophages stably transfected with a luciferase (LUC) reporter gene under the control of iNOS protein. Cells were treated with PPAR agonists, NF-κB inhibitor MG132 or vehicle (DMSO) and stimulated with LPS for six hours. Total RNA was extracted and LUC mRNA was determined by RT-PCR. The results were normalized against GAPDH mRNA. (**d**) Post-transcriptional effects of PPAR agonists on iNOS protein levels. Cells were stimulated with LPS for 10 hours. After the stimulation, the culture medium was changed and the cells were further incubated with PPAR agonists or vehicle (DMSO) without LPS for additional 14 hours. Then, proteins were extracted and the levels of iNOS protein were analysed by Western blotting. β-actin was used as a loading control. Results represent the mean + SEM (n = 3 to 4). In d one representative Western blot is shown. *P <0.05 and **P <0.01 as compared to cells treated with LPS alone.

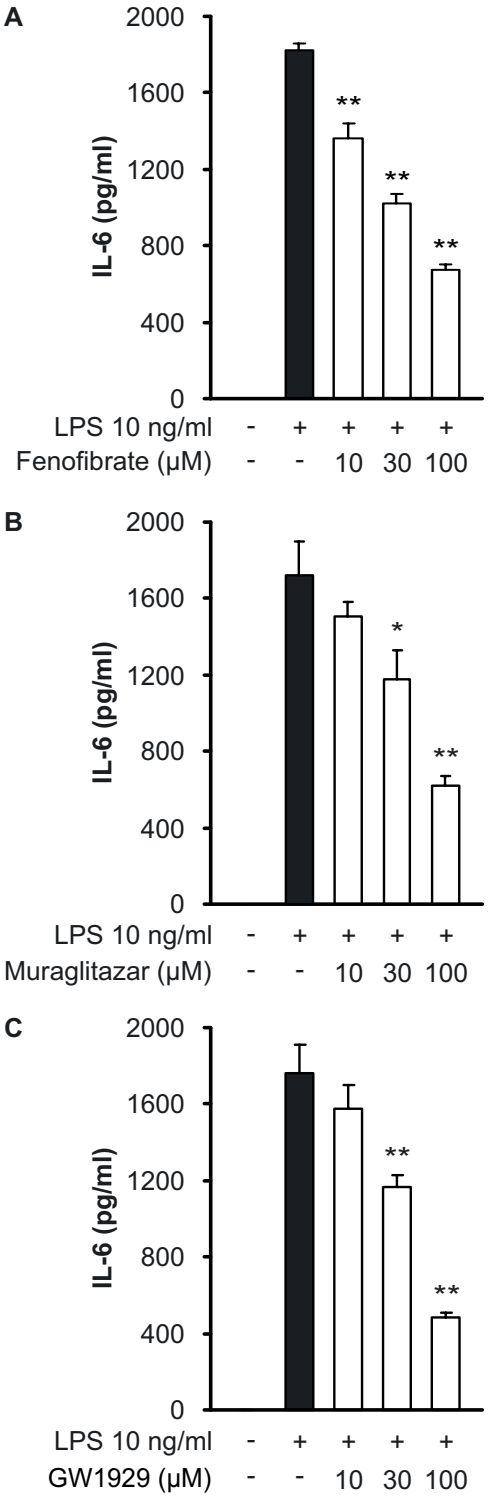

Figure 4 Effects of PPAR agonists on IL-6 production in J774 macrophages. Cells were treated with increasing concentrations of fenofibrate (**a**), muraglitazar (**b**) or GW1929 (**c**) and stimulated with lipopolysaccharide (LPS). After 24 h incubation, IL-6 accumulated into the culture medium was measured by ELISA. Results represent the mean + SEM (n = 4). *P <0.05 and **P <0.01 as compared to cells treated with LPS alone.

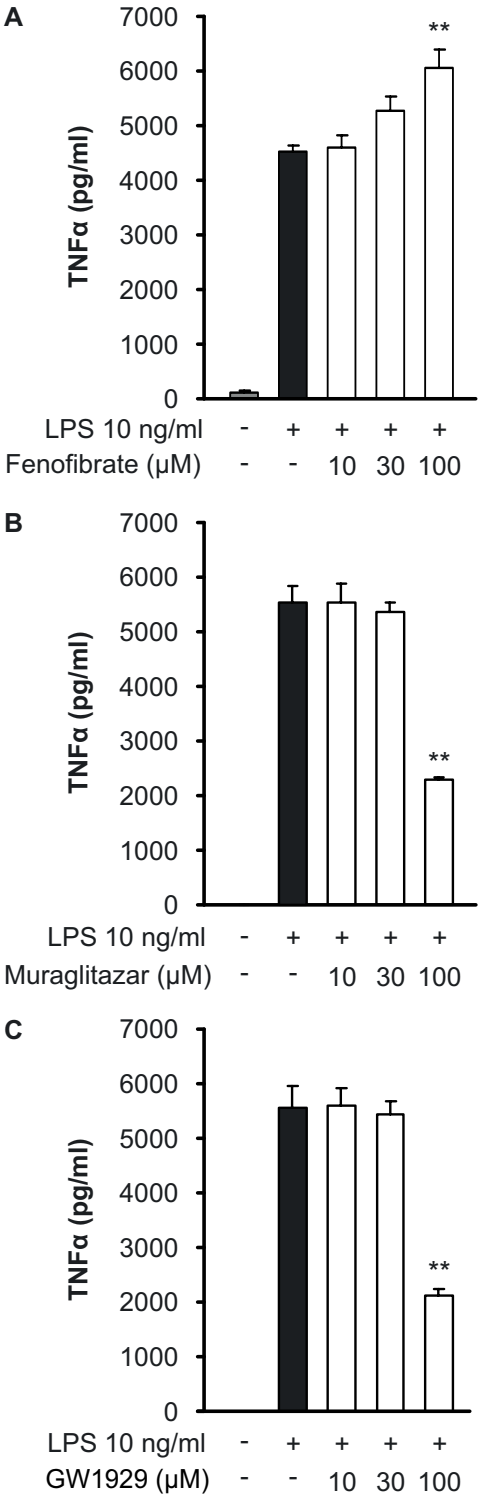

Figure 5 Effects of PPAR agonists on TNFα production in J774 macrophages. Cells were treated with increasing concentrations of fenofibrate (**a**), muraglitazar (**b**) or GW1929 (**c**) and stimulated with lipopolysaccharide (LPS). After 24 h incubation, TNFα accumulated into the culture medium was measured by ELISA. Results represent the mean + SEM (n = 4). *P <0.05 and **P <0.01 as compared to cells treated with LPS alone.

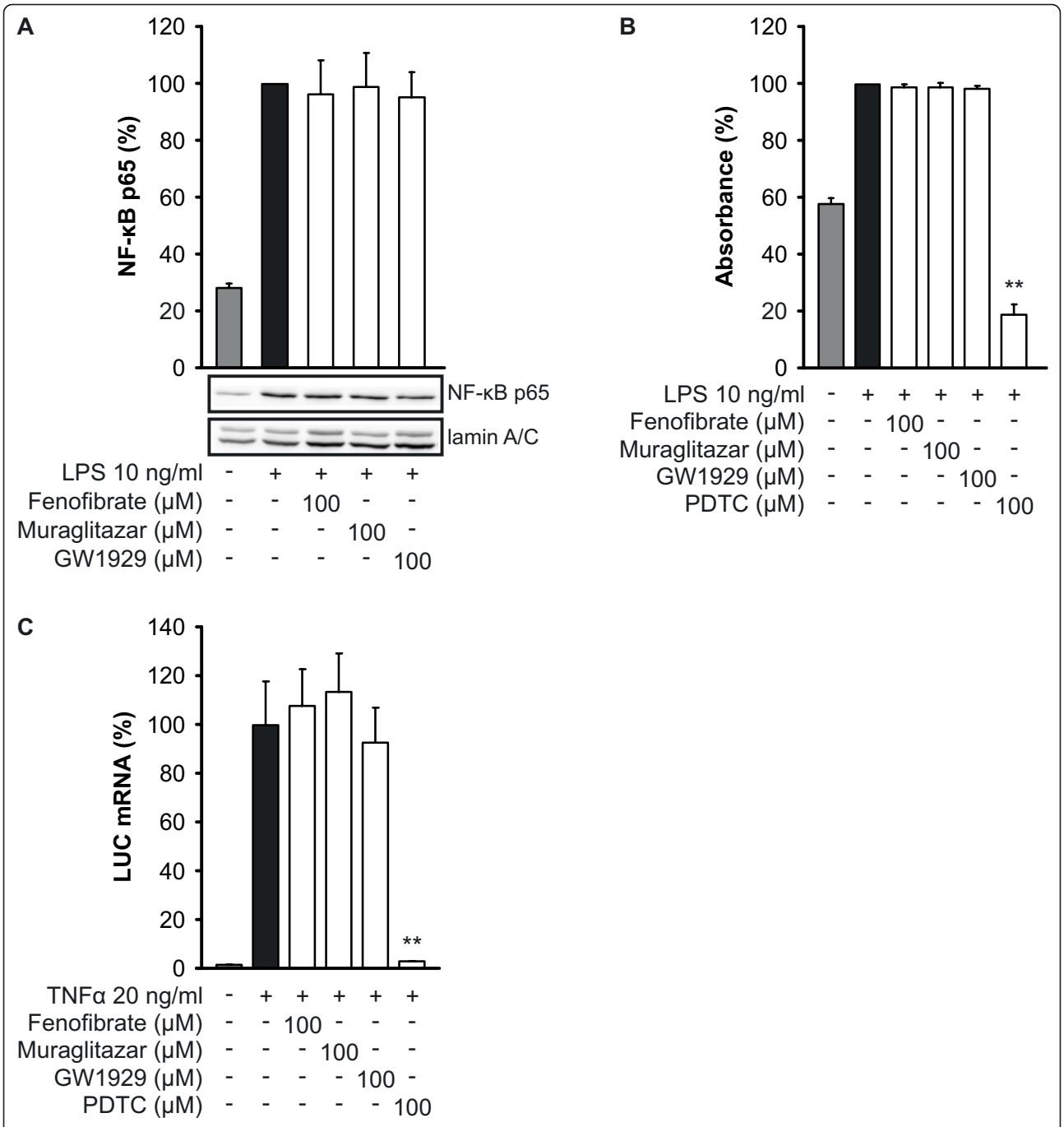

Figure 6 Effects of PPAR agonists on NF-κB activation and NF-κB-mediated transcription. (**a**) Effects of peroxisome proliferator-activated receptor (PPAR) agonists on NF-κB translocation to nucleus. J774 cells were preincubated with PPAR agonists or vehicle (dimethyl sulfoxide, DMSO) for two hours before addition of lipopolysaccharide (LPS). After 30 minutes incubation with LPS, nuclear extracts were prepared. The levels of NF-κB p65 in nuclear extracts were analysed by Western blotting. Lamin A/C was used as a loading control. (**b**) Effects of PPAR agonists on DNA binding activity of NF-κB. J774 cells were preincubated with PPAR agonists, NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) or vehicle (DMSO) for two hours before addition of LPS. After one hour incubation with LPS, nuclear extracts were prepared and the binding activity of NF-κB p65 to DNA was analysed as described in methods. (**c**) Effects of PPAR agonists on NF-κB-mediated transcription activity. HEK293 cells were stably transfected with a luciferase (LUC) reporter gene under the control of NF-κB-responsive promoter. The cells were preincubated with PPAR agonists, NF-κB inhibitor PDTC or vehicle (DMSO) for two hours before addition of TNFα. After six hours' incubation with TNFα, total RNA was extracted and LUC mRNA was determined by RT-PCR. The LUC mRNA levels were normalized against GAPDH mRNA. Results represent the mean + SEM (n = 3 to 4). In a one representative Western blot is shown. **P <0.01 as compared to cells treated with LPS or TNFα alone.

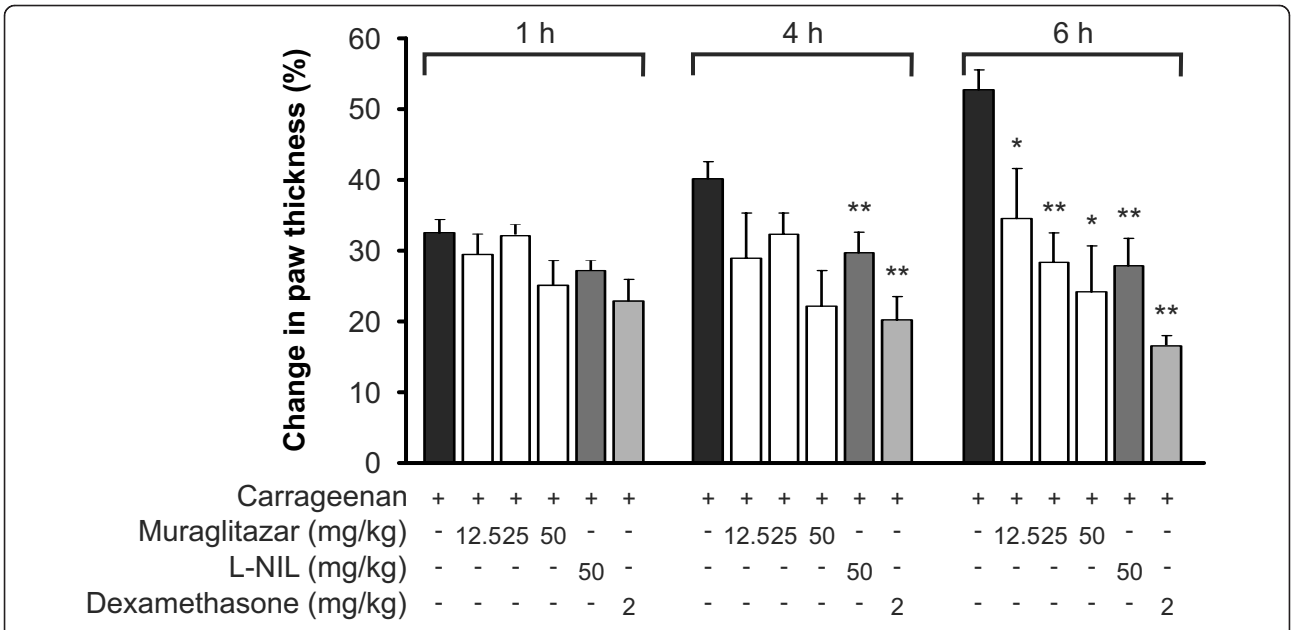

Figure 7 Effect of muraglitazar on carrageenan-induced paw oedema in the mouse. Muraglitazar, inducible nitric oxide synthase (iNOS) inhibitor L-NIL, dexamethasone or vehicle (2.0% CMC) were given to animals per os 1 hour prior to carrageenan injection (1%). Paw oedema was measured before and one, four and six hours after carrageenan injection by micrometer. Oedema is expressed as increase in paw thickness at indicated time points compared to thickness before carrageenan. Values are mean + SEM (n = 6). *P <0.05 and **P <0.01 as compared to mice treated with carrageenan alone.

in carrageenan-induced inflammation. In line with the previous series, muraglitazar attenuated carrageenan-induced paw oedema (Figure 8a). Muraglitazar also decreased the levels of IL-6, TNFα and iNOS mRNA by 80%, 54% and 64%, respectively (Figure 8b-d).

Discussion

The present findings show that muraglitazar has anti-inflammatory properties both in *in vitro* and *in vivo* models. The results suggest that the anti-inflammatory effects of muraglitazar reflect the agonistic action through both PPARα and PPARγ. To our knowledge, this is the first report describing the effects of dual PPARγ/α agonists on inflammatory responses.

In support of the present findings, previous reports also describe the inhibitory effects of PPARγ or PPARα agonists on inflammatory mediators in cell culture experiments [22]. PPARγ and PPARα ligands have been reported to decrease plasma levels of proinflammatory cytokines in diabetic and hyperlipidemic patients [23]. In our experiments, PPARα and PPARγ agonists had somewhat different effects on IL-6, TNFα and iNOS expression. All the agonists reduced IL-6 production in a similar potency. However, fenofibrate was not able to decrease TNFα production or iNOS mRNA levels, contrary to the effects of GW1929 or muraglitazar. On the other hand, unlike GW1929, muraglitazar and fenofibrate were able to decrease iNOS protein levels still at post-transcriptional time points. This suggests that activation of PPARα and PPARγ regulate different pathways in the LPS-activated inflammatory cascades. The effects of PPAR agonists on iNOS expression are supported by our previous findings showing that PPARα agonists WY14643 and GW7647 regulate iNOS expression by enhancing its degradation [19]. The present results imply that the effect of muraglitazar on TNFα production is mediated by the PPARγ component of action and on IL-6 production and iNOS expression by both PPARα and PPARγ components of action. Especially, as for the inhibition of iNOS expression, muraglitazar seems to be a more potent inhibitor than fenofibrate or GW1929 alone. This is explained by the findings that PPARγ (but not PPARα) activation inhibits iNOS transcription and PPARα (but not PPARγ) activation has an effect at post-transcriptional level, while muraglitazar (as expected) has both of those effects. Thus the results suggest that combining the effects of PPARα and PPARγ by using a dual PPARγ/α agonist results in improved anti-inflammatory action as compared to PPARα or PPARγ agonists alone.

In the present study, muraglitazar reduced iNOS mRNA expression and promoter activity but did not affect the activity of NF-κB, which is an important transcription factor for iNOS and IL-6 genes. According to our preliminary experiments, muraglitazar did not affect the activation of STAT1 or IRF1 either. Although the activity of NF-κB was not altered, it is possible that muraglitazar regulates

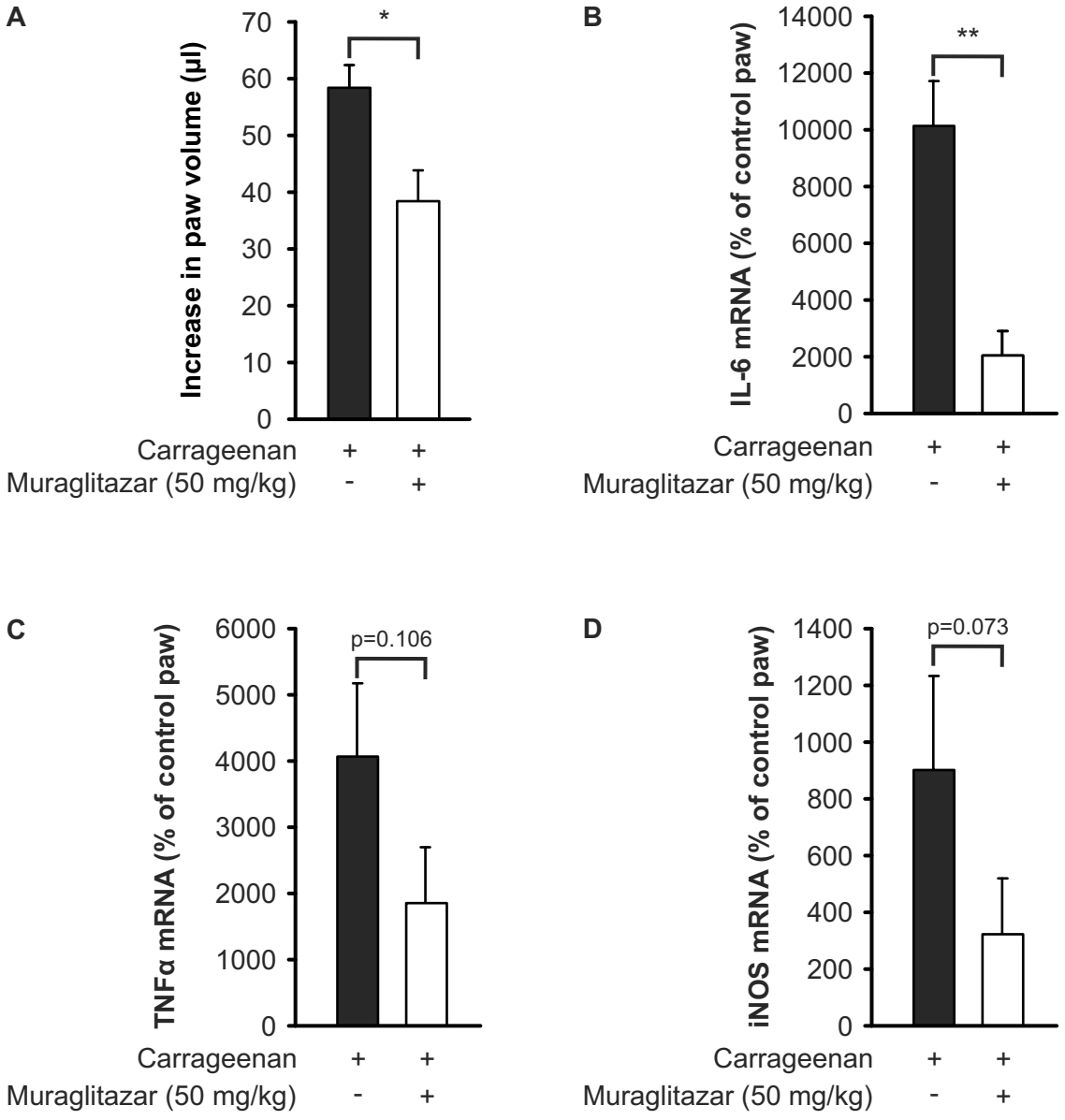

Figure 8 Effect of muraglitazar on carrageenan-induced production of inflammatory mediators in the mouse. Muraglitazar was administrated to animals intraperitoneally two hours prior to carrageenan injection (1.5%). (**a**) Paw oedema was measured before and six hours after carrageenan injection by plethysmometer. Oedema is expressed as difference between carrageenan-treated paw and control paw. (**b-d**) Six hours after carrageenan injection, the animals were sacrificed and total RNA was extracted from subcutaneous connective tissue of carrageenan-injected and control paw. IL-6, TNFα and inducible nitric oxide synthase (iNOS) mRNA were determined by RT-PCR. The results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and mRNA levels in carrageenan-treated paw were compared to those in control paw. Values are mean + SEM (n = 5 to 7). *P <0.05 and **P <0.01 as compared to mice treated with carrageenan alone.

the transcription machinery in a more complicated manner. Still, further studies are needed to uncover the detailed target of muraglitazar in the transcriptional activation of iNOS.

There is evidence suggesting that PPARγ and PPARα agonists attenuate experimentally induced arthritis in murine models. Tomita *et al*. [24] and Sumariwalla *et al*. [25] showed that PPARγ agonists THR0921 and CLX-090717, respectively, reduced clinical signs of synovitis in collagen-induced arthritis in the mouse. THR0921 also decreased the circulating levels of IgG antibody to the collagen used in immunization [24]. Koufany *et al*. [26] reported that thiazolidinediones rosiglitazone and pioglitazone reduced synovial expression of TNFα, IL-1β and basic fibroblast growth factor and clinical signs of synovitis in Freund's adjuvant-induced

arthritis in rats. However, less is known about the possible effects of PPARα agonists in inflammation models. Okamoto *et al*. [12] showed that fenofibrate decreased signs of arthritis and, to a lesser extent, paw oedema in Freund's adjuvant-induced arthritis in rats.

In the present study, we extend the previous data by showing that muraglitazar significantly decreased carrageenan-induced paw inflammation in mice. Carrageenan-induced paw oedema is a widely used model in inflammation research. It represents features of acute inflammation and innate immune responses and is useful for primary screening of therapeutic efficacy of novel anti-inflammatory agents [27]. Carrageenan-induced inflammation has been described to occur in two phases. The first and short-lasting phase starts almost immediately after the injection of carrageenan. The inflammation during this phase is local and the most abundant mediators are histamine, serotonin and kinins. The second phase starts in one to two hours after carrageenan injection and has been described to involve the activation of inflammatory cells, including macrophages. The expression of iNOS and COX-2, and the synthesis of prostaglandins, oxygen-derived free radicals, TNFα, IL-1β and IL-6 are increased in the inflamed tissue [28-30]. In our experiments, muraglitazar did not reduce paw oedema at early time points, that is, during the first phase of inflammation, while the anti-inflammatory effects at later time points were clear. The results of the effects of muraglitazar on paw oedema and inflammatory gene expression in mice are in line with our results *in vitro*, where muraglitazar reduced the production of NO, IL-6 and TNFα and the expression of iNOS. These results show that muraglitazar has anti-inflammatory potency also *in vivo*.

During the last years the safety aspects of PPAR agonists have been discussed. Rosiglitazone was withdrawn from the market in Europe in September 2010, since it had been found to increase the risk of myocardial infarction in diabetic patients [31]. Fibrates, on the other hand, were suggested to increase the risk of myopathy, hepatotoxicity, cholecystitis and deep venous thrombosis [32]. However, in detailed studies, the increased risk of myocardial infarction has specifically been linked to rosiglitazone [33], while the other thiazolidinedione on the market, pioglitazone, seems to be a rather safe drug [34,35]. In addition, fibrates did not show increased risk of serious drug-related adverse events in a recently published meta-analysis when they were used as a monotherapy for the treatment of dyslipidemia [36]. Thus, it seems so far that the side effects of PPAR agonists are compound-dependent and not associated with the mechanism of action, and the advantages of these drugs in diabetes and dyslipidemia are markedly greater than the possible disadvantages. Muraglitazar has been reported to have similar cardiovascular side effects as rosiglitazone, at least when combined with sulfonylureas or metformin [37]. Because of the adverse effects, muraglitazar's developer Bristol-Myers Squibb decided to discontinue further development of the drug in May 2006. Nevertheless, although the safety data published need to be considered, it is worth noticing that only little knowledge of using muraglitazar in nondiabetic patients is available. Thus, further studies are needed to establish the safety of muraglitazar and other dual PPARγ/α agonists in other possible indications than diabetes.

The connection between metabolism and inflammation is interesting and there is lots of research activity going on in that area at this time. As many studies have already suggested, PPARs might be an important link between these two complex systems, and as shown in the present study, PPAR agonists have anti-inflammatory properties in classical inflammatory models. It has been shown that a good treatment of metabolic diseases reduces the low-grade inflammation associated with obesity [38]. But could the modification of metabolic pathways reduce the inflammatory responses associated to classical inflammatory diseases? This is a question we will be interested in answering in the future.

Conclusions

The present study shows that muraglitazar has several anti-inflammatory effects in activated macrophages and in carrageenan-induced inflammation in the mouse, reflecting its activity on both PPARα and PPARγ. The results strengthen the previous evidence of the connection of metabolism and inflammation. Understanding this connection in more detail might open a new avenue in the treatment of chronic inflammatory diseases in the future.

Abbreviations

CMC: carboxymethyl cellulose; DMSO: dimethyl sulfoxide; EI: electron ionization; ESI: electrospray ion source; FTICR: Fourier transform ion cyclotron resonance; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-6: interleukin 6; iNOS: inducible nitric oxide synthase; L-NIL: N^6-(1-iminoethyl)-L-lysine; LPS: lipopolysaccharide; NF-κB: nuclear factor kappa B; NO: nitric oxide; PDTC: pyrrolidine dithiocarbamate; PPAR: peroxisome proliferator-activated receptor; SEM: standard error of mean; TBS: tri-buffered saline; TNFα: tumour necrosis factor α; TZDs: thiazolidinediones

Authors' contributions

EP carried out most of the experiments, participated in the design of the study and interpretation of the results, and drafted the manuscript. TL and ML participated in the experimental analyses and design of the study. MFY and MZA carried out the first series (Figure 7) of the *in vivo* assays. AK and PHA synthesized muraglitazar. EM conceived and coordinated the study, and supervised its design and conduction and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Research Article **Open Access**

Erja-Leena Paukkeri, Antti Pekurinen, Eeva Moilanen

Peroxisome proliferator-activated receptor α and γ agonists differently regulate classical and alternative macrophage activation

Abstract: Peroxisome proliferator-activated receptor (PPAR) agonists, fibrates and thiazolidinediones, are commonly used drugs in the treatment of dyslipidemia and diabetes. Their targets, PPARα and PPARγ, have also been shown to have a role in the regulation of inflammatory responses linking metabolism and inflammation. In the present study we investigated the effects of PPAR agonists on macrophage activation. In addition to the proinflammatory classical activation, we also focused on interleukin (IL) 4 and 13 -induced alternative activation which is a significant macrophage phenotype in tissue repairing processes and in fibrosing diseases. PPARα agonists GW7647 and fenofibrate as well as PPARγ agonist GW1929 inhibited lipopolysaccharide-induced classical macrophage activation and production of the characteristic biomarkers of this phenotype, i.e. IL-6 and nitric oxide, in murine J774 macrophages. Remarkably, the PPARα agonists also inhibited IL-4 and IL-13 –induced expression of alternative activation markers arginase-1, fizz1 and mannose receptor 1 whereas the PPARγ agonist GW1929 enhanced their expression in J774 macrophages. The PPARα agonists GW7647 and fenofibrate also attenuated the production of alternative activation markers chemokine (C-C motif) ligand 13 and platelet-derived growth factor in human THP-1 macrophages. The present findings show that PPARα and PPARγ agonists differently regulate classical and alternative macrophage phenotypes. Furthermore, PPARα activation was introduced as a novel concept to down-regulate alternative macrophage activation indicating that PPARα agonists have therapeutic potential in conditions associated with aberrant alternative macrophage activation such as fibrosing diseases.

Keywords: Macrophage polarization, peroxisome proliferator-activated receptor, fibrosis

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1 Introduction

Macrophages are important effector cells in inflammatory responses. They are involved in bacterial infections and autoimmune diseases but also in the low grade inflammation associated with obesity and metabolic syndrome. Macrophages also have homeostatic functions and they activate healing process associated with inflammation and tissue injury [1-3]. To achieve these multiform tasks, macrophages are programmed to display distinct phenotypes in response to environmental signals [2-5]. For example, Th1 cytokine interferon γ (IFNγ) and toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) trigger classical, i.e. type M1, activation of macrophages, which is typical for bacterial infections and autoimmune reactions [6]. On the other hand, Th2 cytokines interleukin (IL) 4 and 13 trigger alternative, i.e. type M2, activation, which is associated with wound healing but also with the aberrant immune responses associated with over-activation of fibroblasts and subsequent fibrosis [6,7].

Classical activation of macrophages leads to the release of proinflammatory and antimicrobial factors including IL-6, IL-12, tumour necrosis factor (TNF) and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) pathway. On the other hand, alternatively activated macrophages express factors that have been associated with recognition of extracellular pathogens and/or stimulation of fibroblasts. Some of

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these factors are arginase 1, chemokine (C-C motif) ligand 13 (CCL13), platelet-derived growth factor (PDGF), mannose receptor 1 (mrc-1) and, in murine macrophages, found in inflammatory zone 1 (fizz1), for instance [4,8-10]. Likewise, also in clinical settings, alternatively activated macrophages have been linked to defence against extracellular pathogens, and to resolution of inflammation, wound healing and fibrosis [11-13].

In addition to IL-4 and IL-13, peroxisome proliferator-activated receptor γ (PPARγ) ligands have been found to polarize macrophages towards the alternative phenotype [14,15]. PPARs are nuclear receptors that have a crucial physiological role in the regulation of energy homeostasis. PPARγ regulates the import of glucose into cells, and PPARγ agonists thiazolidinediones (TZDs) are widely used as pharmacological agents in the treatment of type 2 diabetes. Another isotype of PPARs, PPARα is linked to the regulation of lipid metabolism. Synthetic ligands include fibrates, which are used to treat dyslipidemia. As compared to PPARγ agonists, less is known about the possible involvement of PPARα agonists in the regulation of immune and inflammatory responses [16-19] and their effects on alternative activation of macrophages remains unknown.

In the present study, we aimed to compare the effects of PPARα and PPARγ agonists on classical and alternative activation of macrophages. We used LPS or a combination of IL-4 and IL-13 to induce macrophage polarization to classical or alternative phenotype, respectively. The results show that PPARα and PPARγ agonists differently regulate macrophage phenotypes, and introduce PPARα activation as a novel concept to down-regulate alternative macrophage activation.

2 Materials and methods

2.1 Materials

Reagents were obtained as follows: GW1929 and GW7647 from Tocris Bioscience (Bristol, UK), rabbit polyclonal β-actin, arginase 1 and STAT6 antibodies and goat HRP-conjugated anti-rabbit and donkey HRP-conjugated anti-goat polyclonal antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). pSTAT6 (Tyr^{641}) antibody was from Calbiochem (Merck Millipore, Billerica, MA, USA) and PPARα and PPARγ antibodies from Alexis Biochemicals (Enzo Life Sciences, Lausen, Switzerland). Recombinant mouse IL-4 and IL-13 and human IL-4 were from R&D Systems (Minneapolis, MN, USA). Other reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise stated.

2.2 Cell culture

Murine J774A.1 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Invitrogen Co., Carlsbad, CA, USA) and harvested with trypsin-EDTA (Invitrogen Co.). Cells were seeded on 24-well plates for RNA extraction, nitrite and ELISA measurements and for preparation of cell lysates for western blotting and on 96-well plates for XTT test. Confluent cultures were exposed to fresh culture medium containing the compounds of interest. PPAR agonists were added to the medium together with LPS or a combination of IL-4 and IL-13 unless otherwise stated.

Human THP-1 promonocytes (American Type Culture Collection) were cultured at 37°C in 5% CO_2 atmosphere in RPMI 1640 (Lonza Group Ltd) adjusted to contain 2 mM L-glutamine (Lonza Group Ltd), 10 mM HEPES (Lonza Group Ltd), 4.5 g/l glucose (Sigma-Aldrich Co.) and 1.5 g/l bicarbonate (Lonza Group Ltd) and supplemented with 10% heat-inactivated foetal bovine serum (Lonza Group Ltd), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B (Invitrogen Co.), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich Co.). The cells were differentiated by adding phorbol ester 12-O-tetradecanoylphorbol-13-acetate (100 nM) (Sigma-Aldrich Co.) at the time of seeding on 24-well plates. Seventy-two hours after the seeding the cultures were confluent and they were exposed to fresh culture medium containing the compounds of interest. PPAR agonists were added to the medium together with LPS or a combination of IL-4 and IL-13 unless otherwise stated.

Cell viability after treatment with combinations of LPS or cytokine mixture and the tested compounds was assessed by modified XTT test (Cell proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.3 Nitrite determination

NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous condition, into the culture medium. The culture medium was collected at indicated time points and nitrite was measured by the Griess reaction [20].

2.4 Preparation of cell lysates for western blotting

At indicated time points, the cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in cold lysis buffer containing 10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 µM n-octyl-β-D-glucopyranoside. After incubation on ice for 15 minutes, lysates were centrifuged (13 400 *g*, 4°C, 10 min), supernatants were collected and mixed 3:1 with SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol). The samples were stored at -20°C until analysed. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [21].

2.5 Western blotting

Prior to western blotting, samples were boiled for 10 minutes and 20 µg of protein was loaded per lane on 8% or 10% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After the transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% of non-fat dry milk or 5% bovine serum albumin at room temperature for 1 h and incubated with the primary antibody in the blocking solution at 4 °C overnight. The membrane was washed with TBS/T, incubated with the secondary antibody in the blocking solution at room temperature for 1 hour and washed. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and ImageQuant LAS 4000 mini imaging system (GE Healthcare). The chemiluminescent signal was quantified with ImageQuant TL 7.0 image analysis software (GE Healthcare).

2.6 RNA extraction and quantitative real-time PCR

At the indicated time points, culture medium was removed and total RNA extraction of the cells was carried out with GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich Co.). 100 ng of total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the reverse transcription reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems).

Primers and probes for arginase 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were optimized according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5'-TCCAAGCCAAAGTCCTTAGAGATTAT-3', 5'-CGTCATACTCTGTTTCTTTAAGTTTTTCC-3', 5'-CGCCTTTCTCAAAAGGACAGCCTCGA-3' (forward and reverse primers and probe for mouse arginase 1) and 5'-GCATGGCCTTCCGTGTTC-3', 5'-GATGTCATCATACTTGGCAGGTTT-3', 5'-TCGTGGATCTGACGTGCCGCC-3' (forward and reverse primers and probe for mouse GAPDH). The expression of mouse PPARα, PPARγ, fizz1 and mrc-1 and human CCL13 and PDGF mRNA was measured by using TaqMan® Gene Expression Assay (Mm00440939_m1, Mm01184322_m1, Mm00445109_m1, Mm00485148_m1, Hs01033504_g1, Hs00966522_m1, Applied Biosystems).

PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2 (arginase 1) or ΔCt method (TaqMan® Gene Expression Assays).

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples were kept at -20°C until assayed. The concentrations of IL-6 and CCL13 in culture medium were determined by ELISA according to the manufacturer's instructions using reagents from R&D Systems Europe (Abingdon, UK).

2.8 Statistics

Results are expressed as mean + standard error of mean (SEM). When indicated, statistical significance was calculated by analysis of variance followed by Dunnett's multiple comparisons test or by unpaired t test with Welch correction. All the statistical analyses were performed

using GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p<0.05$.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 J774 macrophages express PPARs and can represent classical and alternative activation phenotypes

J774 macrophages were found to express PPARα and PPARγ as detected by western blotting and RT-qPCR (Figure 1). As shown in Figures 2 and 3, the activation of the macrophages with LPS increased the expression of classical activation markers including IL-6 and inducible nitric oxide synthase (iNOS) whereas stimulation with a combination of IL-4 and IL-13 induced the expression of alternative activation markers arginase 1, fizz1 and mrc-1. The levels of arginase 1, fizz1 and mrc-1 mRNA were highest at 36 h, 12 h and 12 - 24 h, respectively, after the onset of the interleukin stimulation (Figure 2).

3.2 PPAR agonists reduced the levels of classical activation markers after LPS-stimulation, but had differing effects on alternative activation

PPARα agonists GW7647 and fenofibrate and PPARγ agonist GW1929 attenuated classical macrophage activation as shown by reduced iNOS expression (Figure 3a) and NO and IL-6 production (Figure 3b) in J774 macrophages exposed to LPS. The agonists did not alter the levels of classical activation markers in the absence

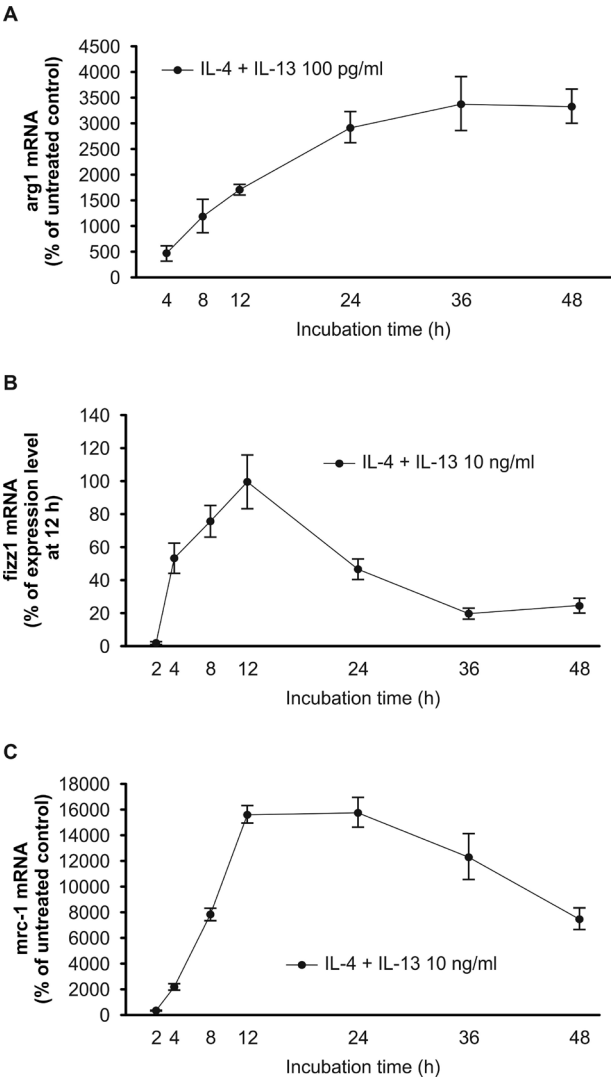

Figure 2 Time-dependent expression of alternative activation markers in J774 macrophages. The cells were stimulated with IL-4 and IL-13 and incubations were terminated at indicated time points and total RNA was extracted. Arginase 1 (a), fizz1 (b) and mrc-1 (c) mRNA were determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean ± SEM (n=4).

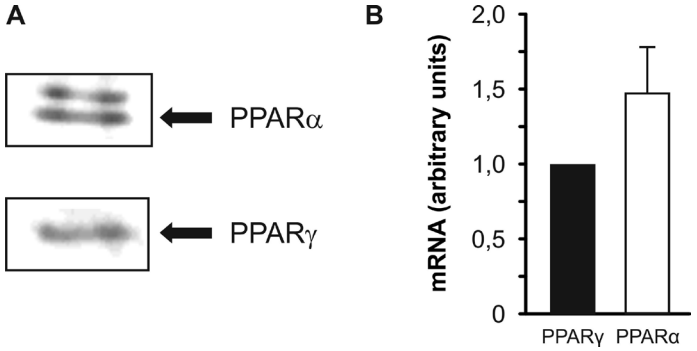

Figure 1 The expression of PPARα and PPARγ in J774 macrophages. (a) Proteins were extracted and PPARα and PPARγ protein expression was determined by western blotting. The gels shown are representatives of three others with similar results. (b) Total RNA was extracted and the levels of PPARα and PPARγ mRNA were analysed by RT-qPCR. The results were normalized against GAPDH mRNA and the level of PPARα mRNA was compared to that of PPARγ (n=6).

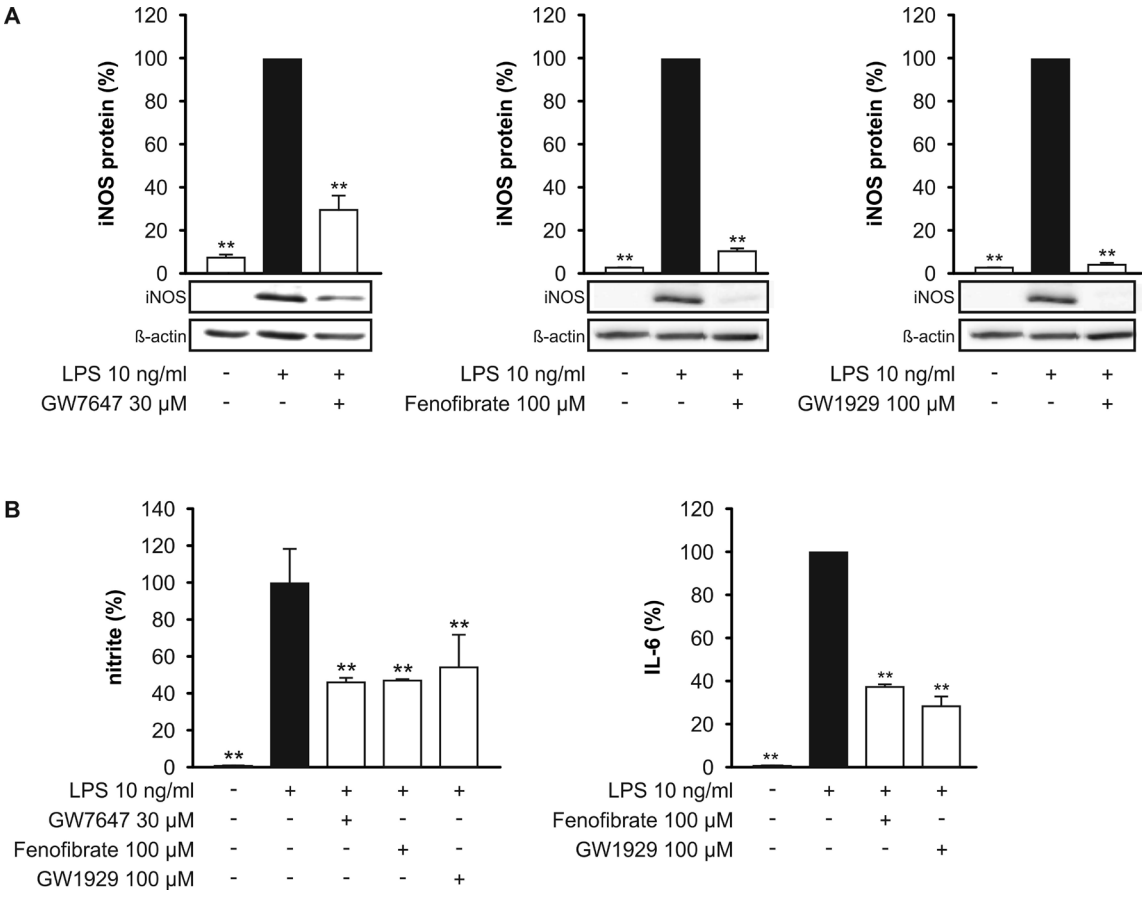

Figure 3 PPARα and PPARγ agonists suppressed classical macrophage activation. (a) Cells were treated with the PPARα agonists GW7647 or fenofibrate or with the PPARγ agonist GW1929 and stimulated with LPS. After 24 h incubation, proteins were extracted and iNOS expression was analysed by western blotting. β-actin was used as a loading control. (b) Cells were treated with the PPARα agonists GW7647 or fenofibrate or with the PPARγ agonist GW1929 and stimulated with LPS. After 24 h incubation, nitrite accumulated into the culture medium was measured by Griess reaction as a marker of NO production. IL-6 in the culture medium was measured by ELISA. Results represent the mean + SEM (n=4). ** = $p<0.01$ as compared to cells cultured with LPS alone.

of LPS or affect cell viability at the concentration used as determined by XTT test (data not shown).

To investigate if PPARs are able to induce alternative activation in macrophages, we tested the effects of the PPAR agonists on the expression of arginase 1. As expected, the PPARγ agonist GW1929 increased the expression of arginase 1 mRNA when measured after 24 h incubation. In contrast, the PPARα agonists GW7647 and fenofibrate decreased the levels of arginase 1 (Figure 4).

Next, we tested the effect of PPAR agonists on IL-4 + IL-13 -induced alternative phenotype in J774 cells. The selective PPARγ agonist GW1929 increased IL-4 + IL-13 -induced protein expression of arginase 1, and mRNA levels of fizz1 and mrc-1, when determined after 24 hour incubation (Figure 5). In contrast, the PPARα agonists decreased IL-4 + IL-13 -induced levels of arginase 1, fizz1 and mrc-1 mRNA, when determined after 24 hour incubation (Figure 6). In line with the results at mRNA level, GW7647 and fenofibrate also decreased the expression of arginase 1 protein (Figure 7).

3.3 PPARα agonists reduced the levels of alternative activation markers also in human THP-1 macrophages

Before evaluating the effects of PPARα agonists on alternative activation in human macrophages, we examined CCL13 and PDGF mRNA transcription following IL-4 stimulation, as markers of alternative activation [3,22]. In THP-1 macrophages IL-4-enhanced CCL13 and PDGF expression, and their mRNA levels continued to increase at least until 36 hours after the addition of IL-4 (Figure 8). GW7647 and fenofibrate clearly decreased IL-4–induced CCL13 expression as shown by reduced cellular levels of CCL13 mRNA and decreased amounts of CCL13 protein in the culture medium (Figure 9a-b). GW7647, and to a lesser

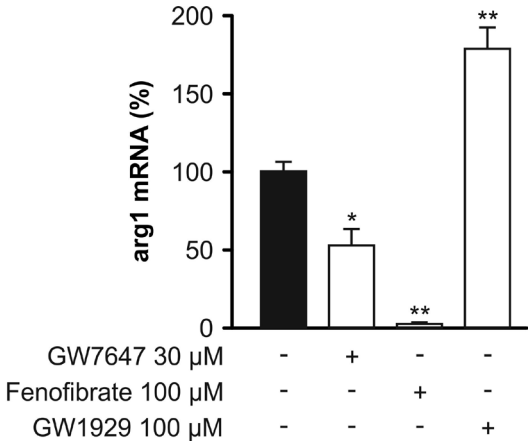

Figure 4 PPARα agonists suppressed, but PPARγ agonists enhanced the expression of the classical marker of alternative activation, i.e. arginase 1, in J774 macrophages. Cells were treated with the PPARα agonists GW7647 or fenofibrate or with the PPARγ agonist GW1929 for 24 hours. Total RNA was extracted and arginase 1 mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). * = p<0.05 and ** = p<0.01 as compared to untreated cells.

extend fenofibrate, also decreased PDGF mRNA levels in THP-1 cells (Figure 9c).

3.4 PPAR agonists did not affect the phosphorylation of STAT6

STAT6 has been shown to be an important cytosolic mediator for transcriptional effects of IL-4 and IL-13. Therefore we investigated the effects of PPAR agonists on STAT6 phosphorylation (i.e. activation). Costimulation with IL-4 and IL-13 induced the phosphorylation of STAT6 peaking at 30 minutes after addition of IL-4 and IL-13 in J774 macrophages (Figure 10a). As seen in Figure 10b-c, neither fenofibrate nor GW7647 modified the phosphorylation of STAT6 in J774 or THP-1 macrophages.

4 Discussion

The present findings introduce PPARα agonists as compounds able to down-regulate alternative macrophage activation, while PPARγ agonists enhanced the alternative phenotype of macrophages. Further, both PPARα and PPARγ agonists attenuated the classical macrophage activation. The alternative activation of adipose tissue macrophages has been proposed to protect lean people from insulin resistance and other obesity-linked metabolic manifestations. Thus, PPARγ agonists and other compounds that polarize macrophages towards the

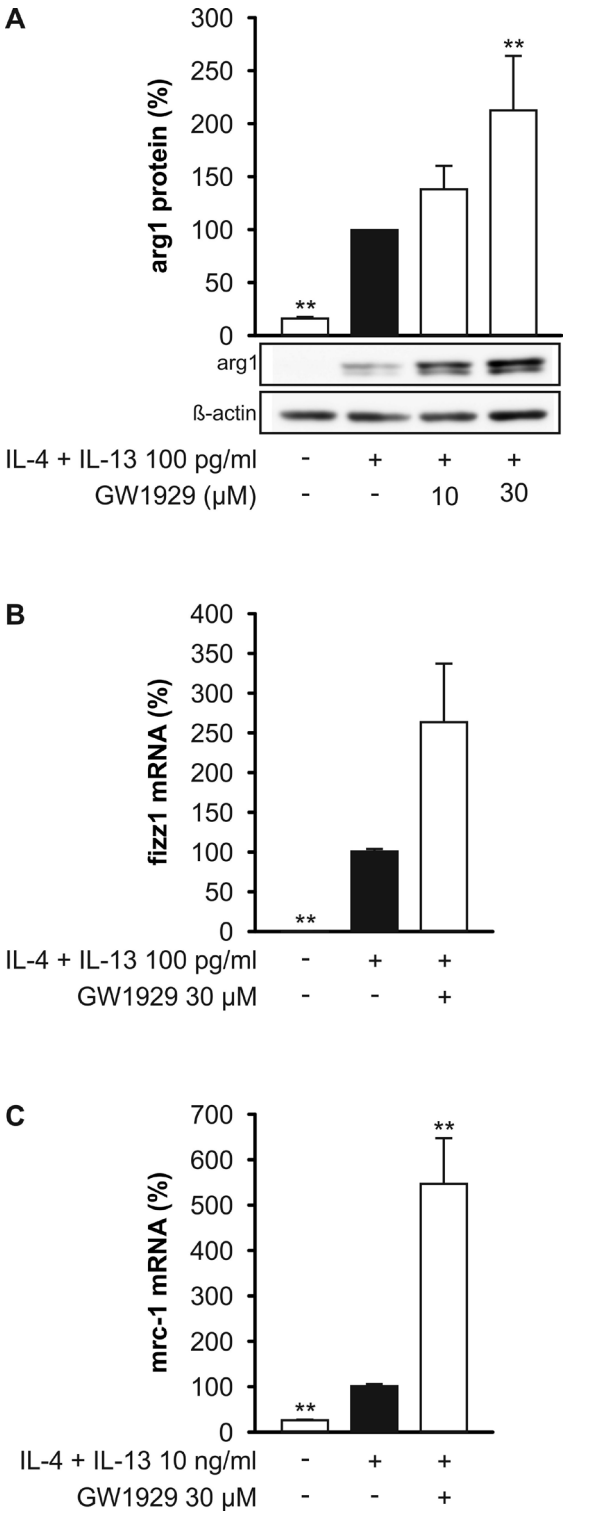

Figure 5 PPARγ agonist GW1929 enhanced the expression of alternative activation markers in J774 macrophages. Cells were treated with GW1929 and stimulated with IL-4 and IL-13 for 24 hours. (a) Proteins were extracted and the levels of arginase 1 were analysed by western blotting. β-actin was used as a loading control. (b-c) Total RNA was extracted and subjected to RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with IL-4 and IL-13.

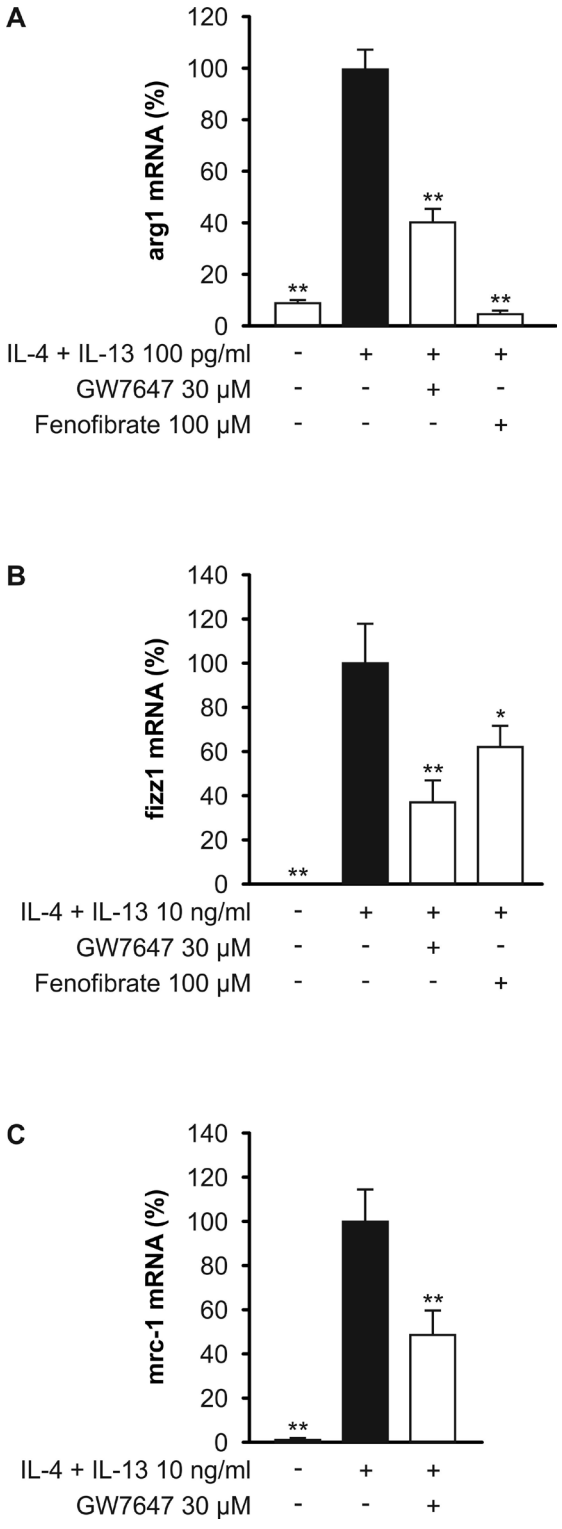

Figure 6 PPARα agonists suppressed the expression of alternative activation markers in J774 macrophages. Cells were treated with GW7647 or fenofibrate and stimulated with IL-4 and IL-13 for 24 hours. Total RNA was extracted and arginase 1 (a), fizz1 (b) and mrc-1 (c) mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean + SEM (n=4). * = p<0.05 and ** = p<0.01 as compared to cells cultured with IL-4 and IL-13.

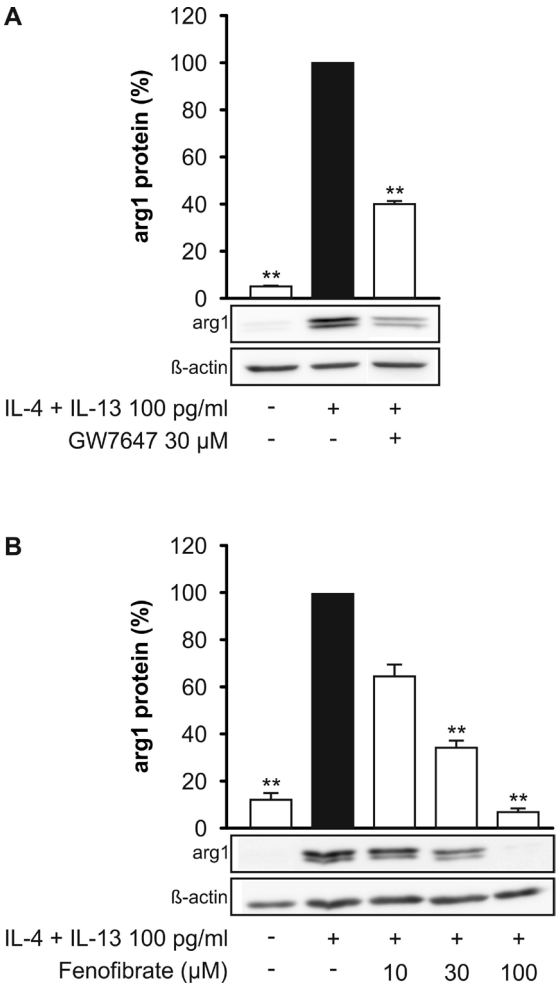

Figure 7 PPARα agonists suppressed arginase 1 protein expression in J774 macrophages. Cells were treated with GW7647 (a) or fenofibrate (b) and stimulated with IL-4 and IL-13 for 24 hours. Proteins were extracted and the levels of arginase 1 were analysed by western blotting. β-actin was used as a loading control. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with IL-4 and IL-13.

alternative phenotype may be beneficial in obesity related metabolic disorders [23,24]. In contrast, compounds able to inhibit alternative macrophage phenotype may have a therapeutic effect in fibrosing diseases, which are complicated with aberrantly enhanced activation of alternative macrophages [25-27].

Alternatively activated macrophages activate fibroblasts to proliferate, migrate to the fibrosing area and to produce extracellular matrix components resulting in fibroblast-populated granulation tissue [28]. However, the mechanisms how this occurs are not known in detail. According to previous reports, systemic sclerosis (SSc) is an example of the diseases where macrophages induce profibrotic action of fibroblasts. Hormer and Herzog showed in 2010 that most of the macrophages in the

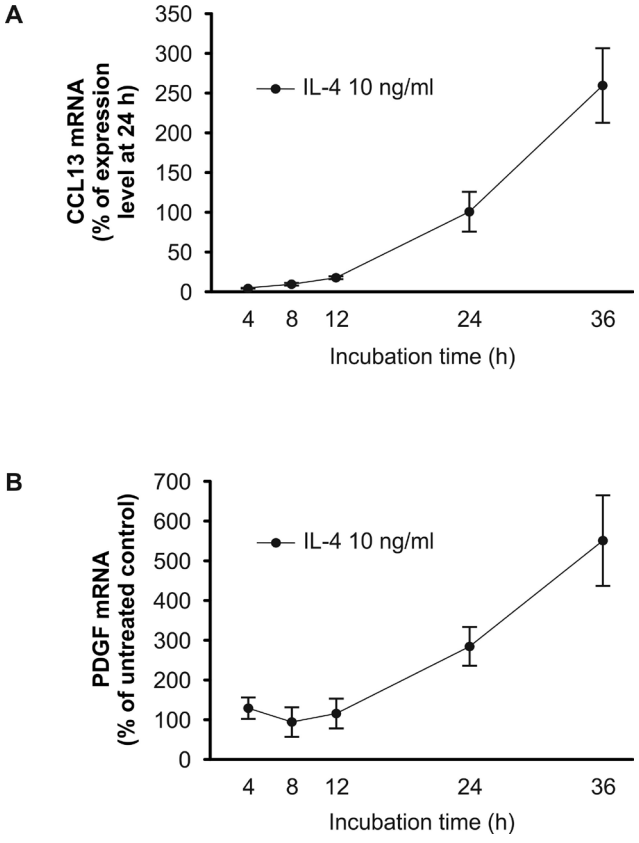

Figure 8 Time-dependent expression of alternative activation markers in THP-1 cells. The cells were stimulated with IL-4 and incubations were terminated and total RNA was extracted at indicated time points. CCL13 (a) and PDGF (b) mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. Results represent the mean ± SEM (n=4).

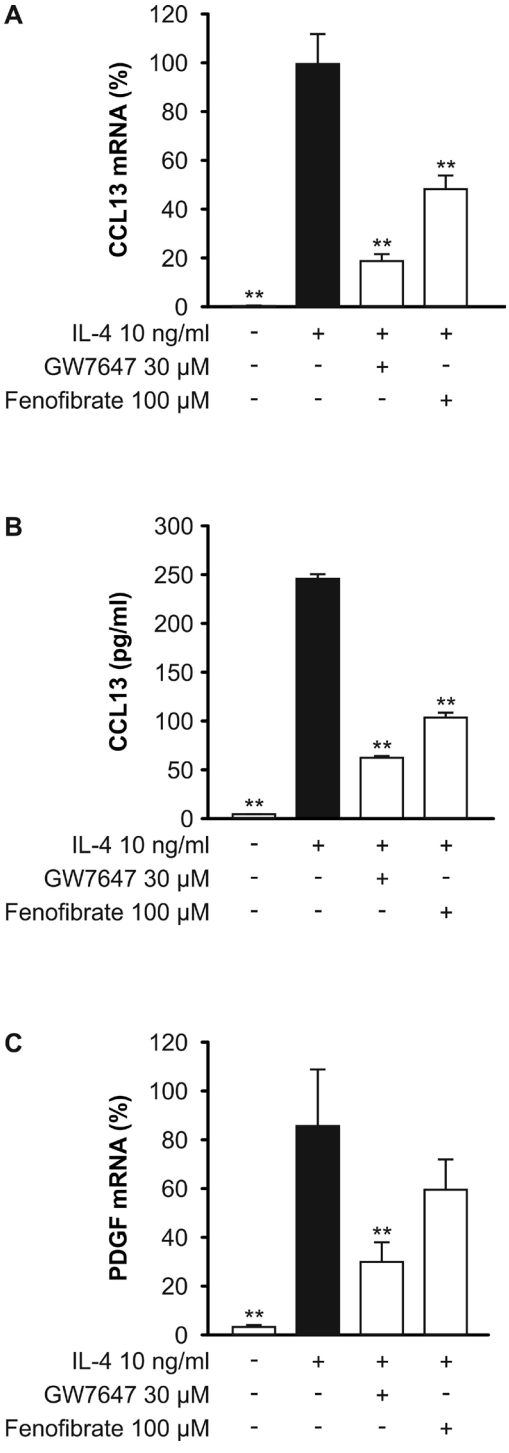

Figure 9 PPARα agonists suppressed the expression of alternative activation markers in THP-1 cells. (a, c) Cells were treated with GW7647 or fenofibrate and stimulated with IL-4 for 24 hours. Total RNA was extracted and CCL13 (a) and PDGF (c) mRNA was determined by RT-qPCR. The results were normalized against GAPDH mRNA. (b) Cells were treated with GW7647 or fenofibrate and stimulated with IL-4. After 24 h incubation, CCL13 accumulated into the culture medium was measured by ELISA. Results represent the mean + SEM (n=4). ** = p<0.01 as compared to cells cultured with IL-4.

lung tissue of patients with SSc-associated interstitial lung disease display markers of alternative macrophage activation [27]. Also in another report, mrc-1 expression was elevated in peripheral blood mononuclear cells (PBMCs) from patients with limited cutaneous SSc and pulmonary hypertension when compared to healthy controls [25]. Additionally, patients with either limited or diffuse cutaneous SSc have been reported to have elevated serum levels of alternative macrophage-related cytokines IL-4, IL-10 and IL-13 [25,26]. IL-13 receptor antagonists also attenuated skin and lung fibrosis in experimental models. Accordingly, treatment with IFN-γ (which drives macrophages towards M1 phenotype) has been shown to improve skin scores in patients with SSc [31]. Most interestingly, an arginase inhibitor, pirfenidone, has been proven to be beneficial in patients with idiopathic pulmonary fibrosis when compared to placebo in two multicentre studies [32]. Pirfenidone was approved for the treatment of idiopathic pulmonary fibrosis in Europe

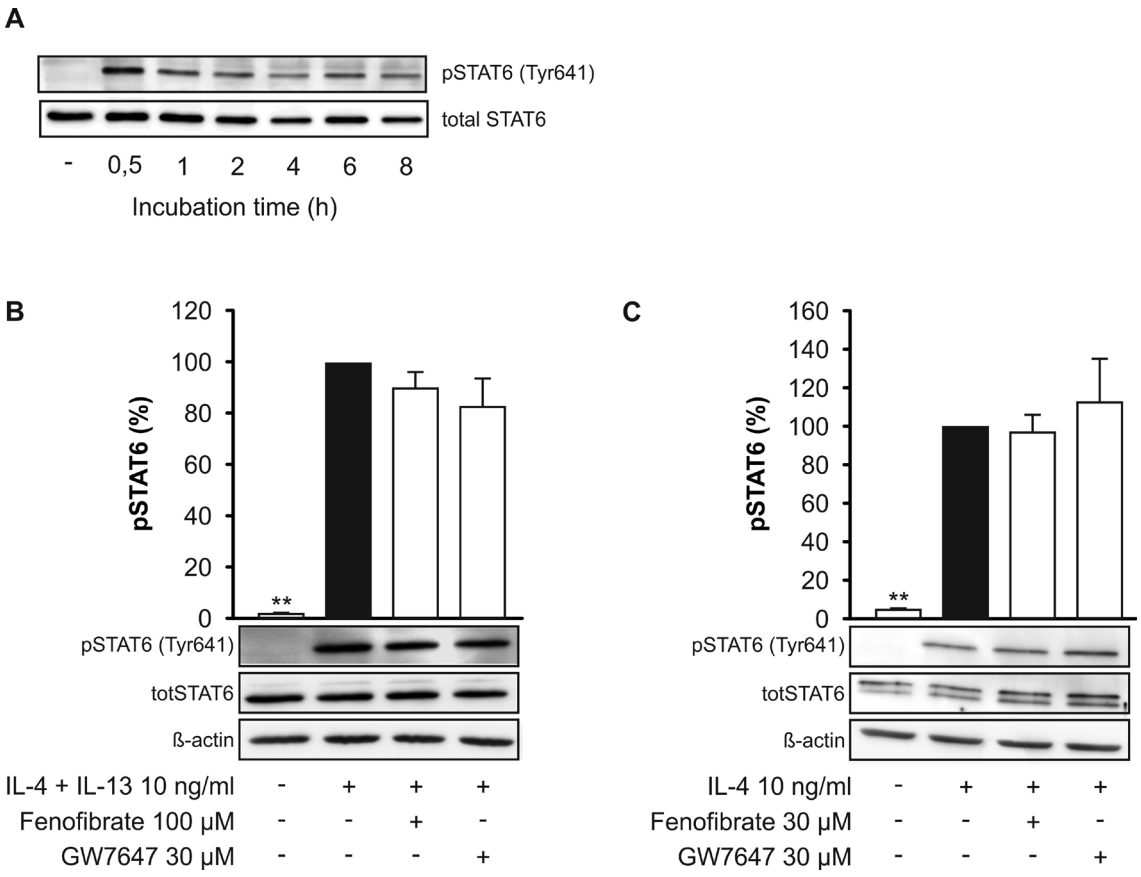

Figure 10 The effects of PPARα agonists on STAT6 phosphorylation in J774 and THP-1 cells. (a) Cells were stimulated with IL-4 and IL-13 and incubations were terminated at indicated time points. Proteins were extracted and the levels of pSTAT6 were analysed by western blotting. Total STAT6 was used as a loading control. The gel shown is a representative of three others with similar results. (b) J774 macrophages were preincubated with the PPARα agonists or the vehicle for an hour before addition of IL-4 and IL-13. After 30 min incubation with IL-4 and IL-13 proteins were extracted and the levels of pSTAT6 were analysed by western blotting. (c) THP-1 cells were preincubated with the PPARα agonists or the vehicle for an hour before addition of IL-4. After 30 min incubation with IL-4, proteins were extracted and the levels of pSTAT6 were analysed by western blotting. (b,c) Total STAT6 and β-actin were used as loading controls. Results represent the mean + SEM (n=4). ** = $p<0.01$ as compared to cells cultured with IL-4 and IL-13 (a,b) or IL-4 (c).

in 2011 and may be beneficial also in other fibrosing diseases including SSc. At the moment, there are no specific treatments targeting the pathophysiology of the fibrosing processes in SSc or related diseases but most of the therapies used act as immunomodulators, like methotrexate, cyclophosphamide and azathioprine, or vasodilators, like calcium channel blockers, prostacyclin analogs, telmisartan, phosphodiesterase inhibitors and endothelin I antagonists. However, the efficacy of these drugs is very limited and better treatment options are critically needed.

PPARα agonists fibrates and PPARγ agonists TZDs are in clinical use for the treatment of metabolic disorders. In line with the present results, both fibrates and TZDs have also shown to decrease the production of IFNγ or LPS –induced proinflammatory mediators typical for classical macrophage activation like iNOS, NO, IL-6 and TNF [17,33,34]. In addition, PPARγ agonists have been reported to shift the polarization of macrophages towards the alternative phenotype [14,15] and that was also seen in the present study. Interestingly, we report here that PPARα agonists have an opposite effect on M2 polarization. To our knowledge, the present study is the first report showing the inhibitory effects of PPARα agonists on the alternative activation of macrophages indicating that PPARα and PPARγ agonists differently regulate alternative macrophage activation.

The diversity of macrophage phenotypes has best been described in mouse macrophages. Some of the known markers of alternative activation, e.g. fizz1, are expressed only in mice [35] and some other markers, like arginase 1, are important *in vivo* but become silenced rapidly in *ex vivo* conditions in human cells [36,37]. Also as reported in this study, J774 macrophages increased

the production of arginase 1, fizz1 and mrc-1 in response to IL-4 and IL-13 treatment showing the suitability of the model to figure alternative activation. During last years, several markers of alternative activation in human cells have been proposed. CCL13 is one of the most widely applied markers, and it was chosen also in the present study to depict alternative activation. CCL13 expression was highly elevated in response to IL-4 to induce alternative activation of macrophages, which is supported by the previous results [22]. Another human marker of alternative activation used in this study was PDGF. PDGF is an essential promoting factor for fibroblast proliferation during wound healing process and fibrosis [38]. It is also one of the direct mediators of wound healing and pro-fibrotic activity of macrophages [3] and a central pathophysiological factor in SSc [39].

Today, fibrates are used in the treatment of hyperlipidemia. The present results introduce fibrates, and PPARα agonists in general, as inhibitors of alternative macrophage activation. Considering that aberrantly enhanced alternative macrophage activation is associated with the pathogenesis of fibrosis, the present findings suggest that fibrates (and other PPARα agonists) would be beneficial also in fibrosing diseases. This hypothesis is supported by the finding reported by Diep et al. that fenofibrate attenuates the angiotensin II -induced expression of pro-fibrotic markers in murine myocardial tissue [40]. Since fibrates are an old group of drugs, the side effects are well known. According to the meta-analysis published recently, fibrates did not show increased risk of any serious drug-related adverse events when they were used as a monotherapy for the treatment of dyslipidemia [41]. So, also from the safety aspect, fibrates or other PPARα agonists hold potential for the treatment of fibrosing diseases including SSc.

5 Conclusions

The present study shows that PPARα agonists suppress alternative activation of macrophages, which is aberrantly enhanced in fibrosis. These results suggest that PPARα agonists might have anti-fibrotic effects, which offers a pharmacological option to prevent the pathological manifestations of fibrosing diseases.

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