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(née Salonen)

Protein Kinase C in the Regulation of Inflammatory Genes iNOS and TTP

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ACADEMIC DISSERTATION
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List of original communications

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-IV.

I Salonen T, Sareila O, Jalonen U, Kankaanranta H, Tuominen R, Moilanen E (2006): Inhibition of Classical PKC Isoenzymes Downregulates STAT1 Activation and iNOS Expression in LPS-Treated Murine J774 Macrophages. Br. J Pharmacol. 147:790-799

II Leppänen T, Jalonen U, Kankaanranta H, Tuominen R, Moilanen E (2008): Inhibition of protein kinase CβII downregulates tristetraprolin expression in activated macrophages. Inflamm. Res. 57: 230-240

III Leppänen T, Jalonen U, Korhonen R, Tuominen RK, Moilanen E (2010): Inhibition of protein kinase Cδ reduces tristetraprolin expression by destabilizing its mRNA in activated macrophages. Eur. J Pharmacol. 628:220-5.

IV Leppänen T, Nieminen R, Korhonen R, Tuominen RK, Moilanen E (2010): Downregulation of protein kinase Cδ inhibits inducible nitric oxide synthase expression through IRF1. (submitted for publication)

Abbreviations

3'UTR	3'–untranslated region
AP-2	activator protein 2
ARE	AU-rich element
BH_4	tetrahydrobiopterin
cAMP	cyclic adenosine 3'5'-monophosphate
CGP53353	5,6-bis[(4-Fluorophenyl)amino]-2H-isoindole-1,3-dione, PKCβII inhibitor
COX-2	cyclooxygenase-2
BMS345541	N-(1,8-Dimethylimidazo[1,2-a]quinoxalin-4-yl)-1,2-ethanediamine hydrochloride
DAG	diacylglycerol
EGR1	early growth response gene-1
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	gamma activated site
GM-CSF	granulocyte-macrophage colony-stimulating factor
GÖ6976	12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole, inhibitor of cPKCs
HDDDE	2,2',3,3',4,4'-Hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether, inhibitor of PKCα and γ
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon response factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KSRP	KH-type splicing regulatory protein
L-NIL	L-N6-(1-iminoethyl)lysine
LPS	lipopolysaccharide
LY333531	(*S*)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16, 21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4,*h*] [1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-d ione, ruboxistaurin, inhibitor of PKCβ
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor κB
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
OA	osteoarthritis

PKA	protein kinase A
PKC	protein kinase C
PDD	phorbol 12,13-didecanoate
PDTC	pyrrolidine dithiocarbamate
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
RACK	receptors for activated C kinase
RO318220	2-[1-(3-(Amidinothio)propyl)-1H-indol-3-yl]-3-(1-methylindol-3-yl)maleimide·methanesulfonate, bisindolylmaleimide IX, inhibitor of PKCβ, γ, and ε
Sp1	specificity protein 1
STAT1	signal transducer and activator of transcription 1
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumour necrosis factor
TTP	tristetraprolin

Abstract

Inflammation is a defence mechanism by which the body reacts against foreign invaders and cell injuries. Activated inflammatory cells produce signalling molecules that regulate the inflammatory response and the progress of inflammation. Inflammation is usually beneficial, but if it is inadequately controlled or inappropriately directed e.g. against the body's own tissues or harmless molecules, it may cause injury or disease. This is the case in some of the most common and disabling human diseases such as rheumatoid arthritis and asthma.

Protein kinase C (PKC) is a family of ten isoenzymes that are crucial in cellular signal transduction. Aberrant PKC activation has been demonstrated in several pathological states including cancer and metabolic disorders. Less is known about the specific effects of PKC isoenzymes on the regulation of inflammatory genes.

Inducible nitric oxide synthase (iNOS) is one of the genes activated in inflammation. The enzyme for which it codes, iNOS, catalyses the production of nitric oxide (NO) from L-arginine and molecular oxygen in inflammatory cells such as macrophages. NO produced by iNOS has beneficial effects in innate immunity, where it functions as a toxic agent towards infectious organisms. However, aberrant iNOS induction seems to be involved in the pathophysiology of human diseases such as asthma, arthritis, and colitis.

Tristetraprolin (TTP) is a factor that can bind to AU-rich elements within the mRNAs of its target genes causing destabilization of the mRNA of several transiently expressed inflammatory genes such as TNFα, GM-CSF, and various interleukins. Studies with TTP knockout mice have shown that TTP deficiency evokes a profound inflammatory syndrome, which has been reported to be mainly due to excessive production of TNF-α. TTP can be thought primarily as an anti-inflammatory or arthritis suppressor gene.

The overall aim of the present study was to investigate the role of PKC isoenzymes in the regulation of protein and mRNA expression of inflammatory genes iNOS and TTP. One aim was to identify which classical PKC isoenzymes are involved and whether the novel isoenzyme PKCδ would take part in the regulation of iNOS or TTP under inflammatory conditions. Another major aim was to characterise the mechanisms involved in the regulation of iNOS and TTP expression by PKC isoenzymes.

Classical PKC isoenzymes were shown to participate in the regulation of the expression of iNOS and TTP. Inhibition of PKCβ reduced the production of NO and

decreased iNOS expression in activated macrophages. This effect seemed to be mediated mainly at the level of transcription, i.e. by affecting the activity of the transcription factor STAT1. PKCβII seemed to be able to also regulate the expression of TTP through the activation of transcription factor activator protein 2 (AP-2).

The novel isoenzyme PKCδ was observed to take part in the regulation of iNOS protein and mRNA expression. Downregulation of PKCδ by PKCδ targeted siRNA or inhibition of PKCδ by rottlerin reduced iNOS expression, most likely through the downregulation of transcription factor interferon response factor 1 (IRF1). PKCδ is believed to be a significant inflammatory pathway also *in vivo*, because inhibition of PKCδ by rottlerin showed anti-inflammatory effects in carrageenan-induced inflammation, as did treatment with an iNOS inhibitor L-NIL. PKCδ seemed to be able to regulate also the expression of TTP by affecting TTP mRNA decay.

Inflammation leads to the activation of PKC signalling pathways and the production of many inflammatory factors, e.g. iNOS, which further enhance the inflammatory process. During inflammation, iNOS produces large amounts of NO, which in addition to its antimicrobial effects, possesses regulatory and proinflammatory/destructive effects. Compounds that inhibit iNOS expression or activity have been claimed to have anti-inflammatory properties in various forms of experimentally–induced inflammation. Here, inhibition of iNOS expression was found after inhibition of PKCβ and PKCδ, suggesting that those two PKC isoenzymes can enhance the inflammatory reaction by upregulating iNOS expression. In the normal inflammatory reaction, also the factors that limit the inflammatory process become activated. One of these factors is TTP, the expression of which was also found to be regulated by PKC in a pathophysiologically relevant manner.

In the present study, the role of different PKC isoenzymes in the regulation of inflammatory genes iNOS and TTP were identified. Novel mechanisms of regulation by PKC isoenzymes were discovered. These findings add to our understanding of the inflammatory process and this information may be useful in the development of novel anti-inflammatory drugs.

Tiivistelmä

Tulehdus on elimistön puolustusmekanismi taudinaiheuttajia ja kudosvauriota vastaan. Tulehdusalueelle kertyneet immuunijärjestelmän solut tuottavat välittäjäaineita, jotka säätelevät tulehdusreaktion voimakkuutta ja etenemistä. Yleensä tulehdus on hyödyllinen reaktio, mutta jos tulehdusreaktion säätely häiriintyy tai reaktio kohdentuu väärin, seurauksena voi olla tulehdustauti, kuten nivelreuma tai astma.

Proteiinikinaasi C (PKC) isoentsyymit ovat seriini/treoniinikinaaseja ja säätelevät kohdeproteiiniensa toimintaa fosforylaation avulla. PKC isoentsyymiperhe koostuu kymmenestä erilaisesta isoentsyymistä, joilla kaikilla on merkittävä rooli solunsisäisessä signaalinvälityksessä. PKC aktivaation tiedetään liittyvän moniin sairauksiin, kuten syöpään ja diabetekseen, mutta PKC isoentsyymien vaikutuksista tulehdusgeenien säätelyssä tiedetään toistaiseksi vähän.

Indusoituva typpioksidisyntaasi (iNOS) on yksi tulehdusreaktiossa aktivoituvista tulehdustekijöistä. Se katalysoi typpioksidin (NO) muodostumista L-arginiinista ja molekulaarisesta hapesta. Näin syntyvällä NO:lla on tärkeä rooli luontaisessa immuunipuolustuksessa taudinaiheuttajien tuhoamisessa. NO:n tiedetään kuitenkin myös voimistavan tulehdusreaktiota ja aiheuttavan kudostuhoa. Tiedetään myös, että lisääntynyt iNOS-välitteinen NO-tuotto liittyy mm. astman, nivelrikon sekä koliitin patofysiologiaan.

Tristetraproliini (TTP) on lähetti-RNA:n stabiiliutta säätelevä tekijä. Se sitoutuu kohdegeeniensä lähetti-RNA:n 3'UTR–alueeseen ja säätelee tällä tavoin useiden tulehdusgeenien, mm. tuumorinekroositekijä-α:n (TNFα) ja granulosyytti-makrofagikasvutekijän (GM-CSF), ilmentymistä. TTP poistogeenisillä eläimillä on havaittu merkittävä tulehduksellinen oireyhtymä, jonka ajatellaan johtuvan pääasiassa lisääntyneestä TNFα:n ja GM-CSF:n tuotosta. TTP:tä voidaankin pitää anti-inflammatorisena tekijänä.

Väitöskirjatyön tarkoituksena oli tutkia PKC isoentsyymien roolia kahden tulehdusgeenin, iNOS:n ja TTP:n, ilmentymisen säätelyssä. Tarkoituksena oli identifioida, mitkä klassisen alaryhmän PKC isoentsyymit osallistuvat iNOS:n ja TTP:n säätelyyn tulehduksessa ja osallistuuko uudentyyppisten isoentsyymien alaryhmään kuuluva PKCδ myös säätelyyn. Lisäksi tarkoituksena oli selvittää, mitkä ovat säätelyn takana olevat molekulaariset mekanismit.

Klassiset PKC isoentsyymit osallistuivat sekä iNOS:n että TTP:n ilmentymisen säätelyyn. PKCβ isoentsyymin esto vähensi NO:n tuottoa ja iNOS:n ilmentymistä

todennäköisesti vähentämällä transkriptiotekijä STAT1:n aktiivisuutta. PKCβII puolestaan osallistui TTP:n ilmentymisen säätelyyn, mahdollisesti transkriptiotekijä AP-2:n aktivaation kautta.

Väitöskirjatyössä osoitettiin myös PKCδ:n osallistuvan sekä iNOS:n että TTP:n ilmentymisen säätelyyn. PKCδ:n ilmentymisen vaimentaminen siRNA-menetelmällä ja PKCδ:n esto rottlerinilla vähensivät iNOS:n ilmentymistä todennäköisesti vähentämällä transkriptiotekijä IRF1:n ilmentymistä. PKCδ on merkittävä signalointireitti tulehduksessa todennäköisesti myös *in vivo*, sillä PKCδ:n esto rottlerinilla, sekä iNOS inhibiittori L-NIL, vaimensivat tulehdusreaktiota myös karrageenilla aiheutetussa hiiren tassun tulehdusmallissa. TTP:n ilmentymisen säätelyyn PKCδ osallistui vaikuttamalla TTP:n lähetti-RNA:n stabiiliuteen.

Tulehdus aktivoi PKC signalointireittejä ja tulehdustekijöiden, kuten iNOS:n, tuottoa. Tulehduksessa iNOS:n kautta muodostuva NO osallistuu luontaiseen immuunipuolustukseen tuhoamalla taudinaiheuttajia. Lisäksi näin muodostuvalla NO:lla on myös tulehdusta voimistavia ja sääteleviä ominaisuuksia. iNOS:n ilmentymistä tai aktiivisuutta estävillä yhdisteillä on todettu olevan tulehdusta vaimentavia vaikutuksia monissa tulehdustautien kokeellisissa malleissa. Tässä tutkimuksessa PKCβ:n ja PKCδ:n eston havaittiin vähentävän iNOS:n ilmentymistä, antaen viitteitä siitä, että kyseessä olevat PKC isoentsyymit saattavat voimistaa tulehdusreaktiota lisäämällä iNOS:n ilmentymistä. Normaalisti etenevässä tulehdusreaktiossa myös tulehdusprosessia rajoittavat tekijät, kuten TTP, aktivoituvat. PKC isoentsyymien havaittiin osallistuvan myös TTP:n ilmentymisen säätelyyn.

Tässä väitöskirjatyössä tutkittiin eri PKC isoentsyymien osuutta kahden tulehdusgeenin, iNOS:n ja TTP:n, ilmentymisen säätelyssä. Tutkimuksessa havaittiin uusia mekanismeja, joilla PKC isoentsyymit voivat vaikuttaa tulehdusreaktioon. Tutkimuksessa saatua tietoa voidaan käyttää hyväksi kehitettäessä uusia anti-inflammatorisia lääkeaineita.

Introduction

Inflammation is the body's protective response against foreign invaders, such as microbes and toxins, which may cause cell injury. Inflammation is also designed to remove the consequences of such cell injury e.g. necrotic cells and tissues. Classical signs of inflammation are redness, swelling, heat, pain, and loss of function.

Inflammation can be either acute or chronic depending on the nature of the stimulus and the intensity of the initial reaction in eliminating the stimulus or the damaged tissues. Acute inflammation is the initial response to tissue injury. It is typically rapid in onset and of short duration. Its principal characteristics are the exudation of fluid and plasma proteins and emigration of leucocytes, mainly granulocytes and monocyte/macrophages. If the acute inflammation naturally eliminates the foreign invaders, then the inflammatory reaction successfully subsides. However, if the acute response fails, then the inflammation may progress to a chronic phase. Chronic inflammation is of longer duration and is associated with the presence of macrophages and lymphocytes, tissue destruction, the proliferation of blood vessels, and fibrosis. Although inflammation is beneficial and required for survival, it can be harmful in some situations. If inflammation is inadequately controlled or inappropriately directed against harmless proteins or host tissues, it may cause injury or disease. This is the case in some of the most common human diseases such as asthma, allergy, rheumatoid arthritis, and inflammatory bowel disease.

The immune response can be divided into innate immunity and adaptive immunity and one major role for macrophages is to function as a link between these two systems. Innate immunity (also called natural immunity) is an inborn defence mechanism evolved to specifically recognise pathogens and protect against infections. This is the first line in defence and it does not require previous contact with the pathogen. Adaptive immunity (also called acquired immunity) develops later, after contact with pathogens, with CD4+ helper T cells playing a major role. T helper cells can be divided into three subsets Th1, Th2, and the recently discovered

Th17, which all play distinct roles. Adaptive immunity is more specific and its mechanisms are capable of recognising specific microbial and nonmicrobial substances called antigens. Autoimmune diseases arise when the immune system recognises substances and tissues normally present in the body as being foreign, and thus the system attacks the body's own cells. (Kumar et al. 2010)

The outcome of inflammatory reaction is very similar, despite its initial cause. Several inflammatory mediators regulate the inflammatory response and the signalling network of immune cells forms a complex phenomena. Inflammatory mediators include nitric oxide (NO) and tristetraproline (TTP). NO is a gaseous signalling molecule involved in physiological and pathophysiological functions in the body. In inflammatory processes, NO is produced primarily by inducible nitric oxide synthase (iNOS) in inflammatory cells such as macrophages. NO is involved in innate immunity as a toxic agent towards infectious organisms. It can also induce or regulate the function and death of antigen-specific immune cells, thereby participating in adaptive immunity. NO may represent also a proinflammatory agent since it can induce toxic reactions against the host tissues. High levels of NO are associated with the generation of certain inflammatory diseases, such as asthma and arthritis. (Tripathi et al. 2007, Kumar et al. 2010)

Tristetraprolin (TTP) is a factor that binds to the mRNAs of some transiently expressed inflammatory genes and regulates the stability of the mRNA. TTP has been shown to destabilize the mRNAs of different factors e.g. tumour necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Blackshear 2002). A role for TTP in inflammation was revealed when TTP knockout mice were demonstrated to develop a severe inflammatory syndrome, including arthritis and autoimmunity, as a consequence of excessive production of TNFα (Taylor et al. 1996a). Thus, TTP can be considered primarily as an anti-inflammatory factor.

Protein kinase C (PKC) is a family of ten isoenzymes that play a crucial role in cellular signal transduction. Studies with PKC knockout animals have revealed that many of the isoenzymes are involved in cell growth, proliferation, and differentiation. However, less is known about the role of PKC in the regulation of the expression of inflammatory genes (Tan and Parker 2003, Lee et al. 2008). The present study investigated the role of protein kinase C isoenzymes in the regulation of inflammatory genes, with iNOS and TTP being used as example genes.

Review of literature

1. Protein kinase C

Protein kinases are enzymes that mediate their effects by phosphorylating their target proteins. There are 518 protein kinases encoded in the human genome (Manning et al. 2002). Serine/threonine kinase protein kinase C (PKC) was discovered in 1977, and it was one of the first protein kinases to be identified. It was first defined as a histone kinase activity from rat brain which was activated by limited proteolysis (Inoue et al. 1977, Takai et al. 1977). Subsequently, it was discovered that this new kinase was also activated by phosphatidylserine and diacylglycerol (DAG) in a Ca^{2+}-dependent manner as reviewed by Gould and Newton (2008) and Steinberg (2008). The first PKCs identified and cloned were the α, β, and γ isoenzymes, which were initially isolated from rat brain cDNA libraries (Coussens et al. 1986, Parker et al. 1986). Further analysis led to the discovery of three additional isoenzymes, δ, ε, and ζ also from rat brain cDNA libraries (Ono et al. 1987). PKC isoenzymes η (Osada et al. 1990), θ (Osada et al. 1992), and ι (λ is the mouse homologue) (Selbie et al. 1993) were found in screens of other tissue cDNA libraries. Today, the mammalian PKC superfamily consists of ten different isoenzymes.

The mammalian isoenzymes have been grouped into three subfamilies on the basis of their domain structure (Figure 1). The best understood and most widely studied of these groups are conventional or classical isoenzymes, cPKCs, which consist of the α, βI, βII, and γ isoenzymes. In the presence of phosphatidylserine, these isoenzymes respond to DAG in a Ca^{2+}-dependent manner. cPKCs are also targets of the tumour promoting phorbol esters. The novel PKCs (nPKCs) consist of δ, ε, η, and θ isoenzymes. These isoenzymes are Ca^{2+}-independent, but respond to DAG or phorbol esters in the presence of phosphatidylserine. The atypical PKC

isoenzymes (aPKCs) ζ and ι/λ are Ca^{2+}-independent and do not respond to DAG or phorbol esters. (Gould and Newton 2008, Steinberg 2008)

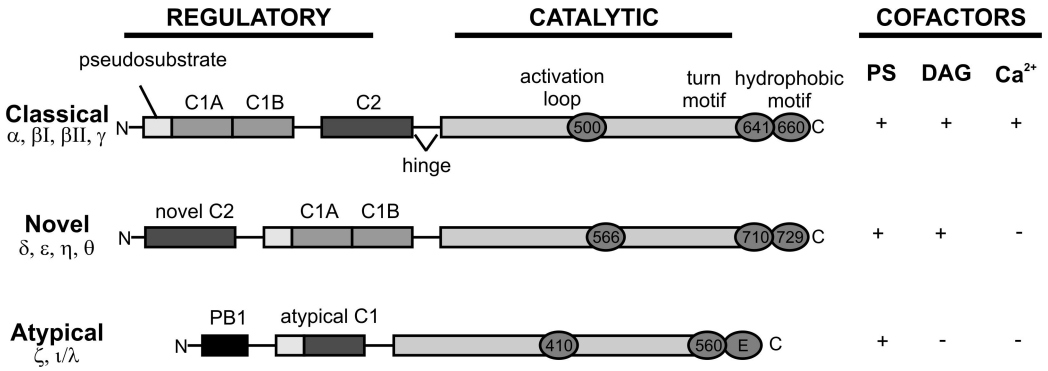

Figure 1. *The structure and cofactor requirements of PKC isoenzymes. Phosphorylation sites for PKCβII, PKCε, and PKCζ are shown as representatives of their own subfamily. PS=phosphatidylserine, DAG=diacylglycerol. (Modified from Newton 2010).*

All PKC isoenzymes (~80 kDa) share a conserved domain structure composed of a carboxyl-terminal kinase core linked by a flexible hinge segment to an amino-terminal region which contains the regulatory modules. These modules confer sensitivity to second messengers with some variation between subgroups. The regulatory moiety, which consists of C1 and C2 domains in cPKCs, maintains the enzyme in an inactive form in the absence of the appropriate second messengers and also targets the enzyme to specific cellular locations and mediates protein-protein interactions. The autoinhibitory pseudosubstrate sequence is located N-terminal to the C1 domain. When PKC is inactive, this peptide sequence occupies the substrate binding cavity. Upon activation of PKC, the pseudosubstrate is released, allowing the binding and phosphorylation of downstream substrates. More detailed structures of isoenzyme subgroups will be described in the following sections. (Steinberg 2008, Newton 2010)

1.1 Overview of protein kinase C signalling pathways

The hallmark of PKC activation is the translocation of the enzyme from the cell cytosol to the membrane compartments (Steinberg 2008). Extracellular signals cause receptor-mediated activation of phospholipase C resulting in the cleavage of

phosphatidylinositol bisphosphate (PIP_2), forming DAG and inositol trisphosphate (IP_3), which releases Ca^{2+} from intracellular storage sites (Hughes and Putney 1988). The second messengers i.e. DAG and Ca^{2+}, initiate the membrane translocation and activation of PKC. In the case of classical isoenzymes, which are the Ca^{2+} dependent isoenzymes, Ca^{2+} binds to the C2 domain and pretargets PKC to the membrane. This allows the C1 domain to bind its membrane embedded ligand DAG, an interaction enhanced by stereospecific binding to phosphatidylserine. The coordinated engagement of both C1 and C2 domains on the membrane provides the energy to release the autoinhibitory pseudosubstrate and then PKC can bind its substrates and initiate downstream signalling (Figure 2). (Steinberg 2008, Newton 2010)

However, before PKC isoenzymes can effectively transduce extracellular signals to downstream targets, they must be properly processed and positioned. PKC isoenzymes are matured in a series of ordered, tightly coupled, and constitutive phosphorylations, that are essential for the stability and catalytic competence of the enzyme (Keranen et al. 1995, Newton 2010). The maturation processes differ to some extent between isoenzymes, but the main stages are similar. The first step is the phosphorylation of the activation loop by the upstream kinase PDK1 (phosphoinositide-dependent kinase 1). Newly synthesized immature PKC is loosely tethered at the membrane in a position that allows PDK1 to bind and phosphorylate the activation loop (e.g. Thr^{500} in PKCβII) (Chou et al. 1998, Dutil et al. 1998, Le Good et al. 1998). This functions as a primer for the subsequent C-terminal autophosphorylations at the turn motif and the hydrophobic motif, which serve to stabilize mature PKC. Rapid autophosphorylation at the turn motif site (e.g. Thr^{641} in PKCβII) is required to maintain catalytic competence of the enzymes (Keranen et al. 1995, Edwards et al. 1999). The final step is autophosphorylation at the C-terminal hydrophobic motif (e.g. Ser^{660} in PKCβII), which influences the subcellular localization and stability of PKC (Keranen et al. 1995, Behn-Krappa and Newton 1999). Once PKC has been processed by phosphorylation, it is released to the cytosol and maintained in a mature inactive conformation (Keranen et al. 1995).

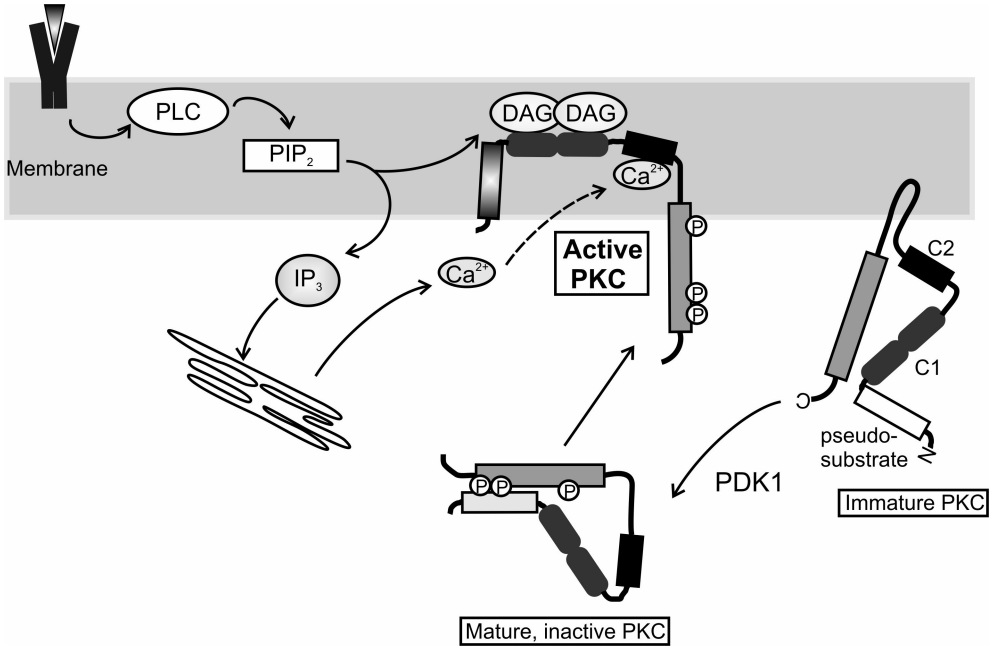

Figure 2. *Schematic presentation of the activation of PKC. Newly synthesised immature PKC is loosely tethered at the membrane in an open conformation. Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop and PKC can be autophosphorylated. PKC is released into the cytosol and maintained in a mature inactive conformation. Second messengers diacylglycerol (DAG) and Ca^{2+} evoke the translocation of mature PKC from the cytosol to the membrane. Binding of DAG to the C1 domain and Ca^{2+} to the C2 domain provides the energy for the release of the pseudosubstrate from the active site to allow downstream signalling. PLC=phospholipase C, PIP_2=phosphatidylinositol bisphosphate, IP_3=inositol trisphosphate*

Phorbol esters, which are analogues of DAG, can also cause translocation and activation of PKC. Phorbol esters are natural products isolated from plant *Euphorbiaceae* and *Thymeleaceae* families. Phorbol esters are tumour promoters, i.e. compounds which are not carcinogenic by themselves, but increase the probability of cells becoming malignant after the initiation event has been triggered by carcinogenic agents. The most potent phorbol ester is phorbol 12-myristate 13-acetate (PMA, also known as TPA from the name 12-o-tetradecanoylphorbol-13-acetate) (Blumberg et al. 1983). Phorbol esters are well known activators of cPKCs and nPKCs. As compared to the transient response of PKC to DAG (which is metabolized rapidly), phorbol ester induced translocation of PKC is prolonged, leaving PKC susceptible to the activity of proteases. Indeed, prolonged treatment with phorbol esters leads to a degradation of PKC, i.e. downregulation of PKC (Huang et al. 1989, Chen 1993). For two decades, PKC was considered the major phorbol ester receptor within cells, and phorbol esters were widely used pharmacological tools in studying PKC activation. However, several non-kinase

targets of phorbol esters, such as chimaerins, Ras guanyl nucleotide-releasing proteins (RasGRPs), and mammalian homologue of uncoordinated-13 protein (Munc-13), have also been described (Kazanietz et al. 2000).

PKC signals in all regions of the cell: plasma membrane, nucleus, Golgi apparatus, mitochondria, and cytosol, and this requires accurate targeting mechanisms. Receptors for activated C kinase (RACKs) have been suggested to function as molecular scaffolds to help to localize the individual PKCs to distinct membranes in close proximity with their activators and intracellular substrates. It has been proposed that cells express a unique RACK for each PKC isoenzyme and that PKC-RACK interactions are essential for isoenzyme specific cellular responses (Mochly-Rosen et al. 1991). To date, proteins with the characteristics of RACKs have been identified for PKCβ and PKCε (Csukai et al. 1997, Ron et al. 1999).

Similar to the translocation of PKC *to* the membrane initiates its activation, translocation *from* the membrane initiates the termination of PKC activity. This process is also tightly regulated. One way to inactivate PKC is to deplete the levels of DAG in the cell through promoting DAG metabolism by diacylglycerol kinases (DGKs) (Crotty et al. 2006). PKC has also been shown to catalyze an activating phosphorylation of DGKγ, leading to a negative feed-back mechanism (Yamaguchi et al. 2006). Activation of PKC allosterically alters the conformation of PKC by removing the pseudosubstrate out of the substrate binding cavity. This leaves PKC susceptible to cleavage by proteases and dephosphorylation by phosphatases. Dephosphorylation of the three processing sites (i.e. activation loop, turn motif, hydrophobic motif) of PKC is known to inactivate the kinase (Lee et al. 1996a, Hansra et al. 1999). Inactivation of PKC through dephosphorylations at the three processing sites has been suggested to precede the degradation of PKC by proteasome (Lee et al. 1996b, Lee et al. 1997, Hansra et al. 1999).

Activation of PKC leads to phosphorylation of its target substrates in an isoenzyme specific manner. PKCs are able to target other protein kinases e.g. protein kinase A (PKA) and mitogen activated protein kinases (MAPKs) as well as G proteins, proto-oncogene products, and nuclear proteins (Liu 1996). Diverse regulatory mechanisms controlling PKC activation and subcellular localization define PKC's isoenzyme-, cell-, and tissue-selective functions. Aberrant PKC activation has been shown to be involved in several pathological states including cancer, metabolic disorders such as diabetes, cardiovascular and pulmonary

disorders, neuronal degeneration such as Alzheimer's disease, pain, central nervous system dysfunction, and inflammatory diseases (Gould and Newton 2008, Yonezawa et al. 2009). In the following sections, the three PKC subfamilies will be discussed in more detail, with the emphasis on their role in inflammation and immunity (Table 1).

Table 1. *Immunological phenotypes of PKC isoenzyme knockout mice*

Isoenzyme	Predominant tissue expression	PKC knockout phenotype	References
cPKC isoenzymes			
PKCα	Ubiquitous, high in T cells	Deficiencies in T cell activation and T lymphocyte immunity	Pfeifhofer et al. 2006
PKCβ	Ubiquitous, high in B cells	BCR signalling and survival defects	Leitges et al. 1996, Saijo et al. 2002
nPKC isoenzymes			
PKCδ	Ubiquitous	Hyperproliferative B cells, self-reactive B cell	Leitges et al. 2001a, Miaymoto et al. 2002
PKCε	Ubiquitous	Macrophage activation defect	Castrillo et al. 2001
PKCθ	T lymphocytes, skeletal muscle, platelets	TCR signalling defect	Sun et al. 2000, Pfeifhofer et al. 2003
aPKC isoenzymes			
PKCζ	Ubiquitous	BCR signalling defect	Leitges et al. 2001b, Martin et al. 2002

BCR=B cell receptor, TCR=T cell receptor

1.2 Classical PKC isoenzymes

cPKCs, α, βI, βII, and γ, are the only calcium-dependent isoenzymes. They are activated by DAG in the presence of phosphatidylserine. The regulatory domain of cPKCs contains tandem C1 domains (C1A and C1B) that bind DAG and phorbol esters, and a C2 domain that binds anionic lipids in a Ca^{2+}-dependent manner. (Steinberg 2008)

PKCα is ubiquitously expressed in all tissues. Its expression is activated by a variety of stimuli, including physical stress like hypoxia and mechanical strain. PKCα plays an important role in the regulation of major cellular functions such as proliferation, apoptosis, differentiation, and cell migration and adhesion. However,

the biological responses are cell type specific. In other words, the output after PKCα activation depends on where and when it is activated, and the substrates on which it acts (Nakashima 2002). A good example is the role of PKCα in cancer. PKCα has been linked to several types of cancer, but whether its role is detrimental or protective seems to depend on the type of the tumour (Martiny-Baron and Fabbro 2007). PKCα is also closely linked to differentiation in several cell types, and it controls e.g. the development of macrophages (Pierce et al. 1998). Despite the ubiquitous expression of PKCα, PKCα knockout mice are fertile, appear healthy and anatomically normal, and enjoy a normal life span (Leitges et al. 2002, Pfeifhofer et al. 2006). However, studies with PKCα knockout animals have revealed a role for PKCα in insulin signalling (Leitges et al. 2002).

PKCβ is also ubiquitously expressed, although preferentially in pancreatic islets, monocytes, and the brain. PKCβI and PKCβII are generated by alternative splicing of the C-terminal exons from a single gene (Coussens et al. 1987, Kubo et al. 1987). Similar to PKCα, PKCβ has been claimed to have a role in proliferation, differentiation, metabolism, and other cell-type specific functions (Kawakami et al. 2002). A role for PKCβ in tumour formation has been demonstrated in several cancer types (Martiny-Baron and Fabbro 2007). More importantly, PKCβ has been implicated in diabetic pathologies. Hyperglycemia has been shown to lead to activation of PKCβ which contributes to diabetic microvascular complications. Inhibition of PKCβ can delay or even reverse diabetic retinopathy, nephropathy, and neuropathy (Suzuma et al. 2002, Martiny-Baron and Fabbro 2007).

In contrast to PKCα and PKCβ, PKCγ is expressed solely in the brain and spinal cord, and its localization is restricted to neurons. Within the brain, PKCγ is most abundant in the cerebellum, hippocampus, and cerebral cortex (Saito and Shirai 2002). PKCγ knockout mice are not visibly abnormal, but experiments testing for fine physiological and behavioural responses have revealed modest impairments of learning and memory as well as reduced neuropathic pain (Abeliovich et al. 1993a, Abeliovich et al. 1993b, Malmberg et al. 1997). There is not much information regarding the role of PKCγ in tumour formation, but the data published so far indicate that PKCγ does not play a significant role in tumour formation (Martiny-Baron and Fabbro 2007).

1.2.1 Classical PKC isoenzymes in inflammation and immunity

PKCα is one of the major PKC isoenzymes expressed in thymocytes and it has been suggested to have a role in their development (Tan and Parker 2003). In PKCα knockout mice, the development of B and T lymphocytes was shown to be unaffected. Relative and total numbers of CD4+ and CD8+ T cells in the lymph nodes and spleen were comparable to wild-type animals. Altogether, loss of PKCα had no apparent effect on T cell development and selection in the thymus. Instead, PKCα seems to be part of a signalling pathway that is necessary for full antigen receptor–mediated T cell activation and T lymphocyte immunity (Pfeifhofer et al. 2006). In addition, PKCα has been linked to the regulation of NO production and/or iNOS expression in rat vascular smooth muscle cells (Li et al. 1998), murine macrophages (Chen et al. 1998b, St-Denis et al. 1998), and murine microglia (Kang et al. 2001), as well as in the regulation of cyclooxygenase-2 (COX-2) expression in murine macrophages (Giroux and Descoteaux 2000).

PKCβ knockout mice are immunodeficient due to the impairment of humoral immune responses and the suppression of B cell responses. Experiments with PKCβ knockout mice have shown that PKCβ is critically important in B cell development and activation. PKCβ knockout mice display a reduced number of splenic B cells, significantly lower number of B-1 lymphocytes, and reduced levels of serum IgM and IgG3. Mutant mice also exhibit defective IgM-induced B cell proliferation. However, T cell proliferation and activation in response to T cell receptor stimulation were normal (Leitges et al. 1996). Studies with PKCβ knockout mice also showed that PKCβ was involved in the B cell receptor mediated nuclear factor κB (NF-κB) activation, since PKCβ deficient B cells displayed defective activation of NF-κB and poor induction of NF-κB-induced cell survival genes (Saijo et al. 2002). The study by Shinji et al. (1994) indicated that PKCβ might play a role also in lipopolysaccharide (LPS) signalling and LPS-induced macrophage function. Together with PKCα, PKCβ has been shown to regulate the production of LPS-induced tumour necrosis factor α (TNFα) in murine macrophages (Foey and Brennan 2004).

Contrary to the PKCα and PKCβ isoenzymes, very little is known about the role of PKCγ in the immune system or inflammation.

1.3 Novel PKC isoenzymes

The novel PKC isoenzymes δ, ε, η, and θ structurally resemble cPKC isoenzymes. However, the activation of nPKCs is calcium-independent, since their C2 domains lack the critical calcium-coordinating residues. These isoenzymes are thus regulated only by DAG, and their affinity for this second messenger is two orders of magnitude higher than that of the cPKCs. (Steinberg 2008, Newton 2010)

PKCδ has been the most widely studied isoenzyme of the novel group and it is known to be ubiquitously expressed in various cells and tissues (Steinberg 2004). Although studies with PKCδ knockout mice indicated that this PKC isoenzyme was not required for cell proliferation during normal development (Leitges et al. 2001a), it has been shown to participate in the regulation of cell proliferation in some transformed cells and cancer cells (Jackson and Foster 2004). A role for PKCδ has been suggested also in the regulation of cell differentiation, as well as in the induction and execution of apoptosis (Brodie and Blumberg 2003).

PKCε is expressed in many tissues and cells, but most abundantly in neuronal, hormonal, and immune cells. Along with PKCδ, PKCε has been the most widely studied novel isoenzyme. Essential roles for PKCε have been found in many signalling systems including cell proliferation, differentiation, muscle contraction, and metabolism, as well as in the functions of nervous, inflammatory, and immune systems (Akita 2002, Roffey et al. 2009). Studies with PKCε knockout mice have revealed its role in the attenuation of pain via nociceptor function in sensory neurons (Khasar et al. 1999), in super-sensitivity of $GABA_A$ receptors and in modified responses to ethanol (Hodge et al. 1999), as well as in the loss of ischemia preconditioning response in the heart (Saurin et al. 2002). Overexpression of PKCε has been detected in tumours from various organ sites (Gorin and Pan 2009).

PKCη is expressed in epithelial tissues, especially in squamous epithelia or epithelia where squamous cell carcinomas arise (skin, tongue, oesophagus, forestomach, trachea, and bronchus) (Osada et al. 1990, Kashiwagi et al. 2002). Disruption of the PKCη gene in mice was associated with defects in wound healing and enhanced tumourigenesis in skin tumour promotion (Chida et al. 2003).

PKCθ is expressed primarily in lymphocytes, skeletal muscle, and platelets. This isoenzyme plays an important role in the regulation of the activation of mature T

cells, and overwhelming majority of studies on PKCθ have focused on its role in T cells (Boschelli 2009). However, some studies have indicated that PKCθ may participate in the regulation of insulin signalling in skeletal muscle and adipocytes, as well as in insulin resistance (Kim et al. 2004, Sampson and Cooper 2006).

1.3.1 Novel PKC isoenzymes in inflammation and immunity

PKCδ knockout mice were generated independently by two groups (Leitges et al. 2001a, Miyamoto et al. 2002). These mice developed and reproduced normally, but displayed defects that revealed critical roles for PKCδ in immune function. The increased number of B cells in the spleen and other peripheral organs led to significant splenomegaly and lymphadenopathy. These mice appeared to have normal B and T cell development in the bone marrow, but they died prematurely due to a severe autoimmune disease, which was characterized by the detection of autoreactive antibodies. Consistently, deficiency in PKCδ was shown to result in the maturation and differentiation of self-reactive B cells, suggesting that PKCδ may play a role in the production of immunological tolerance (Mecklenbräuker et al. 2002).

PKCε seems to possess a unique role in macrophage biology. PKCε knockout mice, developed by Castrillo et al. (2001) appeared normal and were generally healthy. No obvious defects were observed in T cell proliferation or B cell function. However, the ability of the mice to recover from bacterial infections was impaired and the mice displayed dramatically reduced capacities of their peritoneal macrophages to produce NO, TNFα, or interleukin-1β (IL-1β) in response to LPS. In addition, the expressions of iNOS mRNA and protein were severely attenuated. In contrast, the differentiation of monocytes and macrophages from bone marrow precursors was not affected, pointing to a defect in signal transduction. Indeed, macrophages from PKCε knockout mice demonstrated a failure to activate NF-κB and p38 MAPK pathway in response to inflammatory stimuli. Subsequently, PKCε was suggested to be a critical component of the major LPS signalling pathway, toll-like receptor 4 (TLR4) pathway (McGettrick et al. 2006). A role for PKCε as a regulator of iNOS expression has been indicated also in RAW 264.7 macrophages (Díaz-Guerra et al. 1996, Paul et al. 1997) and murine microglia (Kang et al. 2001).

In addition to its predominant expression in squamous epithelia, PKCη is also expressed in pro-B cells and early-stage thymocytes. Indeed, PKCη has been shown to be a key regulator of cell division and cell death in early B cell development (Morrow et al. 1999). PKCη seems to play a role also in LPS-induced NO production and iNOS expression in rat primary astrocytes (Chen et al. 1998a). Perhaps more importantly, PKCη was shown to be essential for LPS-mediated NO production and iNOS expression in human monocytes (Pham et al. 2003a). Pham and coworkers demonstrated that murine macrophage cell lines that express iNOS and produce NO as a response to LPS treatment, express PKCη. However, human monocyte cell lines which do not produce NO in response to LPS treatment, do not express PKCη. When human monocytes were transfected with PKCη, they were found to produce NO in response to LPS treatment. In addition, co-expression of PKCη with iNOS has been linked to severe inflammatory arthritis (Pham et al. 2003b) and severe rheumatoid arthritis (RA) (Heale et al. 2007). On the other hand, lower expression of PKCη has also been associated with pathophysiologic mechanisms of RA (Teixeira et al. 2008).

PKCθ was first identified as a key enzyme in T cell activation and survival (Osada et al. 1992). It has a unique cellular localization in T cells, and it is the only PKC isoenzyme that is translocated to the immunological synapse. This results in the activation of several transcription factors required for T cell activation (Hayashi and Altman 2007, Boschelli 2009). Generation and characterization of PKCθ knockout mice confirmed the essential role of PKCθ in the activation of mature T cells and in T cell survival (Sun et al. 2000, Pfeifhofer et al. 2003). Thymic development was not affected, but the peripheral T cells failed to proliferate normally in response to antiCD3/CD28 stimulation. Interleukin-2 (IL-2) production was markedly diminished, and PKCθ knockout mice displayed impaired receptor-induced activation of transcription factors AP-1, NF-κB, and NFAT (nuclear factor of activated T cells). In addition, PKCθ knockout mice displayed reduced expression of several cytokines (IL-2, IL-4, interferon-γ, and TNFα) after an *in vivo* intraperitoneal challenge with an antiCD3 antibody. These results suggest that PKCθ has an important role in the development of T-cell mediated inflammatory diseases (Anderson et al. 2006).

1.4 Atypical PKC isoenzymes

PKCζ and PKCι/λ comprise the third PKC subfamily, the atypical isoenzymes. These isoenzymes lack the calcium-sensitive C2 domain but contain an atypical C1 domain that binds phosphatidylinositol trisphosphate and ceramide, but not DAG or phorbol esters, and they possess a protein-protein interaction domain PB1. Protein-protein interactions and phosphorylation by PDK1 seem to represent the major driving force for controlling the functions of these isoenzymes within cells. (Gould and Newton 2008, Steinberg 2008)

PKCζ and PKCι/λ exhibit 72% sequence homology at the amino acid level. This, together with the lack of isoenzyme-specific antibodies, has made it difficult to biochemically distinguish between PKCζ and PKCι/λ (Fields and Regala 2007). While PKCι/λ is known to be ubiquitously expressed, the expression of PKCζ seems to be somewhat more restricted (Kovac et al. 2007). PKCζ knockout mice develop essentially normally, exhibiting only subtle immunological deficiencies (Martin et al. 2002), whereas knockout of PKCι/λ is lethal during embryonic period (Fields and Regala 2007). Liver, pancreatic β cell, and muscle specific knockouts of PKCι/λ have indicated that this isoenzyme takes part in the regulation of insulin secretion and actions (Matsumoto et al. 2003, Hashimoto et al. 2005, Farese et al. 2007). PKCι/λ has also been shown to promote transformed growth, invasion, chemoresistance, and tumour cell survival, whereas PKCζ has been shown to inhibit the aspects of transformed phenotype (Fields and Regala 2007).

1.4.1 Atypical PKC isoenzymes in inflammation and immunity

Targeted disruption of the PKCζ gene in mice indicated that the role of this PKC isoenzyme is also specific to B cell function. The relative number and phenotype of splenic B cell subsets in PKCζ knockout mice is similar to that of the wild-type mice, but PKCζ deficient B cells exhibit increased spontaneous apoptosis and impaired B cell receptor dependent proliferation. The defective survival of PKCζ knockout B cells correlated with defects in the activation of the extracellular signal regulated kinase (ERK) pathway and transcription of NF-κB dependent genes (Leitges et al. 2001b, Martin et al. 2002). PKCζ has been also shown to take part in

the regulation of LPS-induced IL-10 production in murine macrophages (Foey and Brennan 2004). Recently, it has been suggested that PKCι/λ is necessary for Th2 cytokine production and optimal T cell proliferation (Yang et al. 2009).

1.5 PKC as a target for drug development

A role for PKC has been indicated in several cellular functions and diseases as reviewed in the previous chapters, making the PKC isoenzymes family a very promising target for drug development. Several PKC selective or PKC isoenzyme selective compounds have been developed, and some have progressed to clinical trials (Table 2) (Lee et al. 2008). However, the selectivity of the compounds is limited and only a few have demonstrated good selectivity for PKC over other kinases or show selectivity for individual isoenzymes. PKC drugs have been targeted mainly against the two regions of the kinase: the catalytic domain and the regulatory domain. In particular, the catalytic domain is conserved with other protein kinases e.g. PKA, which complicate the development of truly PKC selective compounds. In addition, the structural homology between PKC isoenzymes makes the development of PKC isoenzyme selective compounds a challenging task (Gould and Newton 2008).

The general problem with drugs targeted against protein kinases is how to avoid the disturbance of the general homeostasis throughout the whole body. This applies also to compounds targeted against PKC. It seems that PKCs are involved in a complex interplay in which some isoenzymes accelerate disease progression while others are protective. In addition, several PKC isoenzymes may be expressed in the same cells and tissues. Thus, the development of isoenzyme specific compounds is of great importance to prevent unwanted side effects. This might be yield by compounds that target isoenzyme specific activating pathways, membrane interactions or signalling pathways downstream of PKC. In addition, compounds that target certain cell types or PKC isoenzymes whose expression or activity is upregulated in certain pathological situations could prove efficient (Mackay and Twelves 2007, Lee et al. 2008).

Staurosporine is a microbial alkaloid that was identified as a PKC inhibitor over 20 years ago. Although its selectivity for PKC is limited, it has served as a lead

compound from which other PKC inhibitors, e.g. indolocarbazoles and bisindolylmaleimides, have been developed (Mackay and Twelves 2007). PKCβ selective indolocarbazole inhibitors enzastaurin (LY317615) and ruboxistaurin (LY333531) and pan PKC inhibitor midostaurin are all in clinical trials for cancer treatment (Gould and Newton 2008, Lee et al. 2008). Ruboxistaurin has also been tested for use in the treatment of diabetic retinopathy and diabetic macular oedema (Anonymous 2007).

Antisense oligonucleotide aprinocarcen (ISIS 3521) is an inhibitor targeted to the 3'-untranslated region of PKCα mRNA. It has shown activity against a range of tumour types including patients refractory to multiple types of conventional chemotherapy (Rao et al. 2004). The selective PKCδ RACK peptide antagonist KAI-9803 is currently in phase I/II clinical trials for the prevention of reperfusion injury in patients undergoing angioplasty following acute myocardial infarction (Lee et al. 2008, Yonezawa et al. 2009). Bryostatin 1, a macrocyclic lactone, is in phase II trials for the treatment of ovarian cancer and non-Hodgkin's lymphoma. Although bryostatin 1 is an activator of cPKCs and nPKCs, it downregulates PKC isoenzymes through proteasomal degradation (Lee et al. 2008, Roffey et al. 2009).

Rottlerin, also known as mallotoxin, is a natural compound isolated from *Mallotus phillippinensis*. It is an inhibitor of PKCδ (Gschwendt et al. 1994, Keenan et al. 1997). Rottlerin is widely used as a pharmacological tool in *in vitro* studies, but it has not been tested in clinical trials.

Although PKC inhibitors have not been tested in clinical trials for the treatment of inflammatory diseases, several promising results have been obtained from *in vivo* inflammatory disease models. The efficacy of pan PKC inhibitors in inflammatory disease models suggest that PKC is a potential target in the development of novel anti-inflammatory agents (Mulqueen et al. 1992, Kuchera et al. 1993, Birchall et al. 1994, DiMartino et al. 1995, Jacobson et al. 1995, Zhou et al. 1999). This is supported by results from studies with PKC knockout animals. PKCζ knockout mice displayed significant inhibition of ovalbumin-induced allergic airway disease, suggesting that PKCζ could be a therapeutic target in asthma (Martin et al. 2005). Studies with PKCθ knockout animals show that deficiency of PKCθ results in drastically reduced lung inflammation after induction of allergic asthma (Salek-

Ardakani et al. 2004). In addition, PKCθ deficient mice have been shown to be protected from Th1 dependent antigen-induced arthritis (Healy et al. 2006).

Table 2. *PKC inhibitors in clinical trials (www.clinicaltrials.gov, July 2010, Lee et al. 2008)*

Compound	Selectivity	Indication
Aurothiomalate[1]	pan PKC	Lung cancer
Bryostatin 1	cPKC, nPKC	Cancer [2]
Enzastaurin	PKCβ	Cancer [2]
ISIS 3521	PKCα	Lung / breast cancer, melanoma
KAI-9803	PKCδ	Myocardial infarction
Midostaurin	pan PKC	Leukemia
Ruboxistaurin (LY333531)	PKCβ	Associated diseases of diabetes[3]
Sotrastaurin	cPKC, nPKC	Transplantations, psoriasis
Tamoxifen[4]	pan PKC	Bipolar disorder
UCN-01	cPKC > nPKC	Cancer [2]

[1]*Possesses also other mechanisms of action in addition to inhibition of PKC; in clinical use in the treatment of RA*
[2]*Indications include several different types of cancer*
[3]*Includes e.g. diabetic retinopathy, diabetic neuropathy, diabetic macular oedema*
[4]*Studies completed; possesses also other mechanisms of action in addition to inhibition of PKC; in clinical use in the treatment of breast cancer.*

2. Nitric oxide

The discovery of NO as an important vasodilating factor started an era of intensive research work around this small gaseous signalling molecule. Robert Furchgott discovered that the relaxing effect of acetylcholine on the vascular wall was attributed to the release of a diffusible factor from endothelium termed endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). Ferid Murad, for his part, reported that NO and various nitro compounds relaxed the blood vessels by increasing the levels of cGMP (Arnold et al. 1977), a mechanism that was later found to be responsible for the effects of EDRF. In 1987 Furchgott, Louis Ignarro and Salvador Moncada suggested that EDRF and NO were one and the same molecule (Palmer et al. 1987, Ignarro et al. 1987). This was confirmed one year later, when Moncada and his coworkers proved that endothelial cells were able to transform the amino acid L-arginine into NO and citrulline (Palmer et al. 1988). In

1998 Furchgott, Ignarro and Murad were awarded the Nobel Prize for their discoveries concerning NO as a signalling molecule in the cardiovascular system.

Today, NO is known as a molecule that mediates several physiological and pathophysiological functions in the human body. In addition to its role as a regulator of vascular tone, NO prevents leukocytes and platelets from adhering to the vascular wall. It acts as a neuromodulator or neuromediator in some central neurons and in peripheral NANC (non-adrenergic non-cholinergic) nerve endings. NO takes part in both acute and chronic inflammation as well as in host defence mechanisms. (Nijkamp and Parnham 2005, Tripathi et al. 2007)

2.1 Biosynthesis of nitric oxide

The synthesis of NO from L-arginine and molecular oxygen is catalyzed by nitric oxide synthase (NOS) enzymes. NOS enzymes are active as homodimers and the N-terminal oxygenase domain is responsible for their dimerization. The formation of active NOS enzymes also requires binding of two calmodulin molecules, thus creating the NOS tetramer. The oxygenase domain contains two binding sites for cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4) and iron protoporphyrin IX (haem). The C-terminal reductase domain, which is linked by a calmodulin-recognition site to the oxygenase domain, contains binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and nicotineamide adenine dinucleotide phosphate (NADPH). The electrons required for NO synthesis are donated by NADPH to the reductase domain and proceed via redox carriers (FAD and FMN) to the oxygenase domain where the electrons interact with BH_4 and haem to catalyse the reaction of oxygen with L-arginine leading to the formation of citrulline and NO (Figure 3). (MacMicking et al. 1997, Alderton et al. 2001)

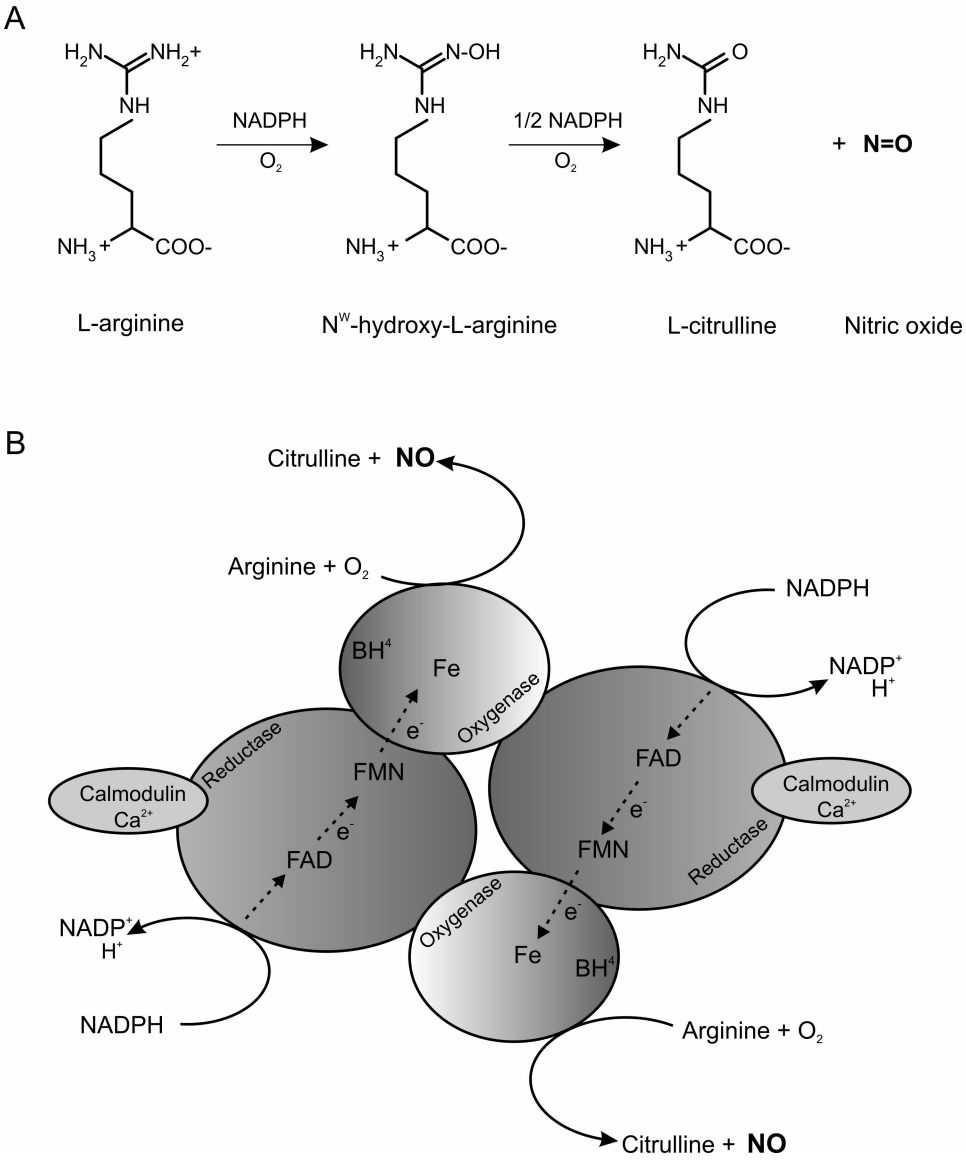

Figure 3. *Biosynthesis of nitric oxide from L-arginine and molecular oxygen (A). Overall reaction and cofactors of NOS (B). NOS catalyses the production of NO and L-citrulline from L-arginine, molecular oxygen, and NADPH derived electrons. Enzymatically active NOS is a tetramer, which contains NOS as a dimer and two molecules of calmodulin. Cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are bound to the reductase domain, whereas tetrahydrobiopterin (BH_4) and haem (Fe) are bound to the oxygenase domain. (Modified from Vuolteenaho et al. 2007).*

Three distinct isoforms of the NOS enzyme have been isolated and represent the products of three different genes with different localization, regulation and catalytic properties. The three human isoforms show approximately 50% homology (Alderton et al. 2001, Tripathi et al. 2007). Neuronal NOS (nNOS, NOS I) was the first isoform found and it was cloned in 1991 by Bredt and coworkers (Bredt et al. 1991). It is expressed predominantly in neurones in the brain and the peripheral nervous system (Zhou and Zhu 2009). The human endothelial NOS (eNOS, NOS

III) was cloned in 1992 (Janssens et al. 1992, Marsden et al. 1992) and it is expressed in endothelial cells, cardiac myocytes, and platelets (Dudzinski and Michel 2007). Both nNOS and eNOS exist in the cells as preformed proteins (and therefore are also called constitutive isoforms); their activity is switched on by the elevation of intracellular Ca^{2+} concentration and the binding of calmodulin in response to neurotransmitters or vasoactive substances. When activated, both constitutive isoforms produce small amounts of NO (Dudzinski and Michel 2007, Zhou and Zhu 2009).

In contrast to the constitutive NOS isoforms, the activation of the third NOS isoform, inducible NOS (iNOS, NOS II), is calcium and calmodulin independent (Tripathi et al. 2007). Murine iNOS was originally and independently purified and cloned from immunoactivated macrophages in 1992 by three groups (Lowenstein et al. 1992, Lyons et al. 1992, Xie et al. 1992), and the respective human isoenzyme was first isolated from primary human hepatocytes and chondrocytes (Charles et al. 1993, Geller et al. 1993). The human iNOS gene is located at cen-q11.2 at chromosome 17. It contains 26 exons spanning over 37 kb and encodes a protein of 131 kDa (1153 amino acids) (Tripathi et al. 2007). iNOS expression in various inflammatory and tissue cells can be induced by proinflammatory cytokines and microbial products, such as LPS. The production of NO through the iNOS pathway is regulated mainly at the level of iNOS expression (Alderton et al. 2001), which is covered in more detail in chapter 2.3.

2.2 Nitric oxide in inflammation

The role of NO in inflammation and immunity was revealed in 1985 after Stuehr and Marletta discovered that LPS activated mouse macrophages produce significant amounts of nitrite and nitrate oxidized from NO (Stuehr and Marletta 1985). Since then, the production of NO has been demonstrated in a number of immune-system cells (e.g. dendritic cells, monocytes, macrophages, and eosinophils) and tissue cells (such as endothelial cells, fibroblasts and chondrocytes) (Bogdan 2001, Tripathi et al. 2007). The actions of NO in inflammation and immunity depend on the environment in which NO is produced and on the amount of NO produced. The enzyme primarily responsible for the production of NO in inflammatory processes is

iNOS. When induced, iNOS can generate large amounts of NO for prolonged periods of time (Zamora et al. 2000, Bogdan 2001).

NO produced by iNOS has beneficial antimicrobial, antiviral, antiparasital, and antitumoral effects. In the presence of equal amounts of NO and superoxide (O_2^-), the effects of NO as a toxic defence molecule against infectious organisms are mainly mediated by the formation of peroxynitrite ($ONOO^-$) (Kumar et al. 2010). NO also regulates the functional activity, growth and death of many immune and inflammatory cell types including macrophages, mast cells, T lymphocytes, and neutrophils (Tripathi et al. 2007). On the other hand, aberrant iNOS induction seems to be involved in the pathophysiology of human diseases such as asthma, arthritis, colitis, psoriasis, neurodegenerative disorders, and tumour development (Bogdan 2001, Kleinert et al. 2003). Indeed, compounds that inhibit iNOS expression or activity have been proved to have anti-inflammatory properties in various forms of experimentally–induced inflammation. The pro- and anti-inflammatory effects of NO are described in more detail in Table 3.

Table 3. *Modulation of inflammation by nitric oxide*

Proinflammatory properties

- Promotes vasodilatation and vascular leakiness
- Reacts with O_2^- to form peroxynitrite[1]
- Activates NF-κB, AP-1[2]
- Upregulates proinflammatory cytokines
- Enhances natural killer cell activity
- Cytotoxic: promotes apoptosis

Anti-inflammatory properties

- Inhibits mast cell degranulation
- Inhibits platalet and neutrophil adhesion to endothelium
- Suppresses antigen presenting cell activity
- Suppresses T and B cell proliferation
- Antimicrobial activity
- Downregulates proinflammatory cytokines

[1] *Excessive production causes protein nitration, DNA damage, apoptosis, and necrotic cell death resulting in cellular/tissue injury.*

[2] *Activation of transcription factors NF-κB and AP-1 leads to the production and release of proinflammatory mediators.*

2.2.1 Nitric oxide in rheumatic diseases

Osteoarthritis (OA), the most common type of joint disease, is characterized by the progressive erosion of articular cartilage. Traditionally OA has been considered to develop as a consequence of mechanical wear and tear of ageing cartilage, but the presence of inflammatory mediators in OA joints indicate that this disease is a local slowly processing inflammatory process (Vuolteenaho et al. 2007, Kumar et al. 2010). RA is a chronic systemic inflammatory disorder that may affect many tissues and organs, but principally attacks the joints leading to an inflammatory synovitis that often progresses to destruction of the articular cartilage (Kumar et al. 2010). The role of NO in both of these diseases has been studied intensively. NO seems to be a proinflammatory and destructive mediator in the cartilage, and it is believed to be involved in the processes leading to chondrocyte death and promote the destruction of articular cartilage (Vuolteenaho et al. 2007, Abramson 2008).

Increased levels of markers of NO production are found in joint fluids and serum from patients with OA and RA (Ersoy et al. 2002, Karan et al. 2003), as well as in serum of patients with juvenile idiopathic arthritis (Bica et al. 2007). Positive correlations between the activity of the disease and the enhanced levels of NO production are seen in patients with RA and juvenile idiopathic arthritis, respectively (Ersoy et al. 2002, Bica et al. 2007). iNOS expression has been demonstrated in RA and OA synoviocytes and chondrocytes (Sakurai et al. 1995, McInnes et al. 1996, Grabowski et al. 1997, Vuolteenaho et al. 2001). Vuolteenaho et al. (2001) also reported that addition of IL-1β, TNFα, and LPS could enhance NO production by OA cartilage in organ culture. NO synthesis was suggested to derive from glucocorticoid-insensitive expression of iNOS, since various NOS inhibitors, but not dexamethasone, suppressed NO production. Recently, the role of adipokines adiponectin and leptin in enhanced NO production in OA cartilage has been reported (Lago et al. 2008, Vuolteenaho et al. 2009).

2.2.2 Nitric oxide in asthma

Asthma is a chronic inflammatory disease of the airways characterized by the presence of activated inflammatory cells, such as eosinophils, macrophages, mast cells, and T-lymphocytes. In asthma, Th2-dependent mechanisms play a critical role

in eosinophil recruitment to the airways, mucus hypersecretion and airway hyperreactivity (Kumar et al. 2010). In experimental animal models of asthma, enhanced NO production and iNOS expression have been linked to bronchial hyperresponsiveness and eosinophilic inflammation (Eynott et al. 2002, Eynott et al. 2003). In addition, acute inhibition of iNOS activity has been shown to inhibit asthma-like responses in a mouse model of asthma (Landgraf et al. 2005). Increased expression of iNOS has been observed also in human airways of asthmatic patients (Hamid et al. 1993), and increased concentrations of NO in the exhaled air have been associated with asthma (Kharitonov et al. 1994). Measurement of exhaled NO in asthmatics can be used as a non-invasive technique and can guide the treatment with inhaled glucocorticoids (Hesslinger et al. 2009). Extended exhaled NO measurement can be used to separately assess alveolar and bronchial inflammation and to determine disease activity, severity, and response to anti-inflammatory treatment in asthma and other inflammatory lung diseases (Lehtimäki et al. 2001a, Lehtimäki et al. 2001b, Lehtonen et al. 2007, Lehtimäki et al. 2010).

2.2.3 Nitric oxide in inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic condition that results from inappropriate immune activation. Crohn's disease and ulcerative colitis are the two disorders that comprise IBD (Kumar et al. 2010) and the involvement of NO has been proposed in both of these diseases. NO per se is not cytotoxic to intestinal tissue, and eNOS derived NO appears to be a homeostatic regulator of several essential functions of the gastrointestinal mucosa. NO is linked to the regulation of microvascular and epithelial permeability, the maintenance of adequate perfusion, and to the major epithelial functions involved in host defence, such as regulation of mucus and epithelial cell fluid production (Cross and Wilson 2003, Kolios et al. 2004). The role of NO in IBD has been evaluated in several studies with somewhat conflicting results. In animal models of intestinal inflammation, the induction of iNOS in acute colitis and the beneficial effects of inhibition of iNOS have been reported (Boughton-Smith et al. 1993b, Kankuri et al. 1999, Kankuri et al. 2001). Patients with ulcerative colitis were reported to have a 100-fold increase in luminal NO levels as compared to healthy controls (Lundberg et al. 1994). iNOS protein

expression has been demonstrated by immunohistochemistry to be present in the epithelial cells of the colonic mucosa of patients with active ulcerative colitis or acute phase infectious colitis (Kolios et al. 1998). In contrast, no iNOS expression was detected in samples from healthy controls or in infectious colitis patients in total remission. Increased iNOS activity has also been demonstrated in the mucosa of patients with ulcerative colitis, but no increase was seen is samples from patients with Crohn's disease (Boughton-Smith et al. 1993a). In contrast, Rachmilewitz et al. (1995) reported 10-fold and 3.8-fold increases in NOS activity and 4.2-fold and 8.1-fold increases in NO generation in patients with ulcerative colitis and Crohn's disease, respectively.

Despite the many studies in humans and animal models, the role of NO in IBD is somewhat controversial. Studies with NOS inhibitors and iNOS knockout mice have reported either improvement or worsening of experimental IBD. The human studies indicate that in IBD, especially in colitis ulcerosa, iNOS expression and activity are upregulated and NO production is enhanced. It is likely that NO is an important mediator in mucosal inflammation, but its role can vary depending on the cells producing it and exposed to it, its interaction with oxyradicals, and the time course and severity of the inflammation (Mashimo and Goyal 1999, Cross and Wilson 2003, Kolios et al. 2004).

2.3 Regulation of iNOS expression

Originally, iNOS enzyme was purified and cloned from a murine macrophage cell line (Lowenstein et al. 1992, Lyons et al. 1992, Xie et al. 1992) and the respective human enzyme was first isolated from primary human chondrocytes and hepatocytes (Charles et al. 1993, Geller et al. 1993). Subsequently, the expression of iNOS enzyme has been shown in various murine and human cell types and cell lines. Marked cell type and species specific differences in the responsiveness of iNOS expression to different stimuli have been reported (Kleinert et al. 2003, Korhonen et al. 2005, Vuolteenaho et al. 2007). Most human cells require multiple synergistically acting cytokines in order to induce detectable iNOS expression and NO synthesis, whereas many mouse cell lines express high levels of iNOS in response to LPS or to a single cytokine. In addition, it has been difficult to induce

iNOS expression in human monocyte or macrophage cell lines *in vitro*, although iNOS expression in macrophages in inflamed tissues has been shown *ex vivo* (McInnes et al. 1996, Moilanen et al. 1997, Korhonen et al. 2005). The level of NO synthesised by iNOS is significantly regulated at the level of iNOS transcription. Depending on the stimulus and the cell type, different signalling pathways activate different transcription factors, activators (e.g. protein kinases) and inhibitors (e.g. protein phosphatases). In addition, the expression of iNOS is also controlled at the post-transcriptional, translational, and post-translational level (Kleinert et al. 2003, Aktan 2004, Korhonen et al. 2005).

Molecular mechanisms for the transcriptional regulation of the iNOS gene have been studied by cloning the murine (Lowenstein et al. 1993, Xie et al. 1993) and the human (Chartrain et al. 1994, de Vera et al. 1996) promoter regions. Murine and human iNOS promoters exhibit homologies to binding sites for several transcription factors, with homology of 55% within the first 1.7 kb of 5'flanking sequence (Rao 2000, Kleinert et al. 2003). Both iNOS promoters contain a TATA box about 30 bp from the transcription starting site. Binding sites for transcription factors NF-κB, nuclear factor interleukin 6 (NF-IL6), octamer factors, and transcription factors induced by TNFα are located near the TATA box. At position –900 bp, murine and human promoters display binding sites for the transcription factors induced by interferon-γ (IFNγ) (Kleinert et al. 2003) (Figure 4).

Most of the transcription regulatory elements of the murine iNOS (miNOS) gene are located within 1.5 kb of the 5'flanking region (Lowenstein et al. 1993, Xie et al. 1993). The miNOS promoter contains two regulatory elements. The proximal region (region I, position –48 to –209) functions as the basal promoter element and mediates the response to LPS through NF-κB and interferon response factor (IRF) binding (Lowenstein et al. 1993, Xie et al. 1993). The distal region (region II, position –913 to –1029) functions as an enhancer element and responds to stimulation by LPS and IFNγ. It contains several transcription binding sites including the NF-κB binding site, gamma-activated site (GAS) element and IRF1 response element (IRE) (Lowenstein et al. 1993, Xie et al. 1993). In the miNOS gene, 1000 bp out of the 1.5 kb promoter confer full inducibility in response to a mixture of IFNγ and LPS in cultured mouse macrophages, RAW 264.7 (Lowenstein et al. 1993, Xie et al. 1993).

In contrast to the miNOS promoter, deletion analysis of the human iNOS (hiNOS) 5'flanking region points to the presence of regulatory elements on the length of 16 kb, and a full-length promoter is required for induction by a cytokine mixture (de Vera et al. 1996). However, contradictory evidence about the presence of regulatory elements in the human promoter has been reported. Nunokawa et al. (1996) reported induction by cytokines when promoter constructs contained the first 3.2 kb. Chu et al. (1998) reported, that only iNOS promoter fractions larger than 3.8 kb exhibit any significant induction with cytokines. In addition, inducibility by cytokines has been shown with a 8.3 kb promoter fragment (Marks-Konczalik et al. 1998, Kristof et al. 2001). The human iNOS promoter is activated by NF-κB. In addition to the proximal NF-κB site, functional NF-κB sites are located also further upstream of the promoter (Marks-Konczalik et al. 1998, Taylor et al. 1998). IFNγ-inducible factors that regulate the activity of the hiNOS promoter are not as well characterised as those that regulate the miNOS promoter. However, two functional GAS sites have been described of which the upsteam site contains overlapping NF-κB and STAT1 (signal transducer and activator of transcription 1) binding sites. Binding of both of these factors to this site is required for full promoter activity (Ganster et al. 2001). In addition, IRF1 has been reported to be involved in hiNOS transcription (Flodström and Eizirik 1997, Tsutsumi et al. 1999).

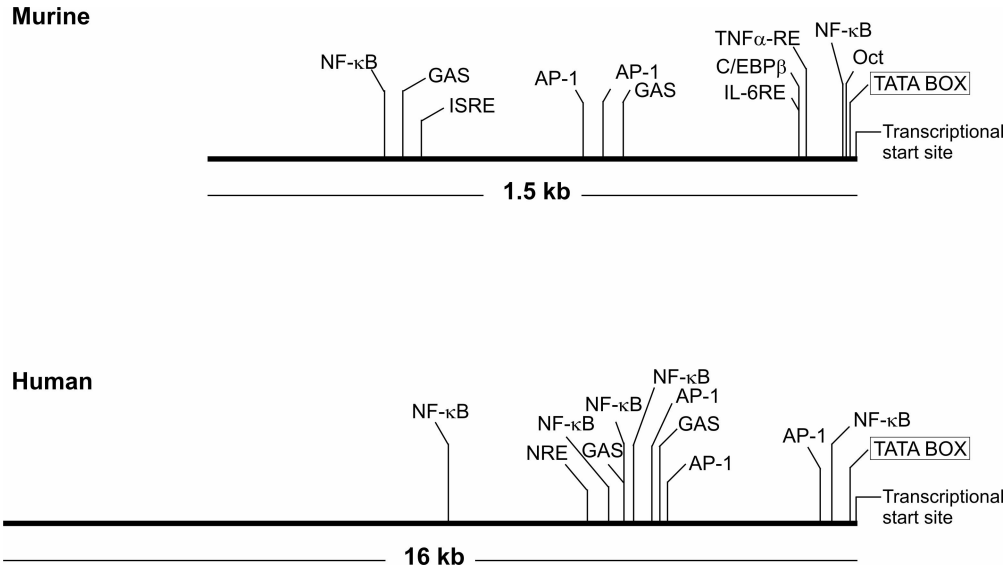

Figure 4. *Schematic presentation of murine and human iNOS promoters and transcription factor binding sites. NF-κB=nuclear factor κB, GAS=gamma-activated site, ISRE=interferon-stimulated response element, AP-1=activator protein 1, IL6-RE=interleukin-6 responsive element, C/EBPβ=CAAT/enhancer binding protein β, TNFα-RE=tumour necrosis factor α responsive element, Oct=octamer factor, TATA=TATA box, NRE=negative regulatory element. (Modified from Kleinert et al. 2003).*

2.3.1 NF-κB pathway

NF-κB is an important transcription factor for iNOS. The NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB (Hayden and Ghosh 2008, Vallabhapurapu and Karin 2009) and many cytokines as well as LPS have been reported to activate the NF-κB pathway. Even trace amounts of LPS are able to activate the innate immunity system via TLR4, leading to the activation of NF-κB and the production of numerous proinflammatory mediators (West et al. 2006). In resting cells, engagement of TLR4 receptors results in the activation of IκB kinase (IKK). The activated IKK complex phosphorylates IκB, which leads to ubiquitination and degradation of IκB proteins. This releases the NF-κB heterodimer (p50/p65), which is further activated by various posttranslational modifications. The NF-κB dimer translocates to the nucleus where it binds to specific DNA sequences and promotes the transcription of the target genes (Figure 5) (Hayden and Ghosh 2008, Vallabhapurapu and Karin 2009).

The importance of NF-κB binding sites for the induction of the iNOS promoter activity has been demonstrated in murine (Lowenstein et al. 1993, Xie et al. 1994) and human cells (Marks-Konczalik et al. 1998, Taylor et al. 1998). The miNOS

promoter contains two NF-κB elements (Xie et al. 1994); the proximal NF-κB site is required for LPS–induced iNOS expression (Lowenstein et al. 1993, Xie et al. 1993), whereas the upstream NF-κB site is required for maximal expression of iNOS gene in macrophages exposed to a combination of LPS and IFNγ (Kim et al. 1997). The hiNOS promoter has several functional NF-κB sites further upstream of the promoter, in addition to the proximal NF-κB site. Conflicting results have been published concerning the importance of the NF-κB binding sites in the human promoter. However, it does seem that at least the proximal binding site seems to be important (Chu et al. 1998, Marks-Konczalik et al. 1998, Taylor et al. 1998). Although NF-κB is not essential for iNOS expression in all cell types, it appears to be a central target for activators and inhibitors of iNOS expression (Rao 2000).

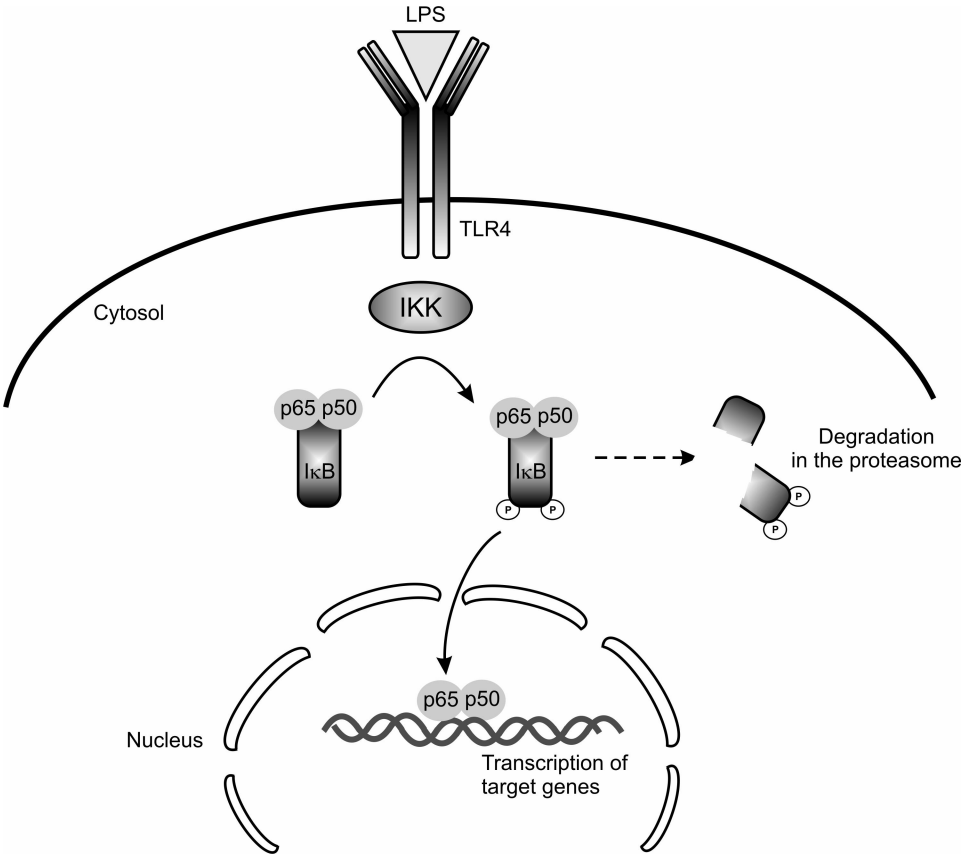

Figure 5. *Activation of the NF-κB pathway by LPS. TLR4=Toll like receptor 4, IKK=IκB kinase, IκB=inhibitory κB, p65 and p50=subunits of NF-κB.*

2.3.2 JAK-STAT pathway

Studies on gene induction by interferons led to the discovery of the Janus kinase (JAK) -STAT pathway (Darnell et al. 1994), which has subsequently been shown to be an important signalling pathway activated by a variety of cytokines. JAKs (four mammalian isoforms; JAK1, JAK2, JAK3, and TYK2) are protein tyrosine kinases that are pre-associated with membrane-proximal regions of cytokine receptors (Ivashkiv and Hu 2004). Cytokine ligation leads to dimerization of plasma membrane cytokine receptors. Receptor dimerization results in the activation of receptor-associated JAKs and phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor. This leads to the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription (Figure 6) (Shuai and Liu 2003, Ivashkiv and Hu 2004). Typically, cytokine stimulation involves the ligation of at least two different receptor subunits, and this leads to association of a pair of different JAKs. For instance, IFNγ activates JAK1 and JAK2. Similarly, certain cytokines preferentially activate particular STATs (out of the seven mammalian STATs), e.g. IFNγ activates STAT1 (Ivashkiv and Hu 2004). The importance of STAT1 as a mediator of INFγ responses has been demonstrated in STAT1 knockout mice (Meraz et al. 1996) where STAT1 deficient bone marrow derived macrophages showed a complete lack of responsiveness to IFNγ.

All mammalian iNOS promoters contain several homologies to STAT1α binding sites (GAS) (Kleinert et al. 2003). Optimal induction of the iNOS gene by IFNγ and LPS requires binding of STAT1α to the GAS of the miNOS promoter (Gao et al. 1997). A requirement for STAT1 in LPS-induced iNOS expression has been demonstrated also in STAT1 knockout mice (Ohmori and Hamilton 2001). JAK inhibitors AG-490 and/or WHI-P154 have also been shown to decrease IFNγ or LPS -induced iNOS expression in murine macrophages (Sareila et al. 2006, Sareila et al. 2008).

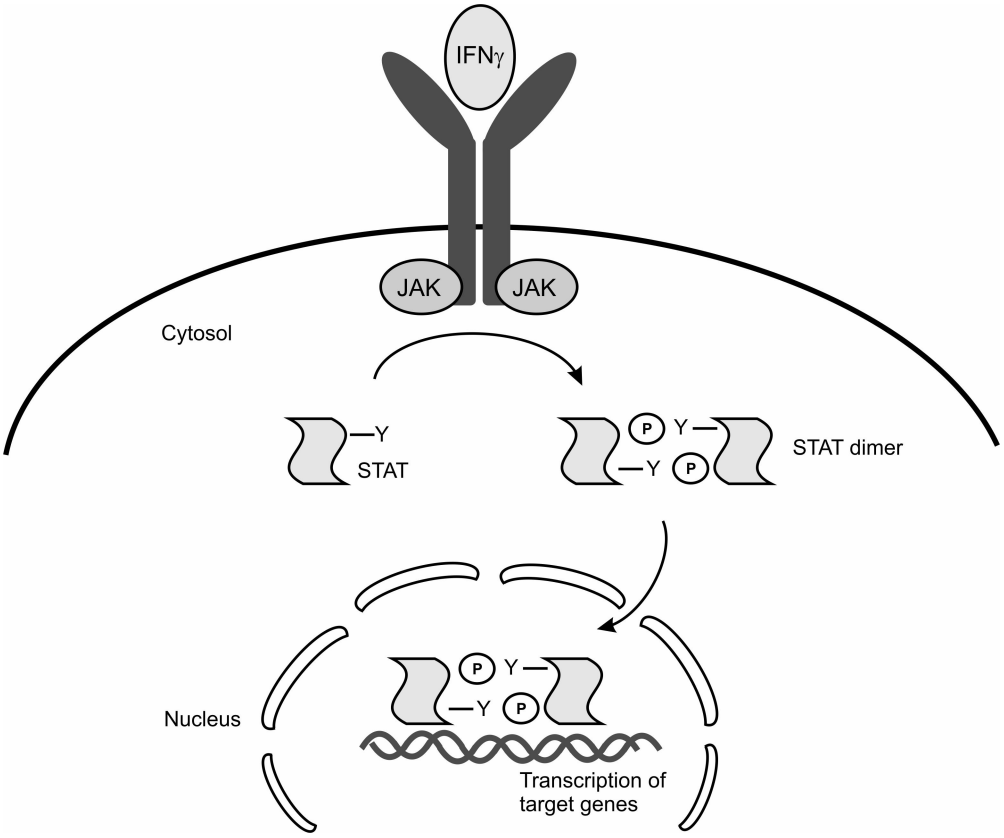

Figure 6. *Activation of the JAK- STAT1 pathway triggered by IFNγ. IFN=interferon, JAK=Janus kinase, STAT=signal transducer and activator of transcription.*

2.3.3 IRF1 pathway

The distal enhancer region in the miNOS promoter contains several interferon regulatory factor binding elements (IRF-E) (Lowenstein et al. 1993, Xie et al. 1994). An essential role in the induction of the iNOS promoter has been demonstrated for one of these IRF1 binding sites (Martin et al. 1994). In reporter assays using deletion constructs mutations of the binding site (positions -913 bp to -923 bp) blocked the IFNγ mediated enhancement of LPS-induced iNOS promoter activity. This data was supported by *in vivo* footprint studies in LPS-stimulated macrophages (Goldring et al. 1996). The requirement for the transcription factor IRF1 was confirmed in macrophages from IRF1 knockout mice and in IRF1 deficient glial cells, where LPS and IFNγ -induced iNOS mRNA expression was markedly reduced (Kamijo et al. 1994, Fujimura et al. 1997). However, Shiraishi et al. (1997) studied the role of IRF1 in iNOS induction in chondrocytes derived from IRF1 knockout mice and found no difference in iNOS expression levels between wild-type and

IRF1 deficient chondrocytes. Thus, it seems that IRF1 has a tissue specific role in the induction of iNOS. In addition, IRF1 has been suggested to be involved in hiNOS transcription (Flodström and Eizirik 1997, Tsutsumi et al. 1999).

2.3.4 iNOS mRNA stability

iNOS expression and NO production can be regulated also at the post-transcriptional level, with one of the most important means being the regulation of the iNOS mRNA stability. Unstimulated cells may show continuous iNOS transcription in nuclear run on assays, and human iNOS promoter constructs have been shown to possess basal activity in colon adenocarcinoma cells (DLD-1) and in liver epithelial cells (AKN-1). However, no iNOS mRNA or protein was detected in those cells under resting conditions, which suggests that iNOS mRNA is highly unstable in the absence of inflammatory stimuli (de Vera et al. 1996, Laubach et al. 1997, Linn et al. 1997).

The first data suggesting the involvement of post-transcriptional mechanisms in the regulation of iNOS expression were provided by Vodovotz et al. in 1993. They demonstrated that in mouse peritoneal macrophages, TGFβ could suppress iNOS expression by decreasing iNOS mRNA stability, reducing iNOS mRNA translation, and increasing degradation of iNOS protein.

The 3'–untranslated region (3'UTR) of human and murine iNOS mRNA contains adenylate- and uridylate- (AU-) rich elements (AREs) (Lowenstein et al. 1992, Lyons et al. 1992, Rodriguez-Pascual et al. 2000), which are known to control mRNA stability of many transiently expressed genes (Caput et al. 1986, Shaw and Kamen 1986). Mouse iNOS 3'UTR contains four to six AUUUA sequences (Lyons et al. 1992), whereas the human iNOS mRNA 3'UTR contains four AUUUA sequences and one AUUUUA sequence (Rodriguez-Pascual et al. 2000). Regulation of the stability of iNOS mRNA seems to be especially important for hiNOS expression and some factors can regulate hiNOS mRNA stability by binding to ARE. HuR has been reported to bind 3'UTR of iNOS mRNA and to stabilize it in human DLD-1 cells (Rodriguez-Pascual et al. 2000). In addition, tristetraprolin (TTP) has been shown to stabilize hiNOS mRNA (Fechir et al. 2005). TTP does not, however, bind to the 3'UTR of iNOS mRNA itself, but it has been shown to mediate

its effects through KH-type splicing regulatory protein (KSRP) (Linker et al. 2005). In contrast, ARE/poly-(U) binding factor 1 (AUF1) was found to bind to 3'UTR of iNOS mRNA and to destabilize it in human DLD-1 cells (Pautz et al. 2009).

Increased iNOS mRNA stability has been shown also after treatment with forskolin or cyclic AMP (cAMP) (Kunz et al. 1994, Oddis et al. 1995) and BH_4 (Linscheid et al. 1998). In addition, activation of certain signalling pathways has been reported to stabilize iNOS mRNA, i.e. c-Jun N-terminal kinase (JNK) (Lahti et al. 2003, Lahti et al. 2006, Korhonen et al. 2007), PKCδ (Carpenter et al. 2001), and polypyrimidine tract-binding protein (PTB) (Pautz et al. 2006). Decreased stability of iNOS mRNA has been described after treatment with dexamethasone (Korhonen et al. 2002), 8-bromo-cGMP (Pérez-Sala et al. 2001), and calcineurin inhibitors (Hämäläinen et al. 2009).

2.3.5 iNOS protein stability

Enhancement or blockage of degradation of the iNOS protein can be considered as another post-transcriptional regulatory mechanism. Inhibition of iNOS expression either by TGFβ1 in primary murine macrophages (Vodovotz et al. 1993) or by dexamethasone in rat mesangial cells (Kunz et al. 1996) was claimed to result from enhanced degradation of iNOS mRNA and protein. Subsequently, the degradation of iNOS protein via the proteasome pathway was demonstrated by using proteasome inhibitor lactacystin in two human intestinal carcinoma cell lines (Felley-Bosco et al. 2000). The role of the proteasome as the primary degradation pathway for iNOS was confirmed when Musial and Eissa (2001) revealed that lactacystin blocked the degradation of iNOS protein in transfected HEK293 human epithelial kidney cells, RT4 human epithelial cells and RAW 264.7 murine macrophages. Later, Kolodziejski et al. (2002) reported that ubiquitination was required for iNOS degradation via the 26S proteasome pathway. Protein degradation through the ubiquitin-proteasome system is the major pathway of non-lysosomal proteolysis of intracellular proteins, and it is responsible for the degradation of more than 80% of intracellular proteins (Wang and Maldonado 2006).

The role of TGFβ in the regulation of iNOS protein stability has been demonstrated in murine chondrocytes and macrophages (Vuolteenaho et al. 2005,

Takaki et al. 2006), and the involvement of proteasome in TGFβ enhanced iNOS protein degradation was detected in RAW 264.7 macrophages (Mitani et al. 2005). Peroxisome proliferator-activated receptor α agonists, zinc protoporphyrin and rapamycin, have also been reported to enhance iNOS ubiquitination and/or proteasomal degradation (Paukkeri et al. 2007, Chow et al. 2009, Jin et al. 2009). Chen et al. (2009) demonstrated that iNOS ubiquitination and subsequent proteasomal degradation was dependent on CHIP, a chaperone-dependent ubiquitin ligase. CHIP has also been demonstrated to have a major role in targeting iNOS protein to the aggresome under circumstances where the ubiquitin-proteasome pathway is overwhelmed (Sha et al. 2009).

Another mechanism to degrade iNOS protein is via the calpain pathway. Calpains are intracellular proteinases that are able to modulate directly the activity and/or function of proteins (Sorimachi et al. 1997). In RAW 264.7 macrophages, the dexamethasone enhanced degradation of iNOS protein was efficiently blocked by calpain inhibitor I (Walker et al. 1997). Subsequently, Walker et al. (2001) reported that the binding of calmodulin to iNOS could play an important role in the regulation of iNOS protein stability and in the degradation by calpain. The role of calpain in iNOS degradation has been suggested also in murine endothelial cells (Liu et al. 2008).

2.4 Inhibitors of iNOS

The expression of iNOS and the overproduction of NO have been linked to many chronic inflammatory diseases, and selective inhibition of iNOS appears to be a promising means for the treatment of inflammatory diseases. However, the development of iNOS inhibitors is a challenging task. The inhibitors should be truly selective for iNOS, in order to prevent the excessive production of NO from iNOS, but at the same time, permit the basal formation of NO by constitutive NOS enzymes (Tinker and Wallace 2006, Hesslinger et al. 2009). Despite intensive research efforts in developing iNOS selective inhibitors and successful animal experiments, no selective iNOS inhibitors have reached the market so far (Hesslinger et al. 2009).

Some of the earliest inhibitors of NOS contained guanidine, amidine or isothiourea moieties, that mimicked the binding mode of the endogenous substrate, L-arginine. Although these inhibitors, such as N^G-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO), and N^G-nitro-L-arginine methyl ester (L-NAME), are potent (Rees et al. 1990), they have poor selectivity between the various NOS isoforms (Tinker and Wallace 2006, Paige and Jaffrey 2007). An amide prodrug L-N6-(1-iminoethyl)lysine (L-NIL) does display more selectivity against iNOS, and it has been reported to be 20-50 -fold more selective towards iNOS than nNOS or eNOS (Moore et al. 1994, Hallinan et al. 2002). L-NIL has been evaluated clinically in man, where it produced a marked inhibition of exhaled breath NO in normal and asthmatic subjects without the side effects observed following the systemic administration of non-selective NOS inhibitors (Hansel et al. 2003).

Arginine competitive, NADPH-dependent inhibitors GW274150 and GW273629 are potent, time-dependent and highly selective inhibitors of iNOS, with 80-100 -fold selectivity against nNOS and eNOS (Young et al. 2000, Alderton et al. 2005). GW274150 has proven to be effective in animal models (Dugo et al. 2004, De Alba et al. 2006), although it failed to affect airway hyperreactivity or inflammatory cell numbers in airways after allergen challenge in human asthmatic patients (Singh et al. 2007).

Isothioureas have been reported to be potent and somewhat selective inhibitors of iNOS, however they were too toxic for *in vivo* evaluation (Garvey et al. 1994). The work with bisisothioureas led to the discovery of a more promising agent, 1400W, which was claimed to possess 32-fold selectivity against nNOS and > 5000-fold selectivity against eNOS (Garvey et al. 1997, Paige and Jaffrey 2007).

AR-C102222, a 3,4-dihydro-1-isoquinolinamine, appears to be a potent inhibitor of iNOS (Beaton et al. 2001). It also possesses high oral bioavailability and reasonable selectivity against nNOS (30-fold) and eNOS (3000-fold). AR-C102222 has also shown excellent efficacy in animal models of inflammation following oral administration (Tinker et al. 2003).

The latest L-arginine site inhibitors are a novel class of compounds based on an imidazopyridine backbone. BYK191023, a highly potent inhibitor of iNOS, shows 200-fold and 2000-fold selectivity over nNOS and eNOS, respectively (Strub et al. 2006). Imidazopyridine compounds did not show any toxicity in various human cell

lines even up to high micromolar concentrations (Strub et al. 2006), and BYK191023 has proven to be effective in *in vivo* rat systemic inflammation models (Lehner et al. 2006). Thus imidazopyridines appears to be promising candidates for future therapeutics.

Since NOS isoforms require dimerization for their enzymatic function, compounds that disrupt or prevent the formation of the iNOS dimer might represent a way to obtain isoform-selective inhibitors. Some dimerization inhibitors, that are potent and selective for iNOS, have already been developed (Tinker and Wallace 2006, Paige and Jaffrey 2007).

During the writing process of this thesis, only one iNOS inhibitor was found to be in an ongoing phase II/III clinical trial. This compound, SD6010, is being developed by Pfizer and currently in tests evaluating its efficacy in the treatment of knee OA. (www.clinicaltrials.gov, July 2010)

3. Tristetraprolin (TTP)

Tristetraprolin (TTP) was first discovered 20 years ago in screens for genes that were rapidly turned on by exposure of cultured fibroblasts to insulin, serum or tumour promoting phorbol esters (Lai et al. 1990). The name tristetraprolin derives from the presence of three PPPPG amino acid repeats that are conserved across species. Three other groups also described the same sequence, therefore TTP is also known as nuclear protein 475 (Nup475) (DuBois et al. 1990), TPA-induced sequence 11 (TIS11) (Varnum et al. 1989, Varnum et al. 1991) and G0/G1 switch gene 24 (G0S24) (Heximer and Forsdyke 1993). TTP is encoded by an immediate-early response gene *Zfp-36* (refering to zinc finger protein 36) in mice and the equivalent human gene *ZFP-36* in man (Taylor et al. 1991).

The TTP family is composed of three members in mammals; TTP, TIS11b (also known as ZFP36L1, BRF1), (Gomperts et al. 1990, Varnum et al. 1991, Barnard et al. 1993) and TIS11d (also known as ZFP36L2, BRF2) (Varnum et al. 1991, Nie et al. 1995). The fourth member has been identified in frogs (XC3H-4) (De et al. 1999) and fish (CTH1) (Stevens et al. 1998), and it seems to be restricted to maternal mRNA pool. TTP is known to be a factor that binds to AREs within the mRNAs of its target genes, and causes destabilization of the mRNA and decreased

formation of the protein. All four members of the TTP protein family exhibit ARE-binding activity and the three mammalian members also exhibit mRNA-destabilizing capabilities in intact cells (Lai et al. 2000).

The importance of mRNA stability in the regulation of gene expression is well recognized, and it is now established that mRNA degradation is a tightly regulated process. One important element controlling the mRNA half-life is the ARE element found in the 3'UTR of many unstable mammalian mRNAs. This was identified as an mRNA-destabilizing element more than 20 years ago (Caput et al. 1986, Shaw and Kamen 1986). In addition to its role as a regulator of mRNA stability *in vivo*, ARE is a potent stimulator of decapping (Gao et al. 2001) and deadenylation (Xu et al. 1997) processes. Functional AREs have been categorized based on their sequence characteristics and decay kinetics. Class I AREs contain non-adjacent copies of AUUUA in the context of other U-rich sequences. Class II AREs contain tandemly repeated copies of the AUUUA motif, whereas class III AREs have U-rich sequences in the absence of the AUUUA motif (Chen and Shyu 1995, Xu et al. 1997). The known TTP binding sites in the 3'UTR of cytokine transcripts resemble class II AREs, whereas the TTP binding site found in the c-fos 3'UTR belongs to the class I AREs (Raghavan et al. 2001). TTP seems to bind to ARE sequences as a complex that contains also other components of the cellular mRNA decay machinery; 3'-5' exonuclease Xrn1 and the exosome component PM-scl75 (Hau et al. 2007). However, in dendritic cells from normal human donors, immunoprecipitation studies indicate that TTP could also interact with and regulate the expression of non-ARE-containing mRNAs (Emmons et al. 2008).

3.1 TTP in inflammation

TTP knockout mice were generated by Perry Blackshear and his colleagues in the mid 90s (Taylor et al. 1996a). The mice appeared normal at birth, but within 1-8 weeks developed a characteristic systemic phenotype that included loss of body weight and body fat, patchy alopecia, dermatitis, severe polyarticular erosive arthritis, myeloid hyperplasia, autoimmunity, and glomerular mesangial thickening (Taylor et al. 1996a). The nature of this systemic phenotype resembled the phenotype caused by chronic administration of TNFα (Keffer et al. 1991). When

newborn TTP knockout mice were treated with repeated injections of a specific mAb to mouse TNFα, the development of TTP deficiency phenotype was prevented (Taylor et al. 1996a) indicating the involvement of TNFα in the development of the inflammatory phenotype. In a subsequent study, the role of TNFα receptor (TNFR) subtypes in the TTP deficiency phenotype was investigated (Carballo and Blackshear 2001). The study revealed that TNFR1 is responsible for the development of arthritis and cachexia in the absence of TTP. TNFR2 may well have a protective role in this syndrome, because the TTP deficiency syndrome appeared to be exacerbated in TTP/ TNFR2 knockout mice.

In a subsequent study, macrophages derived from TTP knockout mice were shown to secrete elevated levels of TNFα in response to LPS as compared to cells from wild-type mice (Carballo et al. 1997). This effect was seen in cultured TTP deficient macrophages derived from the peritoneal cavity or from the bone marrow of adult TTP knockout mice or in cells from foetal liver, but not in cultured fibroblasts or T and B lymphocytes. The enhanced levels of TNFα protein were accompanied by increased levels of TNFα mRNA. The mechanism of this effect was investigated by Carballo and coworkers (1998) who evaluated the influence of TTP deficiency on the stability of TNFα mRNA after LPS treatment. The half-life of TNFα mRNA in the macrophages derived from the bone marrow of TTP knockout mice was significantly increased compared to that observed in the cells from wild-type mice, suggesting that TTP could regulate TNFα mRNA expression post-transcriptionally. These results indicate that the increase in the half-life of TNFα mRNA in macrophages derived from TTP knockout mice is likely to be responsible for the enhanced secretion of TNFα from TTP deficient macrophages and also for the TNFα excess that characterizes the TTP knockout mice.

Carballo and coworkers (1998) studied the possibility that TTP might be able to bind directly to the TNFα mRNA, which could then lead to its instability. Using RNA cross linking and gel mobility shift techniques, they were able to show that TTP bound directly to the ARE within the 3'UTR of TNFα mRNA. A single mutation of one of the key cysteine or histidine residues within either of the TTP zinc fingers completely prevented TTP binding to TNFα ARE (Lai et al. 1999). Hence, the RNA binding site in TTP was identified as the tandem zinc finger

domain and the binding site for TTP within the target mRNA was recognized as the ARE (Lai et al. 2000).

After those findings, the question arose whether TTP could regulate also the stability of other mRNAs containing characteristic class II AREs. In primary cultures of bone marrow stromal cells derived from TTP knockout mice, the absence of TTP resulted in increased secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the presence of LPS (Carballo et al. 2000). As compared to cells derived from wild-type animals, TTP deficiency led also to increased steady-state levels of GM-CSF mRNA after stimulation with LPS or TNFα, and increased half-life of GM-CSF mRNA after stimulation with LPS. In addition, TTP deficiency evoked an almost complete absence of the deadenylated form of GM-CSF mRNA.

IL-2 was reported to be a target for TTP in a study by Ogilvie et al. (2005). They showed that splenocytes or purified T cells from TTP knockout mice overproduced IL-2, and IL-2 mRNA was more stable in TTP deficient splenocytes as compared with wild-type cells. Binding of TTP to IL-2 ARE was demonstrated by gel shift assays. In a subsequent study from Ogilvie and coworkers (2009), the role of TTP as a mediator of IFNγ mRNA decay was investigated. Using T cells from TTP knockout mice, they noted that overexpression of IFNγ mRNA was due to stabilization of IFNγ mRNA. In a UV cross-linking assay TTP was also shown to mediate the rapid degradation of IFNγ transcript by binding to the IFNγ ARE.

Immediately early response 3 (Ier3) has also been recognized as a target for TTP mediated mRNA decay in embryonic fibroblasts from TTP knockout mice (Lai et al. 2006). In that study, microarray analysis of RNA from wild-type and TTP deficient fibroblast cell lines identified 250 mRNAs apparently stabilized in the absence of TTP. Of these, 23 contained conserved binding sites for TTP, with nine of them appearing to be stabilized. The transcript encoding for Ier3 was the most dramatically affected. In a recent study, IL-10 mRNA was shown to be a target for TTP mediated decay in primary macrophages from TTP knockout mice (Stoecklin et al. 2008). Overall, the results obtained from studies with TTP deficient cells indicate that TTP acts to downregulate TNFα, GM-CFS, IL-2, IL-10, IFNγ, and Ier3 gene expression through ARE-mediated mRNA decay.

Other possible targets for TTP have been proposed by alternative approaches. Overexpression studies have indicated that IL-3, IL-6, COX-2, and TTP itself may be destabilized by TTP (Stoecklin et al. 2000, Raghavan et al. 2001, Stoecklin et al. 2001, Sawaoka et al. 2003, Brooks et al. 2004). Later it was shown that peritoneal macrophages derived from TTP knockout mice overexpressed COX-2 protein (Phillips et al. 2004). Knockdown of TTP in macrophages by siRNA was shown to result in increased production of IL-6, IL-12, and macrophage inflammatory protein-2 (a homologue of human IL-8) (Jalonen et al. 2006), whereas macrophage inflammatory protein-3α was produced at lower levels than in control cells. In contrast, Fechir et al. (2005) reported that TTP could positively regulate the expression of human iNOS by enhancing the stability of human iNOS mRNA. TTP does not bind directly to iNOS mRNA, but interacts with KSRP which interacts with the 3'UTR of human iNOS mRNA. The interaction between TTP and KSRP is suggested to result in dislodgement of the KSRP/exosome complex from the iNOS mRNA, thus allowing the binding of HuR, which in turn leads to increased iNOS mRNA stability and enhanced iNOS expression (Linker et al. 2005). Recently, it was reported that increased expression of TTP in cystic fibrosis cells resulted in reduced stability and enhanced deadenylation of IL-8 mRNA (Balakathiresan et al. 2009), in line with the results obtained by Jalonen et al. (2006).

Evidence from mice with altered cytokine mRNA stability, along with human data, suggests that imbalance between the decay and stability of the inflammatory cytokine mRNAs could represent a basic mechanism leading to autoimmunity. Indeed, agents that enhance TTP expression or regulate the function or activity of TTP e.g. by phosphorylation of TTP, may have potential therapeutic value for the prevention or treatment of inflammation-related diseases.

3.2 Regulation of TTP expression

TTP mRNA and protein are expressed in several mouse tissues including thymus, liver, intestine, kidney, lung and spleen (DuBois et al. 1990, Lai et al. 1990, Cao et al. 2004, Lu and Schneider 2004). A similar expression pattern has been shown in rat tissues (Smoak and Cidlowski 2006). A number of agents have been reported to increase TTP mRNA and/or protein levels in mammalian tissues. These include

growth factors (e.g. insulin and insulin-like growth factor I) (DuBois et al. 1990, Lai et al. 1990), cytokines (e.g. TNFα, IFNγ, GM-CSF) (Varnum et al. 1989, DuBois et al. 1990, Carballo et al. 1998, Sauer et al. 2006), tumour promoters (Varnum et al. 1989, Lai et al. 1990) and the bacterial endotoxin, LPS (Carballo et al. 1998, Cao et al. 2004, Rigby et al. 2005).

Promoter regions of mouse, human and rat TTP have been demonstrated to include several different transcription factor binding sites. Mouse, human, and rat promoter regions contain consensus sequences for binding of activator protein-2 (AP-2) and specificity protein 1 (Sp1) (DuBois et al. 1990, Heximer and Forsdyke 1993, Lai et al. 1995, Kaneda et al. 2000). Mouse and human TTP promoter regions also contain binding sites for early growth response gene-1 (EGR1) (DuBois et al. 1990, Heximer and Forsdyke 1993, Lai et al. 1995). EGR1, AP-2 and TTP promoter element 1 were observed to contribute to the serum inducibility of mouse TTP (Lai et al. 1995). The TTP intron region was also found to be important for full serum inducibility. Some years later, Lai and coworkers (1998) reported that the mouse TTP intron sequence displayed binding sites for Sp1, AP-2, and NF-κB, as well as an NF-κB-like binding site, and that Sp1 might contribute to the full serum inducibility of TTP. Subsequently, a binding site for NF-κB has been located also in human TTP promoter and intron regions (Smoak and Cidlowski 2006).

Originally TTP was proposed to function as a transcription factor due to its nuclear localization in mouse fibroblasts (DuBois et al. 1990). However, stimulation of quiescent mouse fibroblasts with serum or other mitogens was shown to cause a rapid phosphorylation of serine residues and translocate TTP from nucleus to cell cytosol (Taylor et al. 1995, Taylor et al. 1996b). Later, it was shown that nuclear localization of TTP in normal and stimulated cells was actually very minimal, with a high abundance in dividing cells (Cao et al. 2004). TTP was primarily found to be localized in cell cytosol in THP-1 monocytic cells (Carballo et al. 1998, Brooks et al. 2002) and mouse tissues and cells (Cao et al. 2004), as well as in cells transfected with GFP-tagged TTP or cells overexpressing TTP (Lai et al. 1999, Cao 2004). Nuclear export of TTP is mediated by a functional leucine-rich nuclear export sequence in both mouse and rat (Murata et al. 2002, Phillips et al. 2002). Its transfer to the nucleus requires the presence of the tandem zinc finger domain, though it occurs independently of zinc finger RNA-binding ability (Phillips et al. 2002). In

transfected fibroblasts, the extent of cytoplasmic localization of TTP was increased by association with 14-3-3 protein (Johnson et al. 2002). This shuttle activity was also partially affected by phosphorylation at Ser^{178} but not at Ser^{220} in mouse TTP (Taylor et al. 1996b, Johnson et al. 2002). The overexpression studies conducted by Cao (2004) indicated that the nuclear TTP was able to bind TNF mRNA ARE in a manner similar to that of cytosolic TTP.

In normal tissues and in stimulated cells TTP exhibits a larger molecular mass on SDS gels than the predicted size (~43 kDa) due to extensive phosphorylation the TTP molecule (Cao et al. 2004). A number of agents have been reported to be able to phosphorylate TTP, e.g. LPS (Carballo et al. 2001, Cao et al. 2004), phorbol esters, serum, platelet derived growth factor and fibroblast growth factor (Taylor et al. 1995). Phosphorylation assays have revealed that the phosphorylation of TTP at least *in vitro* may occur via several different protein kinase cascades. These include p42 MAPK (Taylor et al. 1995, Cao et al. 2003, Cao 2004), p38 MAPK (Carballo et al. 2001, Zhu et al. 2001, Cao et al. 2003, Cao 2004), JNK (Cao et al. 2003), MAP kinase-activated protein kinase 2 (MK2) (Mahtani et al. 2001, Chrestensen et al. 2004, Stoecklin et al. 2004), PKA, PKB, and PKC (Cao and Lin 2008). Phosphorylation of serine residues has been shown to modulate several functions of TTP such as the mRNA ARE binding activity of TTP in mouse macrophages (Carballo et al. 2001, Hitti et al. 2006). It may also affect TTP's subcellular localization (Taylor et al. 1996b, Johnson et al. 2002), stability (Cao et al. 2004), and autoregulation (Brooks et al. 2004, Tchen et al. 2004) as well as its association with the exosome (Chen et al. 2001), stress granules (Stoecklin et al. 2004), and other proteins (Twizere et al. 2003, Carman and Nadler 2004).

3.2.1 Pharmacological control

TTP expression may also be regulated by different pharmaceutical agents. The role of glucocorticoids has been examined in a few studies. First it was shown that in murine macrophages, dexamethasone and a dissociated steroid, RU24858, could reduce LPS-induced TTP mRNA and protein expression (Jalonen et al. 2005). This was suggested to happen in a glucocorticoid response element -independent mechanism, possibly through histone deacetylation and transcriptional silencing.

Later, Smoak and Cidlowski (2006) showed that in unstimulated human A549 lung epithelial cells and various rat tissues, glucocorticoids could enhance TTP mRNA and protein expression via transcriptional mechanisms. However, they were unable to observe this enhancing effect in murine RAW 264.7 macrophages. Dexamethasone reduced TNFα mRNA levels and this was prevented by TTP siRNA. The researchers concluded that TTP was critical for the inhibitory effect of glucocorticoids on TNFα mRNA expression. A similar enhancing effect of glucocorticoids on TTP expression was seen in unstimulated human bronchial epithelial cells (Ishmael et al. 2008). In the same study, when mouse embryonic fibroblasts from wild-type and TTP knockout mice were compared, it was noted that glucocorticoid-mediated gene expression was absent in cells from TTP knockout mice. This is further support for a role of TTP as a mediator of the post-transcriptional effects of glucocorticoids. Altogether, it seems that the effects of glucocorticoids on TTP expression might be tissue and/or species specific since the induction of TTP by glucocorticoids was seen in human cell lines and rat tissues but not in mice. The difference might also stem from different treatment of cells, i.e. untreated cells versus cells treated with an inflammatory stimulus.

Another group of drugs that seem to regulate the expression of TTP are agents that increase the intracellular levels of cAMP. These include the cAMP analog, dibutyryl cAMP, the adenylate cyclase activator forskolin, and compounds that activate G_s-receptors (such as β_2-agonists). Initial results were obtained when two research groups showed that dibutyryl cAMP and forskolin could elevate TTP mRNA levels (DuBois et al. 1990, Kaneda et al. 1992). Subsequently, Jalonen et al. (2007) reported that β_2-agonists (salbutamol, terbutalin and formoterol), forskolin, and cAMP analogs increased TTP expression in murine J774 macrophages and human THP-1 monocytes and this was mediated partly through the activation of transcription factor AP-2. In a further study, Jalonen and coworkers (2008) examined the effects of these agents on TTP expression in murine macrophages exposed to an inflammatory stimulus (LPS). This study revealed that cAMP elevating agents had a decreasing effect on LPS-induced TTP mRNA expression, and they significantly reduced TTP protein levels as compared to LPS treatment alone. Therefore it seems that in macrophages TTP partly mediates the anti-

inflammatory effects of β_2-agonists and their effects on cytokine production, but this effect is reversed under inflammatory conditions.

3.2.2 MAPK signalling pathway

The studies on the regulation of TTP expression by different kinase pathways have focused mainly on the role of MAP kinases. In mouse fibroblasts overexpressing TTP, TTP Ser^{220} residue was shown to be phosphorylated by p42 MAPK *in vitro* and this was claimed to regulate TTP's function (Taylor et al. 1995). The p38 MAPK pathway has been reported to regulate the expression and posttranslational modification of TTP in LPS-stimulated RAW 264.7 macrophages (Mahtani et al. 2001). The same study also indicated that MK2 could phosphorylate recombinant TTP *in vitro* i.e. it could mediate the effects of p38 on TTP expression. Similar results were obtained by Brook and coworkers (2006), who reported that in LPS-stimulated RAW 264.7 macrophages p38 and ERK pathways synergistically regulated TTP expression at the translational and posttranslational levels. Inhibition of p38 destabilized endogenous TTP protein, reducing its half-life from >4 h to 30 min. This was shown to occur via MK2-mediated phosphorylation of Ser^{52} and Ser^{178}. Simultaneous inhibition of p38 and ERK was shown to enhance TTP protein degradation. The inhibition of p38 also evoked a rapid dephosphorylation of TTP and increased its nuclear localization. Simultaneous inhibition of p38 and ERK pathways resulted in enhanced nuclear accumulation and a greater degree of dephosphorylation, as compared to p38 inhibition alone. Subsequently, it was reported, that both p38 and ERK activation were required to inhibit TTP function and to stabilize TNFα mRNA in activated macrophages (Deleault et al. 2008). In addition, the p38 pathway has been shown to regulate TTP mRNA stability in murine macrophages (Tchen et al. 2004), TTP protein expression in human monocytes and murine macrophages (Brooks et al. 2004, Jalonen et al. 2005), TTP ARE binding activity in bone marrow macrophages from TTP knockout mice (Carballo et al. 2001), phosphorylation of TTP in murine macrophages (Zhu et al. 2001), and localization of TTP to stress granules (Rigby et al. 2005). ERK and JNK pathways have also been suggested to participate in the regulation of TTP expression (Brooks et al. 2004, Jalonen et al. 2005). MK2 was shown to be essential

for stabilization of TTP mRNA in bone marrow macrophages from TTP knockout mice (Hitti et al. 2006). Phosphorylation of TTP by MK2 led to increased TTP protein stability, and reduced ARE binding affinity. It was concluded that MK2 could inhibit the mRNA destabilizing activity of TTP. Phosphorylation of TTP by MK2 has been reported also in mouse fibroblasts *in vivo*, where Ser^{52} and Ser^{178} were identified as the putative MK2 phosphorylation sites (Chrestensen et al. 2004).

3.2.3 PKC signalling pathway

Phorbol esters, known activators of PKC, have been shown to increase TTP expression (Varnum et al. 1989, Lai et al. 1990). However, the signalling pathways responsible for PMA-mediated TTP induction and the role of PKC in it are not known in detail. In mouse fibroblasts treated overnight with 1.6 µM PMA to downregulate PKC isoenzymes, the elevation of TTP induced by insulin and serum remained unchanged, but this overnight treatment did abolish the induction of TTP mRNA by PMA (Lai et al. 1990). In rat fibroblasts and hepatoma cells, overnight treatment with 16 µM PMA did not change the levels of insulin induced TTP or cfos mRNAs (Stumpo et al. 1994). However, in a screen of the effects of several kinase inhibitors on the expression of different immediate early genes in mouse 3T3-L1 cells, PKC seemed to regulate *tis11* gene expression (Inuzuka et al. 1999). The PKC inhibitor RO320432, which according to the manufacturer, inhibits PKCα, PKCβI and PKCε decreased *tis11* gene expression as well as *c-fos, jun-B, egr-1* and some other immediate early genes, as did the MEK inhibitor, PD98059. Murata et al. (2000a) demonstrated that PMA caused an inactivation of TIS11 transcriptional activity and this was blocked by PKC and MEK inhibitors. In addition, the PMA stimulated promoter activity of TTP was shown to be mediated through PKC and MEK cascades (Murata et al. 2000b). Overall, these results indicate that PKC isoenzymes might have a role in the regulation of the expression of TTP, however no studies have actually determined which PKC isoenzymes are responsible for this putative regulation.

Aims of the study

Increased iNOS expression and NO production in macrophages and other inflammatory cells have been implicated in the pathogenesis of several inflammatory diseases. TTP expression is also increased in inflammation, but it is thought to act primarily as an anti-inflammatory factor by destabilising the mRNAs of various inflammatory genes and thus attenuating their expression. PKC pathways are major signalling mechanisms in cell differentiation and growth, but less is known about their specific effects on the regulation of inflammatory genes. The aim of the present study was to investigate from the drug development perspective the role of PKC isoenzymes in the regulation of inflammatory genes with iNOS and TTP as examples of a proinflammatory and anti-inflammatory factors, respectively.

The detailed aims of the present study were:

1. To evaluate if classical PKC isoenzymes are involved in the regulation of NO production and iNOS expression as well as in the regulation of TTP expression in activated macrophages using PKC inhibitors and phorbol esters as pharmacological tools. In addition, to identify which classical isoenzymes are involved in this phenomenon. (I, II)
2. To evaluate whether the novel isoenzyme PKCδ is involved in the regulation of NO production and iNOS expression, and in the regulation of TTP expression using a PKCδ specific siRNA and the PKCδ inhibitor rottlerin as investigational tools. (III, IV)
3. To characterize the mechanisms involved in the regulation of NO production and iNOS expression (I, IV) and of TTP expression (II, III) by PKC isoenzymes.

Materials and methods

1. Materials

Reagents were purchased as follows: DMEM and FBS were from Lonza Verviers SPRL (Verviers, Belgium); penicillin, streptomycin, amphotericin B, trypsin-EDTA, and Lipofectamine 2000 were from Invitrogen (Paisley, UK); RO318220, phorbol 12,13-didecanoate (PDD) and LY333531 were from Alexis Biochemicals (Lausen, Switzerland); GÖ6976, HBDDE and recombinant PKCγ were from Calbiochem (La Jolla, CA, USA); LPS (*Escherichia coli* 0111:B4), PMA, rottlerin and all other reagents were from Sigma Chemical Co (St Louis, MO, USA). Protein kinase C inhibitors and the other pharmacological compounds used in this study are listed in Table 4.

2. Cell culture

Murine J774A.1 macrophages (I, II and III) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and murine J774.2 macrophages (IV) were obtained from European Collection of Cell Cultures (Porton Down, Wiltshire, UK). Both cell lines were cultured at 37 ºC in 5% CO_2 atmosphere and grown in Dulbecco's modified Eagle's medium (DMEM) with Ultraglutamine 1 supplemented with 10% (J774A.1) or 5% (J774.2) heat-inactivated foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml). J774A.1 macrophages were harvested with trypsin-EDTA.

Murine L929 fibroblasts (IV) (CCL-1; ATCC) were cultured at 37 ºC in 5% CO_2 atmosphere and grown in Eagle's minimum essential medium with L-glutamine

containing 10% heat-inactivated foetal bovine serum and supplemented with sodium bicarbonate (0.15%), non-essential amino acids (1mM each), sodium pyruvate (1mM) and 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B.

Murine L929 fibroblasts were stably transfected with NF-κB responsive luciferase reporter gene to investigate NF-κB mediated transcription (IV). In experiments to prepare L929-pNFκB(luc)neo reporter cell line, L929 cells were stably transfected with a luciferase reporter construct [pNFκB(luc)neo] which contained five NF-κB binding sites to drive luciferase expression. The luciferase reporter construct was kindly provided by Professor Hartmut Kleinert at the Johannes Gutenberg University (Mainz, Germany). The plasmid contained also a neomycin resistance gene under the control of TK promoter for mammalian selection. To create a stable transfection, L929 cells were transfected with pNFκB(luc)neo reporter plasmid using Lipofectamine 2000 according to the manufacturer's instructions. Transfected cells were selected with G 418 disulphate salt (800 µg/ml) treatment. After the selection, the surviving clones were pooled to give rise to the L929 pNF-κB cell line and then further cultured in the presence of 400 µg/ml of G 418.

Cells were seeded on 96-well plates for cell viability assays, 24-well plates for siRNA and RT-PCR experiments and nitrite and ELISA measurements, on 6-well plates for extraction of whole cell lysates, and on 10 cm dishes for extraction of nuclear proteins and for PKC translocation studies. Cells were grown for 48 h (L929) or 72 h (J774) to confluence prior to the experiments.

3. Cell viability assays

Cytotoxicity of the tested compounds was examined by measuring cell viability using Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany). This test measures the metabolic activity of viable cells, i.e. the ability of cells to metabolize XTT to formazan via the activity of mitochondrial dehydrogenase. Cells were incubated with stimulants and tested compounds for 20 h before the addition of sodium 3'-[-1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)

benzene sulphonic acid hydrate (XTT) (final concentration 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulphate (an electron coupling reagent, final concentration 2.5 µg/ml). After 3 h incubation, the amount of formazan accumulating in the culture medium was assessed spectrophotometrically. Triton X-100 treated cells were used as a positive control of cytotoxicity. If the treatment with a tested compound led to a decreased mitochondrial dehydrogenase activity (20% or greater difference as compared to cells treated with stimulant only), the compound was excluded from further studies at that toxic concentration.

Table 4. *PKC inhibitors and other pharmacological compounds used in this study*

Compound		**Supplier**	**Reference**
CGP53353	PKCβII inhibitor	Sigma Chemical Co	Chalfant et al. 1996
GÖ6976	cPKC inhibitor	Calbiochem	Martiny-Baron et al. 1993
HBDDE	PKCα and PKCγ inhibitor	Calbiochem	Kashiwada et al. 1994
LY333531	PKCβI and PKCβII inhibitor	Alexis Biochemicals	Jirousek et al. 1996
RO318220	PKCβ, PKCγ, PKCε inhibitor	Alexis Biochemicals	Davis et al. 1992, Wilkinson et al. 1993
Rottlerin	PKCδ inhibitor	Sigma Chemical Co	Gschwendt et al. 1994
Actinomycin D	Inhibitor of transcription	Sigma Chemical Co	
BMS345541	Inhibitor of IκB kinase (NF-κB inhibitor)	Sigma Chemical Co	
PDD	Phorbol ester	Alexis Biochemicals	
PDTC	NF-κB inhibitor	Sigma Chemical Co	
PMA	Phorbol ester	Sigma Chemical Co	

4. Nitrite assays

The effects of the tested compounds on the ability of the cells to produce NO was determined by measuring the accumulation of nitrite, a stable metabolite of NO, in the culture medium by the method of Griess (Green et al. 1982). Briefly, after 24 h incubation, 100 µl of culture medium was collected and incubated with 100 µl of

Griess reagent (0,1% naphthylethylenediamine dihydrochloride, 1% sulfanilamine, 2,4% H_3PO_4). Absorbance was measured at 540 nm. The concentration of nitrite was calculated using sodium nitrite as the standard. The detection limit was 0.313 µM.

5. Western blotting

Western blotting was performed using protein extracts from whole cell lysates or, when translocation of transcription factors was being studied, with nuclear extracts.

For the preparation of whole cell lysates, 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 EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 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 for 15 min on ice, lysates were centrifuged (13 400 x *g*, 4 ºC, 10 min), supernatants were collected and stored in sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, 5% β-mercaptoethanol) in -20 ºC. An aliquot of the supernatant was used to determine the protein concentration by the Coomassie blue method (Bradford 1976).

In the preparation of nuclear extracts, 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 (DTT), 0.2 mM PMSF, 1 mM Na_3VO_4, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF, 0.1 mM EGTA). After incubation for 10 min on ice, cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 ºC, 21 000 x *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 $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na_3VO_4, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF, 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 ºC, 21 000 x *g* for 2 min. The protein contents of the nuclear extracts were measured by the Coomassie blue method (Bradford 1976). Supernatants were collected and stored in SDS sample buffer at -20 ºC.

Prior to Western blotting, proteins were boiled for 10 min with SDS sample buffer and an equal amount of protein was used per lane on 8% (iNOS and STAT1α Western blot), 12% (TTP Western blot) or 10% (all other Western blots) SDS-polyacrylamide gels. Actin was used as a loading control for proteins in whole cell extracts and lamin A/C for nuclear proteins. After electrophoresis, the proteins were transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences UK, Ltd, Little Chalfont, Buckinghamshire, UK). Following transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature and incubated with primary antibody (Table 5) in the blocking solution at 4 ºC overnight (for anti-EGR1, anti-NF-κB p65, and anti-Sp1 milk was replaced with 5% BSA). The membrane was washed with TBS/T and incubated with the secondary antibody (Table 5) in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using Super Signal® West Pico or Dura chemiluminescent substrate (Pierce, Rockford, USA) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, I-III) or Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB, IV).

Table 5. *Antibodies used in this study*

	MW (kDa)	Supplier
Primary antibodies		
Anti-actin sc-1616R	43	Santa Cruz Biotechnology
Anti-AP-2 sc-184	50	Santa Cruz Biotechnology
Anti-EGR1 #4152	80	Cell Signaling Technology
Anti-iNOS sc-650	130	Santa Cruz Biotechnology
Anti-lamin A/C sc-20681	69/62	Santa Cruz Biotechnology
Anti-NF-κB p65 #3034	65	Cell Signaling Technology
Anti-PKCα sc-8393*	80	Santa Cruz Biotechnology
Anti-PKCβI sc-209	80	Santa Cruz Biotechnology
Anti-PKCβII sc-210	80	Santa Cruz Biotechnology
Anti-PKCδ sc-213	80	Santa Cruz Biotechnology
Anti-PKCγ sc-211	80	Santa Cruz Biotechnology
Anti-Sp1 sc-17824*	106	Santa Cruz Biotechnology
Anti-STAT1α sc-345	91	Santa Cruz Biotechnology
Anti-TTP	43	A kind gift from Dr. Perry Blackshear, NIEHS, Research Triangle Park, NC, USA
Secondary antibodies		
Goat anti-rabbit SC-2004		Santa Cruz Biotechnology
Goat anti-mouse # 1858413		Pierce

Primary antibodies are rabbit polyclonal antibodies
**mouse monoclonal antibody*

6. PKC translocation studies

The activation and downregulation of PKC isoenzymes were studied by determining their translocation from cell cytosol to cell membrane by a Western blot assay. The cytosolic and membrane fractions were prepared as follows: at the predetermined time points, cells were rapidly washed with ice-cold PBS and solubilized in cold buffer A (20 mM Tris-base pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.5 mM PMSF, 2 mM Na_3VO_4, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1.25 mM NaF). After incubation for 15 min on ice, the lysates were centrifuged at 100 000 x *g* for 1 h at 4 ºC, supernatants were collected and marked as the cytosolic fraction. Pellets were resuspended in cold lysis buffer B (20 mM Tris-base pH 7.4, 10 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5 mM PMSF, 2 mM Na_3VO_4, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1.25 mM NaF, 10 µM n-octyl-β-D-glucopyranoside). After

incubation for 2 h on ice, lysates were centrifuged at 100 000 x *g* for 1 h at 4 ºC, supernatants were collected and marked as the membrane fraction. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford 1976). Samples were stored in SDS sample buffer at -20 ºC until analysis. Western blot analysis was performed as described above.

7. Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA extraction was carried out with the use of RNeasy® kit (QIAGEN GmbH, Hilden, Germany) or GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA). Briefly, cells were incubated with the compounds of interest for indicated times. Thereafter, the cells were washed twice with PBS, lysed and total RNA was extracted according to the manufacturer's instructions. The amount of RNA was measured spectrophotometrically and purity was confirmed via the absorbance ratio at A_{260}/A_{280}.

Total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription Reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). The parameters for the reverse-transcriptase reaction were as follows: incubation at 25 ºC for 10 min, reverse transcription at 48 ºC for 30 min, and inactivation of reverse transcriptase at 95 ºC for 5 min.

cDNA obtained from the reverse-transcriptase reaction (corresponding to approximately 2.5 ng of total RNA) was subjected to PCR using TaqMan Universal Master Mix and ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The primer and probe sequences were designed using Primer Express Software, version 2.0.0 (Applied Biosystems) as listed in Table 6. Concentrations for primers and probes were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were 300 nM and 150 nM, respectively. All probes contained 6-FAM (6-carboxy-fluorescein) as the 5'-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as the 3'-quencher. The expression of IRF1 mRNA was measured using TaqMan® Gene Expression Assay (Applied Biosystems). For luciferase mRNA experiments,

total RNA was treated with DNAse I (Fermentas UAB, Vilnius, Lithuania) prior to conversion to cDNA.

PCR reaction parameters were as follows: incubation at 50 ºC for 2 min, incubation at 95 ºC for 10 min, and thereafter 40 cycles of denaturation at 95 ºC for 15 s and annealing and extension at 60 ºC 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. Briefly, total RNA was isolated from stimulated cells and reverse transcribed as described earlier. Standard curves for all quantified genes in the present study were created using dilution series of cDNA corresponding to approximately 1 pg to 10 ng of total RNA in PCR. The threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels were then calculated using the standard curve. The relative amount of gene transcript present was calculated and normalized by dividing the calculated value of the gene of interest by the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) value in each sample.

8. Actinomycin D assay

Actinomycin D assay was performed to study the decay of iNOS, TTP or TNFα mRNA. Cells were incubated with the stimulants and the compounds of interest for 4 h (TTP), 6 h (iNOS) or 9 h (TNFα) before the addition of actinomycin D, an inhibitor of transcription. Thereafter, RNA was extracted at predetermined time points and subjected to quantitative RT-PCR to measure the remaining mRNA. Time points were selected from the expression curve of the mRNA in question.

Table 6. *Primers and probes used in this study*

Gene	Oligonucleotide	Sequence 5'→ 3'
GAPDH	Forward primer	GCATGGCCTTCCGTGTTC
	Reverse primer	GATGTCATCATACTTGGCAGGTTT
	Probe	TCGTGGATCTGACGTGCCGCC
iNOS	Forward primer	CCTGGTACGGGCATTGCT
	Reverse primer	GCTCATGCGGCCTCCTT
	Probe	CAGCAGCGGCTCCATGACTCCC
Luciferase	Forward primer	AAAAAGTTGCGCGGAGGAG
	Reverse primer	TTTTTCTTGCGTCGAGTTTTCC
	Probe	TGTGTTTGTGGACGAAGTACCGAAAGGTCTTAC
TNFα	Forward primer	AATGGCCTCCCTCTCATCAGTT
	Reverse primer	TCCTCCACTTGGTGGTTTGC
	Probe	CTCAAAATTCGAGTGACAAGCCTGTAGCCC
TTP	Forward primer	CTCAGAAAGCGGGCGTTGT
	Reverse primer	GATTGGCTTGGCGAAGTTCA
	Probe	CCAAGTGCCAGTTTGCTCACGGC

9. Downregulation of PKCδ by siRNA

PKCδ expression was downregulated using Dharmacon ON TARGET plus siRNA oligos (Dharmacon, Lafayette, CO, USA). The transfection conditions were chosen based on preliminary tests in order to archieve good downregulation and to avoid cytotoxicity. J774 macrophages or L929 cells were seeded at 1 x 10^5 cells per well in 24-well plates 24 h before the transfection with siRNA oligos targeted to murine PKCδ or non-targeting control siRNA using DharmaFECT4 (J774) or DharmaFECT1 (L929) transfection reagent according to the manufacturer's instructions (Dharmacon). Cells were incubated for 6 h (J774A.1), 24 h (J774.2), or 48 h (L929) with siRNA duplexes and the transfection reagent. Subsequently, the medium was replaced with fresh culture medium and cells were further incubated for 42 h (J774A.1) or 24 h (J774.2) before the experiments were started and stimulants and tested compounds were added in fresh culture medium. Downregulation of PKCδ by siRNA was determined by Western blotting from samples extracted at the beginning of the experiments. The transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green indicator).

10. Enzyme linked immunosorbent assay

The concentrations of IL-6 and TNFα in the culture medium were determined by enzyme linked immunosorbent assay (ELISA) by using reagents from R&D Systems Europe Ltd (Abingdon, UK) according to the manufacturer's instructions.

11. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was used to study the activation of NF-κB transcription factor. For EMSA, nuclear extracts were prepared as described above. Protein concentrations were determined by the Coomassie blue method (Bradford 1976) and samples were stored at -70 ºC until analyzed. Single-stranded oligonucleotides that contain the consensus NF-κB binding sequences (5'-AGTTGAGGGGACTTTCCCAGGC-3', 3'-TCAACTCCCCTGAAAGGGTCCG-5, Amersham Pharmacia Biotech, Piscataway, NJ, USA) were 5'-^{32}P-end-labeled with DNA 5'-End Labeling Kit (Boehringer Mannheim Indianapolis, IN, USA). In the binding reactions, 5 µg of nuclear extract was incubated in 20 µl of total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM DTT, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 40 mM KCl, and 10% glycerol for 20 min at room temperature. ^{32}P-labeled oligonucleotide probe (0.2 ng) was added and the reaction mixture was incubated for 10 min. Protein - DNA complexes were separated from the free probe by electrophoresis on a native 4% polyacrylamide gel. The gel was dried and autoradiographed using an intensifying screen at –70 °C. The quantitation of densities of specific bands was carried out using FluorChem™ software version 3.1.

12. Carrageenan-induced inflammation in mice

The anti-inflammatory effects of rottlerin *in vivo* were studied in carrageenan-induced paw oedema in male 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 three study groups, i.e. control group, L-NIL-treated group (50 mg/kg) (Kondapaneni et al. 2008), and rottlerin-treated group (10 mg/kg) (Ohno et al. 2010), with six mice in each group. Two hours before carrageenan, the mice were treated with the same volume of normal saline or the drug by intraperitoneal injection. The mice were anesthetized with an intraperitoneal injection of 0.5 mg/kg of medetomidine (Domitor® 1 mg/ml. Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland) and thereafter dosed with a 30 µl intradermal injection of normal saline containing λ-carrageenan (1.5 %) into one hindpaw. 30 µl of saline was injected into the contralateral paw and it was used as the control. The paw volume was measured before and three hours after carrageenan injection by use of a plethysmometer (Ugo Basile) to determine the development of inflammatory oedema. Oedema was expressed in µl as the difference between the change in the paw with inflammation and the change in the control paw.

13. Statistics

Results are expressed as mean + standard error of mean (SEM). Statistical significance of the results was calculated by the analysis of variance supported by Dunnett's or Bonferroni's post test. Differences were considered significant at $P<0.05$.

Results

1. Activation and downregulation of PKC isoenzymes in J774 macrophages

J774 macrophages expressed cPKC isoenzymes α, βI, βII, as well as the novel isoenzyme δ. As expected, PKCγ expression was not detected in J774 macrophages (Figure 7).

Translocation of PKC isoenzymes from the cell cytosol to the membrane fractions can be considered as the hallmark of PKC activation (Steinberg 2008). In J774 macrophages, treatment with phorbol ester PMA (100 nM) for 10 min led to the activation of PKCα, PKCβI, PKCβII, and PKCδ. The activation was measured by the translocation of isoenzymes from the cell cytosol to the cell membrane by Western blotting (Figure 7A-B). In contrast, prolonged treatment of cells with higher concentrations of PMA is known to cause downregulation of cPKCs and nPKCs, probably as a result of proteolysis (Huang et al. 1989, Chen 1993). In J774 macrophages, 6 h pretreatment with 1 µM PMA led to the downregulation of PKCα, PKCβI, and PKCβII (Figure 7A). Downregulation of PKCδ required a longer pretreatment period (24 h) with 1 µM PMA (Figure 7B).

The siRNA technique is a more efficient and selective way to downregulate PKC isoenzymes. PKCδ specific siRNA downregulated PKCδ expression by more than 80% in J774 macrophages (Figure 7D) and L929 fibroblast (Figure 7E).

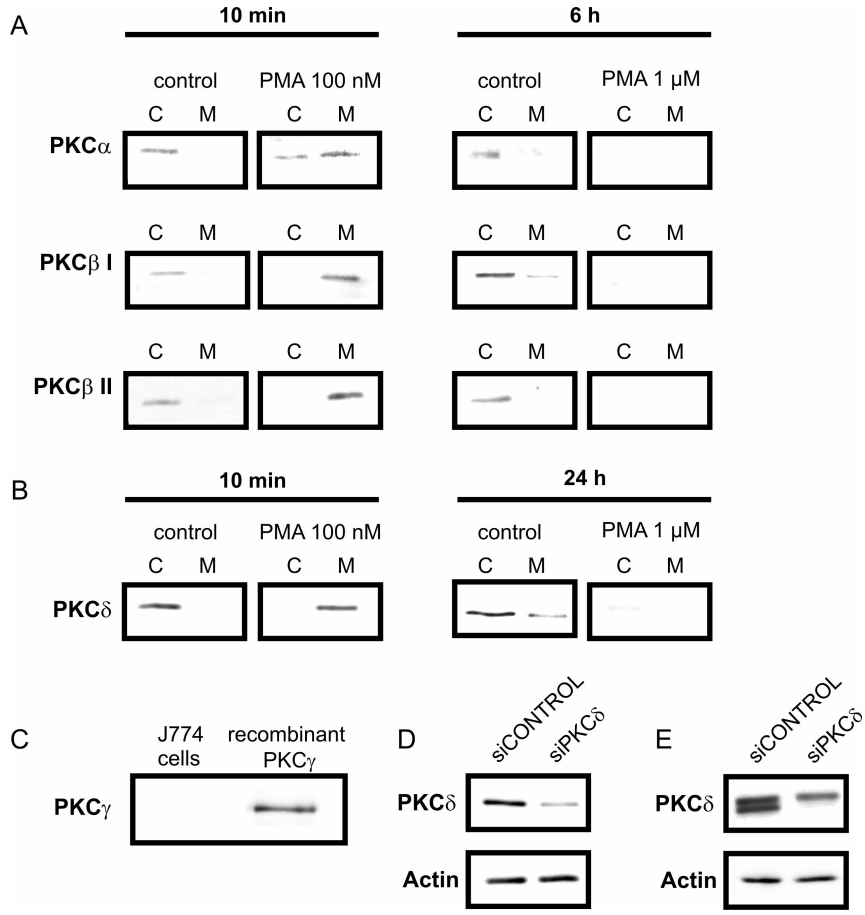

Figure 7. *Activation and downregulation of cPKC isoenzymes (A, C) and PKCδ (B, D, E) in macrophages. (A and B) J774 cells were treated with 100 nM PMA or 1 µM PMA for the indicated times. The expression of individual isoenzymes was assessed by Western blot with isoenzyme specific antibodies. (C) The expression of PKCγ in resting J774 macrophages was tested by Western blot using recombinant human PKCγ as a positive control. (D) J774.2 cells and (E) L929 cells were transiently transfected with PKCδ specific siRNA. Non-targeting siRNA (siCONTROL) was used as a control. The gels shown are representatives of three others with similar results. C = cytosolic fraction, M = membrane fraction. (Reprinted with permission from Salonen et al. 2006, Br J Pharmacol 147:790-799 © John Wiley & Sons Ltd, modified and Leppänen et al. 2010, Eur J Pharmacol 628:220-225 © Elsevier Ltd, modified).*

2. The effects of PKC isoenzymes on NO production and iNOS expression (I, IV)

Bacterial endotoxin LPS was used to activate cells through a TLR4 dependent manner which is known to induce NO production and iNOS expression in J774 macrophages. In L929 fibroblasts, NO production and iNOS expression were induced by a mixture of cytokines IFNγ, IL-1β, and TNFα. The concentrations of LPS and cytokines were selected from dose response curves.

In the present study, the role of PKC isoenzymes in the regulation of NO production was studied by utilizing three approaches: by using PKC selective

inhibitors, by activating and downregulating PKC with PMA, and by downregulating PKCδ with siRNA. First, to determine the effects of PKC inhibitors with different selectivity profiles on NO production and iNOS expression in J774A.1 macrophages, LPS-induced NO production and iNOS expression were measured in the presence of PKC inhibitors RO318220 (inhibits isoenzymes β, γ, ε) (Davis et al. 1992, Wilkinson et al. 1993), GÖ6976 (inhibits all cPKCs) (Martiny-Baron et al. 1993), LY333531 (inhibits PKCβI and PKCβII) (Jirousek et al. 1996), and HBDDE (inhibits PKCα and γ) (Kashiwada et al. 1994). Except for HBDDE all inhibitors reduced LPS-induced NO production and iNOS expression in J774 macrophages in a dose dependent manner (Table 7 and Figure 8). Secondly, the role of cPKCs was further studied using phorbol ester PMA. When cells were treated with 100 nM PMA, i.e. cPKC isoenzymes were activated (Figure 7A), LPS-induced NO production and iNOS expression were enhanced (Table 7). On the other hand, when cells were pretreated for 6 h with a higher concentration of phorbol esters PMA or PDD (phorbol 12,13-didecanoate), i.e. cPKCs were downregulated (Figure 7A), LPS-induced NO production and iNOS expression were inhibited (Table 7).

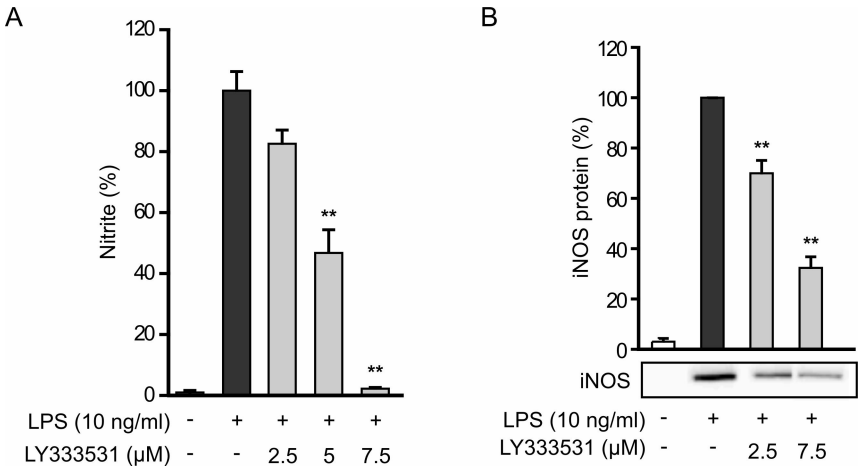

Figure 8. *The effects of PKCβ inhibitor LY333531 on LPS-induced NO production (A) and iNOS expression (B). NO production was determined after 24 h incubation by measuring the nitrite concentrations (metabolite of NO) in the culture medium by Griess reaction (n=3, mean + SEM). iNOS protein expression was measured by Western blot analysis after 24 h incubation (n=3, mean + SEM).** p<0.01. (Reprinted with permission from Salonen et al. 2006, Br J Pharmacol 147:790-799 © John Wiley & Sons Ltd, modified).*

The role of the novel isoenzyme PKCδ was determined by using a selective inhibitor rottlerin (Gschwendt et al. 1994) and by using PKCδ specific siRNA. In both cell types tested (J774.2 macrophages and L929 fibroblasts), rottlerin reduced NO production and iNOS expression in a dose dependent manner (Table 7), and PKCδ specific siRNA had a similar effect (Figure 9). In addition, rottlerin had no effect in cells in which PKCδ had been downregulated by siRNA (Figure 9).

Table 7. *Effects of PKC inhibitors on NO production and iNOS expression*

Treatment	Conc. (μM)	NO production (%)	iNOS expression (%)
J774 cells			
LPS 10 ng/ml		100	100
+ RO318220	1	66.4 ± 2.5 **	77.7 ± 10.5
	3	25.9 ± 1.1 **	50.3 ± 11.1 **
+ GÖ6976	0.1	8.9 ± 0.7 **	46.6 ± 4.7 **
	1	4.0 ± 0.4 **	7.6 ± 1.6 **
+ HBDDE	30	112.5 ± 2.7	nd
	100	114.7 ± 7.7	nd
+ rottlerin	3	71.8 ± 1.3 **	18.2 ± 2.2 **
	10	20.6 ± 1.2 **	10.4 ± 2.5 **
+ PMA	0.1	224.3 ± 9.0 **	175.3 ± 14.3 **
	1[§]	23.2 ± 2.1 **	36.1 ± 10.1 **
+ PDD	0.1	nd	207.2 ± 53.9
	1[§]	nd	24.1 ± 5.1 **
L929 cells			
Mixture of cytokines		100	100
+ rottlerin	1	78.7 ± 1.4 **	70.2 ± 5.3 **
	3	42.6 ± 0.8 **	23.9 ± 4.0 **

*The cells were treated with LPS 10 ng/ml and tested inhibitors for 24 h before NO production or iNOS expression were determined. Results are expressed as mean ± SEM, n=3-4. ** indicates p<0.01 as compared to cells treated with the stimulant alone.*
[§]*To downregulate PKC expression, the cells were pretreated with 1 μM PMA or PDD for 6 h prior to stimulation with LPS 10 ng/ml.*
Concentrations of the compounds were selected from dose response curves.
nd = not determined

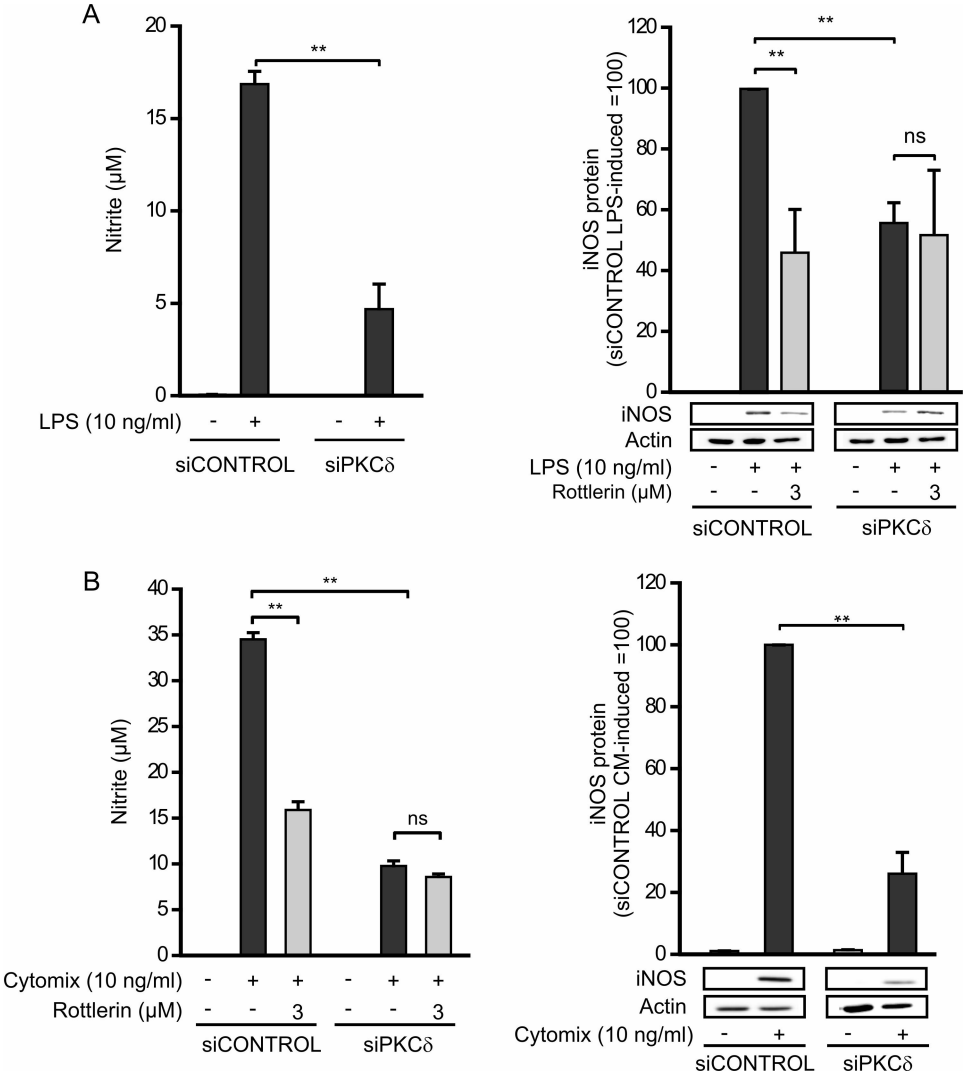

Figure 9. *The effects of downregulation of PKCδ by siRNA on NO production and iNOS expression in J774.2 macrophages and L929 fibroblasts. (A) In J774.2 macrophages NO production and iNOS expression were induced by LPS. NO production was determined after 24 h incubation by measuring the nitrite concentrations (metabolite of NO) in the culture medium by Griess reaction (n=3, mean + SEM). iNOS protein expression was measured by Western blot after 24 h incubation (n=3, mean + SEM). (B) In L929 fibroblasts, NO production and iNOS expression were induced by a mixture of cytokines containing IFNγ, IL-1β, and TNFα, 10 ng/ml each (n=7 mean + SEM for NO, n=3 mean + SEM for iNOS), ** p<0.01.*

2.1 Transcriptional regulation of iNOS expression by PKCβ - the role of STAT1 (I)

The results from the studies with cPKC inhibitors on NO production and iNOS protein expression indicated that the classical isoenzymes, especially PKCβ, participate in the regulation of LPS-induced iNOS expression. This concept was further supported by the results from the phorbol ester studies.

Next, the effects of RO318220 and GÖ6976 on the expression of iNOS mRNA were studied. In J774 macrophages, LPS induced a transient iNOS mRNA expression, which peaked at 6 h after LPS addition. Both RO318220 and GÖ6976 inhibited the expression of iNOS mRNA when measured 2 h before and after the 6 h peak (Figure 10A). However, neither inhibitor affected the half-life of iNOS mRNA when this was measured by the actinomycin D assay (Figure 10B). These results indicated that the effects of cPKCs on LPS-induced iNOS expression are mediated at the level of iNOS transcription rather than at the level of iNOS mRNA stability.

In order to evaluate whether the effects of cPKC inhibitors on the expression of iNOS mRNA could be a consequence of their effects on transcription factors, their effects on the activation of NF-κB and STAT1 were studied. Both NF-κB and STAT1 are important transcription factors for iNOS expression (Xie et al. 1994, Gao et al. 1997). Neither RO318220 nor GÖ6976 affected the LPS-induced NF-κB activation as measured by EMSA (Figure 10C). However, both inhibitors, as well as the PKCβ inhibitor LY333531, inhibited the activation of STAT1 as measured by the translocation of STAT1α from the cell cytosol to the nuclei by Western blot analysis (Figure 10D-E). These data suggested that the effects of classical isoenzymes on LPS-induced iNOS expression were NF-κB independent, but may have been mediated through the activation of STAT1. In addition, the results obtained with LY333531 indicated that PKCβ could be the classical isoenzyme responsible for this regulation.

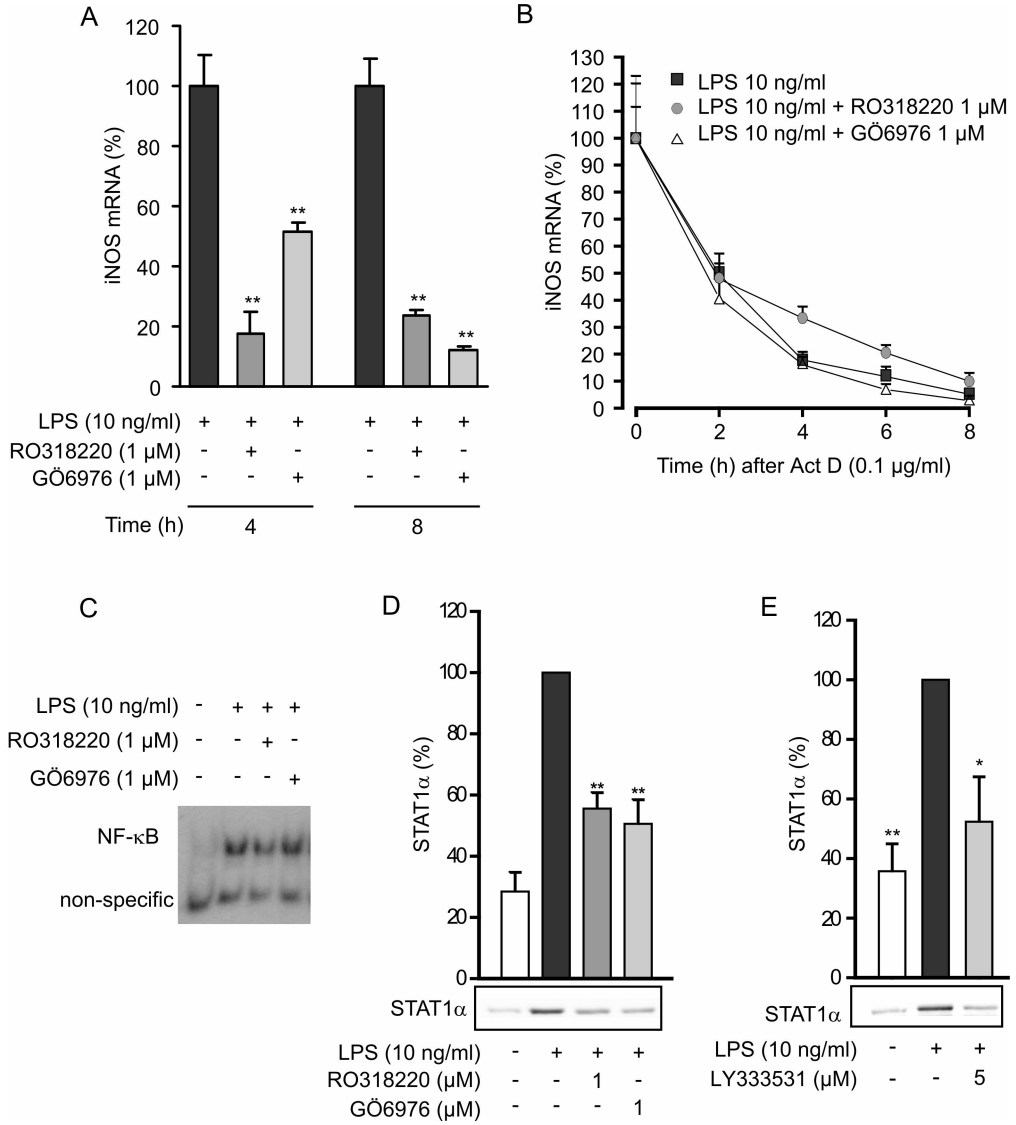

Figure 10. *Effect of cPKC inhibitors on iNOS mRNA expression (A), iNOS mRNA decay (B), NF-κB activation (C), and STAT1 activation (D-E). iNOS mRNA expression was measured after 4 h and 8 h incubation by RT-PCR (n=3, mean + SEM). iNOS mRNA decay was measured by the actinomycin D assay (n=3, mean + SEM). Activation of transcription factor NF-κB was determined by EMSA at the 30 min time point, the experiment is a representative of three other experiments with similar results. Activation of transcription factor STAT1 was measured as translocation of STAT1α by Western blot at 6 h time point (n=3, mean + SEM). *p<0.05, **p<0.01. (Reprinted with permission from Salonen et al. 2006, Br J Pharmacol 147:790-799 © John Wiley & Sons Ltd, modified).*

2.2 Transcriptional regulation of iNOS expression by PKCδ – involvement of IRF1 (IV)

In J774.2 macrophages and L929 fibroblasts downregulation of PKCδ by siRNA or inhibition of PKCδ by rottlerin reduced NO production and iNOS expression. These results indicated that, in addition to cPKC isoenzymes, also PKCδ participates in the regulation of iNOS expression. Since rottlerin affected neither NO production nor iNOS expression when used under the conditions when PKCδ was downregulated by siRNA, it seemed likely that the effects of rottlerin were being mediated by PKCδ.

When the effects of rottlerin or PKCδ siRNA on iNOS mRNA expression were studied it was observed that, in contrast to the cPKC inhibitors, inhibition of PKCδ did not affect iNOS mRNA expression when measured at the early time points (4 h in L929, 3 h in J774), but it reduced iNOS mRNA expression when measured at later time points (10 h in L929, 9 h in J774) (Figure 11A-B). However, as with the cPKC inhibitors, no effect on iNOS mRNA decay was observed (Figure 11C).

PKCδ inhibition did not affect the activity of transcription factor NF-κB as measured by EMSA (J774) or on NF-κB mediated transcription in L929 cell line stably transfected with NF-κB responsive reporter (L929-pNFκB cell line) (Figure 12A-B). This result was in line with the finding that inhibition of PKCδ did not affect iNOS mRNA levels when measured at the early time points. Instead, when measured at the 4 h time point, inhibition of PKCδ by rottlerin and downregulation of PKCδ by siRNA reduced the expression of IRF1 mRNA (Figure 12C-D). IRF1 is an important transcription factor for iNOS, but in contract to NF-κB, IRF1 has been shown to act as a later phase transcription factor (Kamijo et al. 1994, Fujimura et al. 1997). In order to test whether downregulation of PKCδ by siRNA could affect the production of other IRF1 responsive inflammatory mediators, the effect of PKCδ siRNA on IL-6 production was measured. In J774.2 macrophages downregulation of PKCδ by siRNA reduced also LPS-induced IL-6 production (Figure 12E).

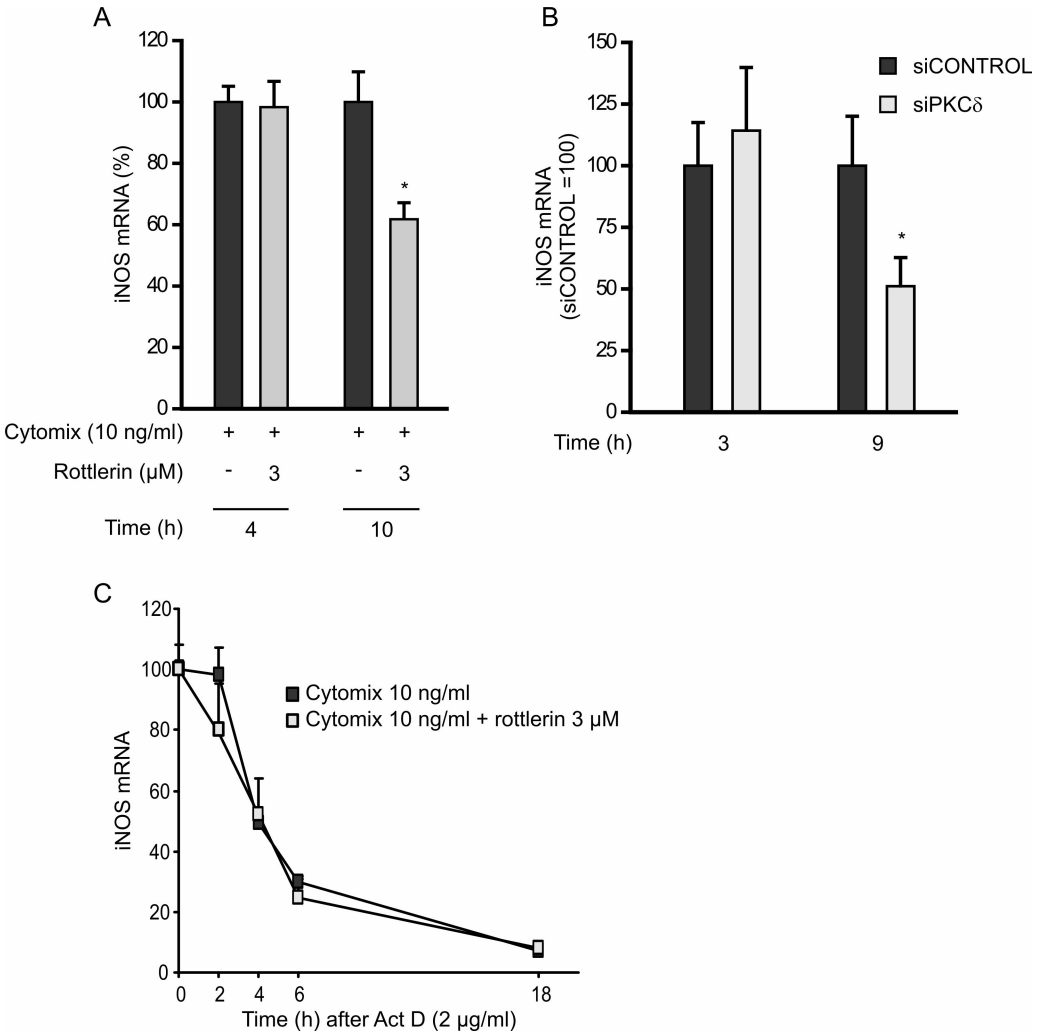

Figure 11. *Effects of PKCδ downregulation on iNOS mRNA expression. (A) Effects of PKCδ inhibitor rottlerin on iNOS mRNA expression in cytokine-induced L929 fibroblasts were measured by RT-PCR after 4 h and 10 h incubation (n=3, mean + SEM). (B) Effects of PKCδ siRNA on LPS-induced iNOS mRNA expression in J774.2 macrophages were measured by RT-PCR after 3 h and 9 h incubation (n=3, mean + SEM). (C) Effects of PKCδ inhibitor rottlerin on iNOS mRNA decay in L929 fibroblasts were measured by the actinomycin D assay (n=3, mean + SEM). *p<0.05.*

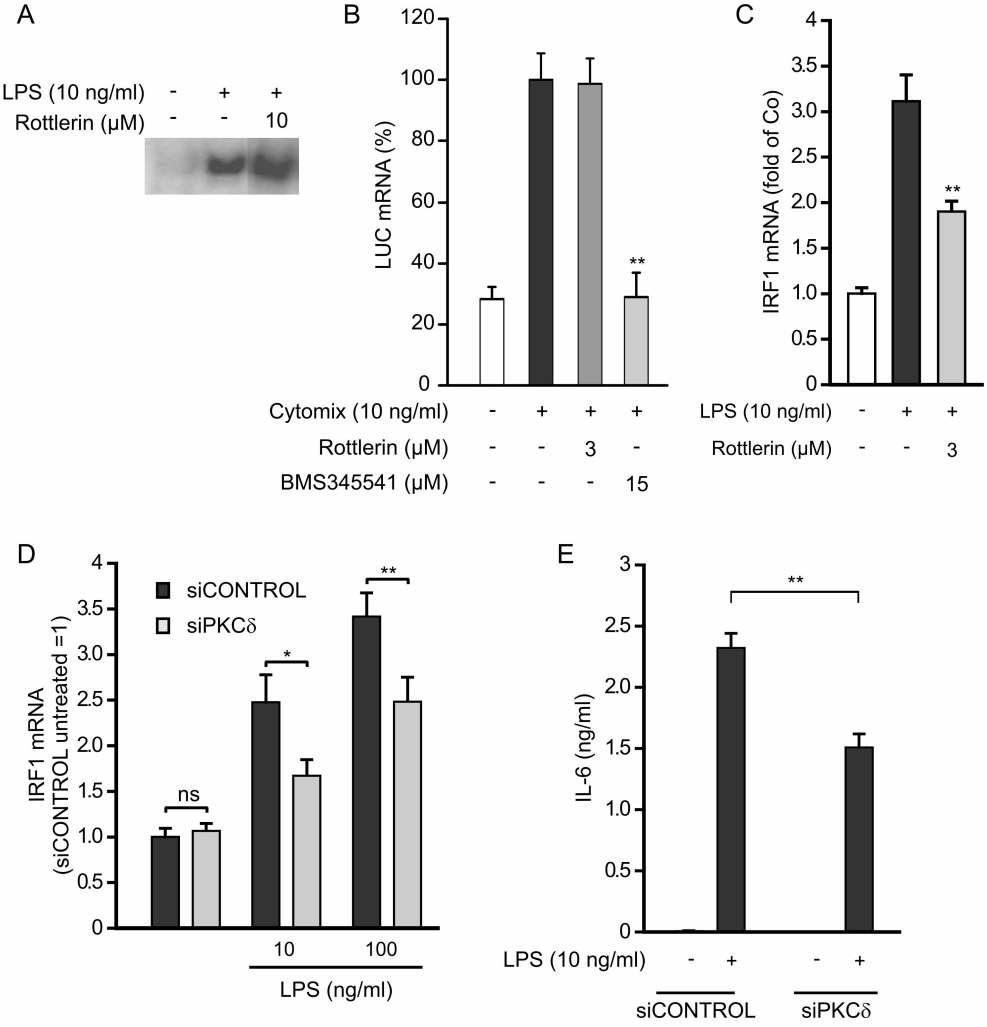

Figure 12. *Effects of PKCδ inhibition/downregulation on transcription factors and on IL-6 production. (A) Effects of PKCδ inhibitor rottlerin on NF-κB activity in J774A.1 macrophages as measured by EMSA at 30 min time point; this experiment is a representative of three others with similar results. (B) Effects of PKCδ inhibitor rottlerin on NF-κB mediated transcription in L929 pNFκB cell line at 1 h time point as measured by RT-PCR. BMS3445541 is an inhibitor of IκB kinase and is used here as a control agent (n=4, mean + SEM). (C) Effects of PKCδ inhibitor rottlerin on LPS-induced IRF1 mRNA expression in J774.2 macrophages as measured by RT-PCR after 4 h incubation (n=3, mean + SEM). (D) Effects of PKCδ siRNA on LPS-induced IRF1 mRNA expression in J774.2 macrophages as measured by RT-PCR after 4 h incubation (n=6, mean + SEM). (E) Effects of PKCδ siRNA on LPS-induced IL-6 production in J774.2 macrophages as measured by ELISA (n=6, mean + SEM). *p<0.05, **p<0.01.*

3. TTP expression in J774 macrophages (II, III)

Resting J774A.1 macrophages expressed very low levels of TTP protein. When the cells were treated with LPS (10 ng/ml), TTP expression was significantly enhanced. Treatment of cells with LPS together with PMA (100 nM), further enhanced the expression of TTP. However, PMA alone did not induce any marked TTP expression (Figure 13A). TTP protein expression peaked at 9 hours after treatment

with LPS alone or after treatment with the combination of LPS and PMA (Figure 13B).

TTP mRNA expression was measured by quantitative RT-PCR. Again, PMA alone was not able to induce any significant effects on the TTP mRNA. Similarly with TTP protein expression, LPS alone induced TTP mRNA expression and the addition of PMA further enhanced this effect (Figure 14B).

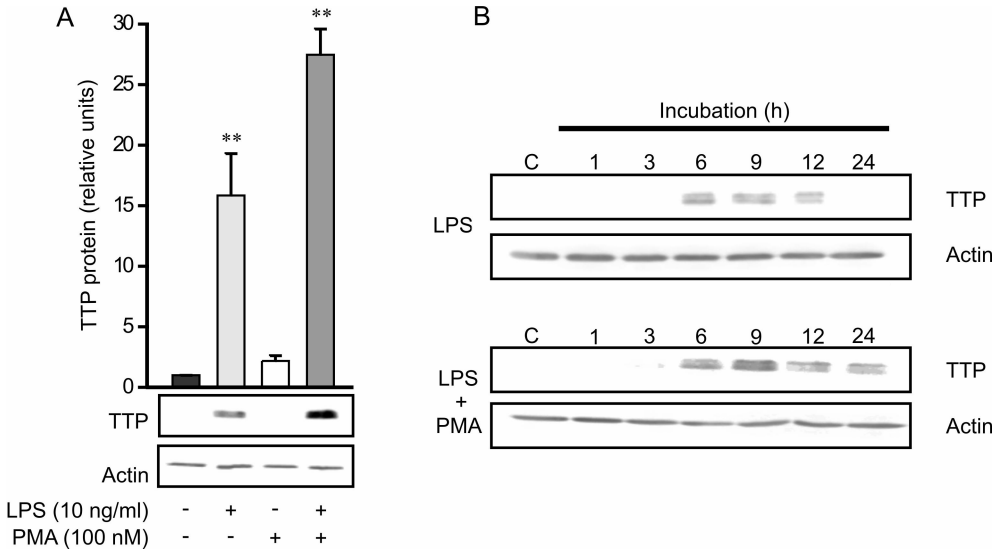

Figure 13. *The expression of TTP in J774A.1 macrophages. (A) Cells were treated with LPS (10 ng/ml), PMA (100 nM), or their combination for 9 h and the expression of TTP protein was determined by Western blot (n=3, mean + SEM) **p<0.01. (B) Time curve of TTP protein expression after treatment with LPS (10 ng/ml) or LPS (10 ng/ml) and PMA (100 nM). Each experiment is a representative of three others with similar results. (Reprinted with permission from Leppänen et al. 2008, Inflamm Res 57:230-240, © Birkhäuser Verlag GmbH, modified).*

4. Effects of PKC inhibition or downregulation by phorbol esters on TTP expression (II, III)

Since PMA (100 nM), a known activator of PKC, together with LPS (10 ng/ml) induced TTP expression, the effects of PKC isoenzymes on the expression of TTP were studied. Firstly, since PMA can function as a PKC activator and downregulator (Figure 7), it was used as a tool to study the effects of PKC isoenzymes on TTP expression. Cells were pretreated for 24 h with 1 µM PMA to downregulate PKC expression. Thereafter LPS (10 ng/ml), PMA (100 nM), or their combination were added into the cell culture. After 9 h, the cells were harvested for the determination

of TTP expression by Western blot. Pretreatment with 1 µM PMA did not alter the TTP expression induced by LPS alone. However, it abolished the enhancing effect of 100 nM PMA on LPS-induced TTP expression (Figure 14A). A similar expression pattern was also seen in TTP mRNA levels. Again, pretreatment with 1 µM PMA had no effect on LPS-induced TTP mRNA, but it abolished the enhancing effect of 100 nM PMA on LPS-induced TTP mRNA expression (Figure 14B).

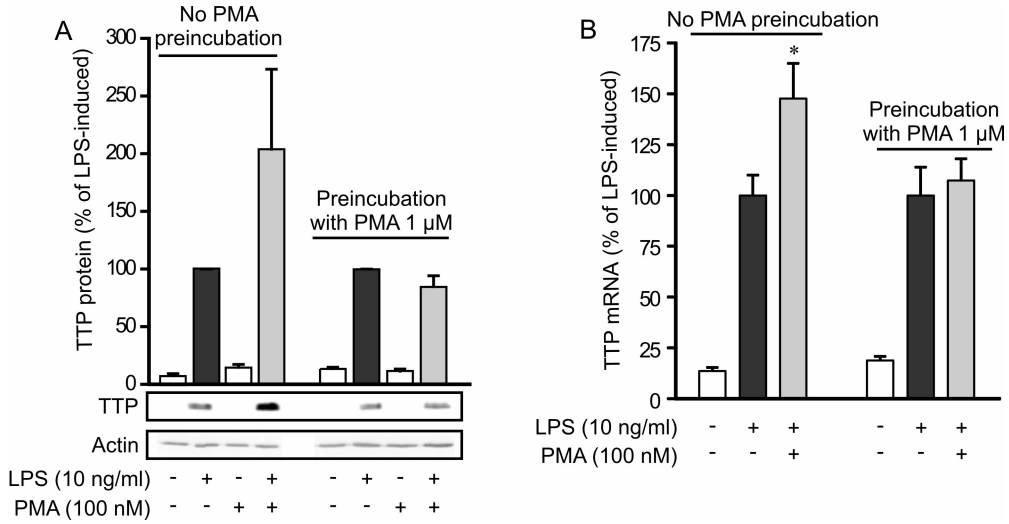

Figure 14. *The effects of PKC activation and downregulation on TTP protein (A) and TTP mRNA (B) expression. J774A.1 macrophages were pretreated with vehicle (no preincubation) or with 1 µM PMA for 24 h to downregulate PKC expression. Thereafter the cells were stimulated by adding LPS (10 ng/ml), PMA (100 nM), or combination of LPS and PMA. The expression of TTP protein was determined at 9 h time point by Western blot analysis and the expression of TTP mRNA was determined at 6 h time point by RT-PCR (n=3, mean + SEM) *p<0.05, **p<0.01. (Reprinted with permission from Leppänen et al. 2008, Inflamm Res 57:230-240, © Birkhäuser Verlag GmbH, modified).*

The effects of PKC isoenzymes on the regulation of LPS and PMA –induced TTP expression were further studied by using PKC inhibitors with varying selectivity profiles. All inhibitors were added to the cell culture 30 min prior to the addition of the combination of LPS and PMA. RO318220 and GÖ6976 inhibited TTP protein and mRNA expression (Table 8). In addition, both PKCβ selective inhibitors, PKCβI and βII selective LY333531 (Jirousek et al. 1996) and PKCβII selective CGP53353 (Chalfant et al. 1996), reduced TTP protein and mRNA expression (Figure 15A-B). In contrast, HBDDE, an inhibitor of PKCα and γ, did not affect TTP protein or mRNA expression (Table 8).

Table 8. *Effects of PKC inhibitors on TTP protein and mRNA expression*

Treatment	Conc. (µM)	TTP protein (%)	TTP mRNA (%)
LPS 10 ng/ml + PMA 100 nM		100	100
+ RO318220	0.3	103.2 ± 20.0	nd
	1	61.3 ± 8.4 *	23.3 ± 3.0 **
+ GÖ6976	0.3	83.1 ± 10.1	nd
	1	39.6 ± 6.4 **	46.1 ± 2.1**
+ HBDDE	100	nd	118.3 ± 7.9
+ rottlerin	3	74.7 ± 4.2 *	nd
	10	48.2 ± 1.8 **	71.1 ± 5.0 **

*The tested inhibitors were added to the cell culture 30 min prior to the addition of LPS (10 ng/ml) and PMA (100 nM). Cells were incubated for 9 h prior to TTP protein determination and 6 h prior to TTP mRNA determination. Results are expressed as mean ± SEM, n=3. * indicates p<0.05 and ** indicates p<0.01 as compared to cells treated with the stimulant alone. nd = not determined*

Rottlerin, an inhibitor of PKCδ, reduced TTP expression (Table 8) and therefore also the effects of PKCδ downregulation by siRNA on TTP expression were studied. Treatment with PKCδ-targeting siRNA caused a significant reduction in PKCδ expression. Under these conditions, the expression of TTP was significantly lower than in cells treated with non-targeting control siRNA (Figure 15C). In addition, in cells that were treated with PKCδ-targeting siRNA, rottlerin had no effect on TTP expression.

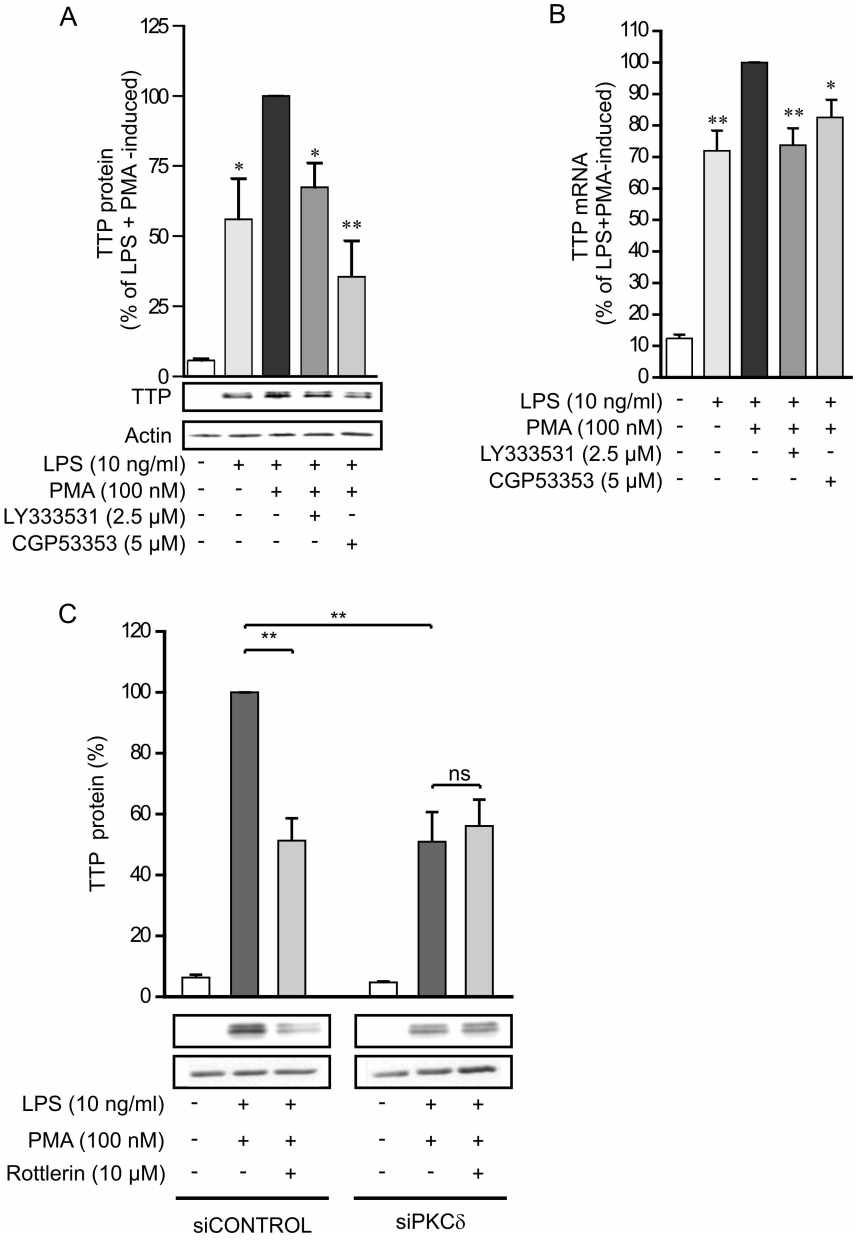

Figure 15. *Effects of PKCβ inhibitors on TTP protein expression (A), PKCβ inhibitors on TTP mRNA expression (B), and PKCδ siRNA on TTP protein expression (C). TTP protein expression was measured by Western blot after 9 h incubation, n=3 (A), n=6 (C). Expression of TTP mRNA was determined by RT-PCR after 6 h incubation, n=9. Values are mean + SEM, *p<0.05, **p<0.01. (Reprinted with permission from Leppänen et al. 2008, Inflamm Res 57:230-240, © Birkhäuser Verlag GmbH, modified and Leppänen et al. 2010, Eur J Pharmacol 628:220-225 © Elsevier Ltd, modified).*

4.1 Transcriptional regulation of TTP by PKCβ (II)

Since both of the PKCβ inhibitors used reduced TTP protein and mRNA expression in a similar manner, the studies were continued with the PKCβII selective inhibitor CGP53353. To evaluate whether the effects of PKCβII on TTP expression were being mediated at the transcriptional level, the effects of CGP53353 on the activity of transcription factors were determined. AP-2, EGR1, NF-κB, and Sp1 have been reported to have at least one binding site in the TTP promoter or intron region (DuBois et al. 1990, Lai et al. 1995, Lai et al. 1998) and therefore suggested to be important for TTP. CGP53353 was added to the culture medium 30 min prior to the addition of LPS (10 ng/ml) and PMA (100 nM). After 30 min (AP-2) or 1 h (EGR1, NF-κB, Sp1) incubation, the nuclear proteins were extracted and the activation of transcription factors was determined in terms of their translocation from the cell cytosol to the nuclei by Western blot analysis. CGP53353 had no effect on the activation of EGR1, NF-κB, or Sp1 (Figure 16A-C), but it reduced the activation of AP-2 (Figure 16D). In addition, PKC downregulation by pretreatment with 1 μM PMA totally abolished the LPS + PMA –induced activation of AP-2 (Figure 16E).

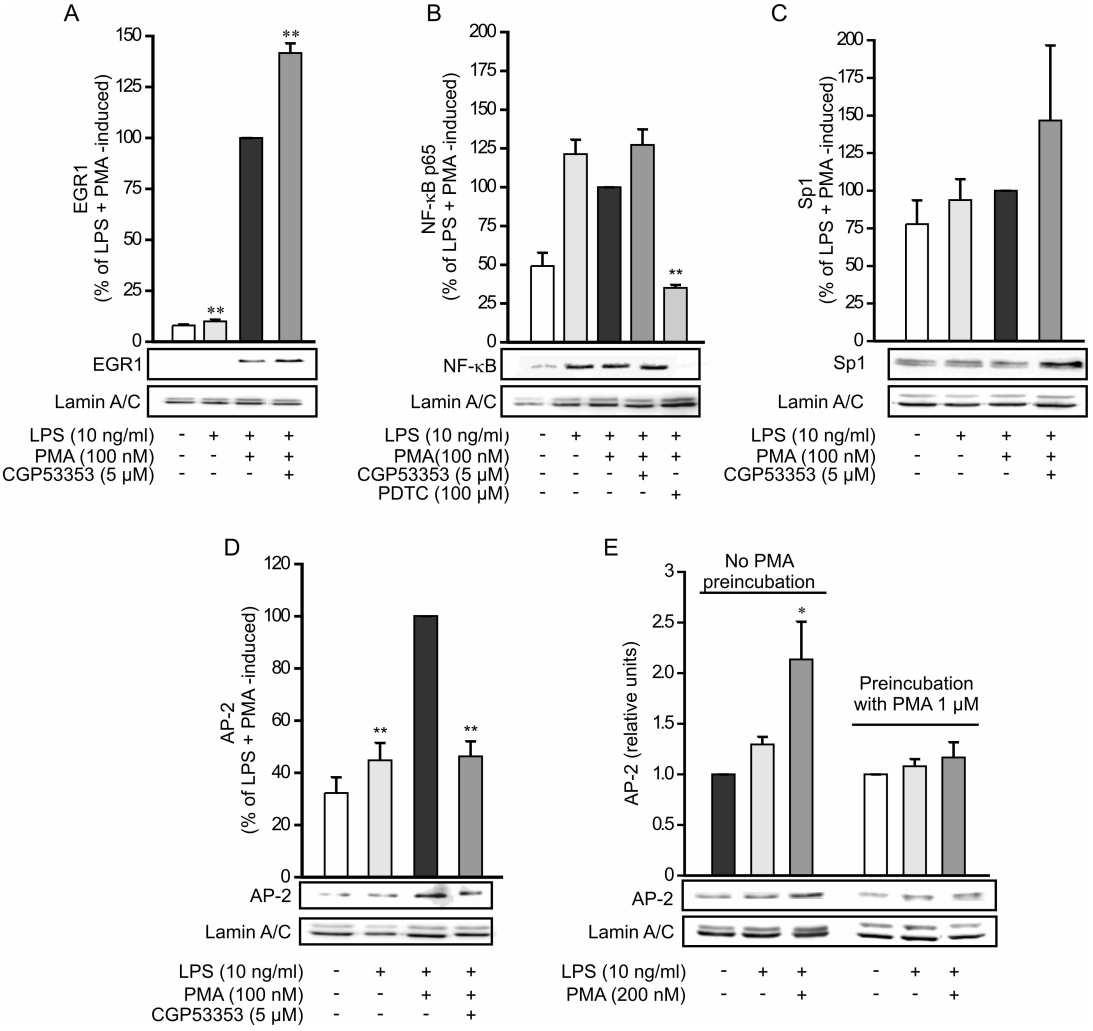

Figure 16. *Effects of CGP53353 on (A) EGR1, (B) NF-κB p65, (C) Sp1, and (D) AP-2 activation, and (E) the effect of downregulation of PKC by 1 µM PMA pretreatment on AP-2 activation in LPS+PMA –induced J774A.1 macrophages. PDTC is an inhibitor of NF-κB and is used as a control agent (n=3-4, mean + SEM), *p<0.05, **p<0.01. (Reprinted with permission from Leppänen et al. 2008, Inflamm Res 57:230-240, © Birkhäuser Verlag GmbH, modified).*

4.2 Post-transcriptional regulation of TTP by PKCδ (III)

Rottlerin reduced TTP expression in control cells, but not in cells in which PKCδ had been downregulated with PKCδ siRNA. This indicated that the effect of rottlerin on TTP expression was most likely being mediated through inhibition of PKCδ and the studies were continued with rottlerin. In contrast to the PKCβII inhibitor CGP53353, rottlerin did not affect the activation of the transcription factors AP-2, ERG1, NF-κB, or Sp1 (Table 9). Instead, when measured by the actinomycin D assay, it decreased the half-life of TTP mRNA to about half of that found in control cells (Figure 17A).

Table 9. *Effects of rottlerin on LPS + PMA-induced activation of transcription factors in J774A.1 cells*

Transcription factor	Effect of 10 µM rottlerin (%)
AP-2	91.2 ± 14.9
EGR1	80.9 ± 13.7
NF-κB p65	136.0 ± 20.2
Sp1	112.8 ± 33.3.

Results are expressed as mean ± SEM.

In macrophages from TTP knockout mice, the absence of TTP has been shown to increase the half-life of TNFα mRNA (Carballo et al. 1998, Lai et al. 1999). In order to study whether the downregulation of TTP expression by rottlerin was sufficient to alter TNFα mRNA decay, the effect of rottlerin on TNFα mRNA half-life was measured by means of the actinomycin D assay. The half-life of TNFα mRNA was increased by 2 h in cells treated with rottlerin as compared to the cells treated with the stimulants only (Figure 17B). In addition, rottlerin enhanced LPS and PMA –induced TNFα production as measured by ELISA (Figure 17C). These results indicate that the downregulation of TTP expression by rottlerin might have functional significance.

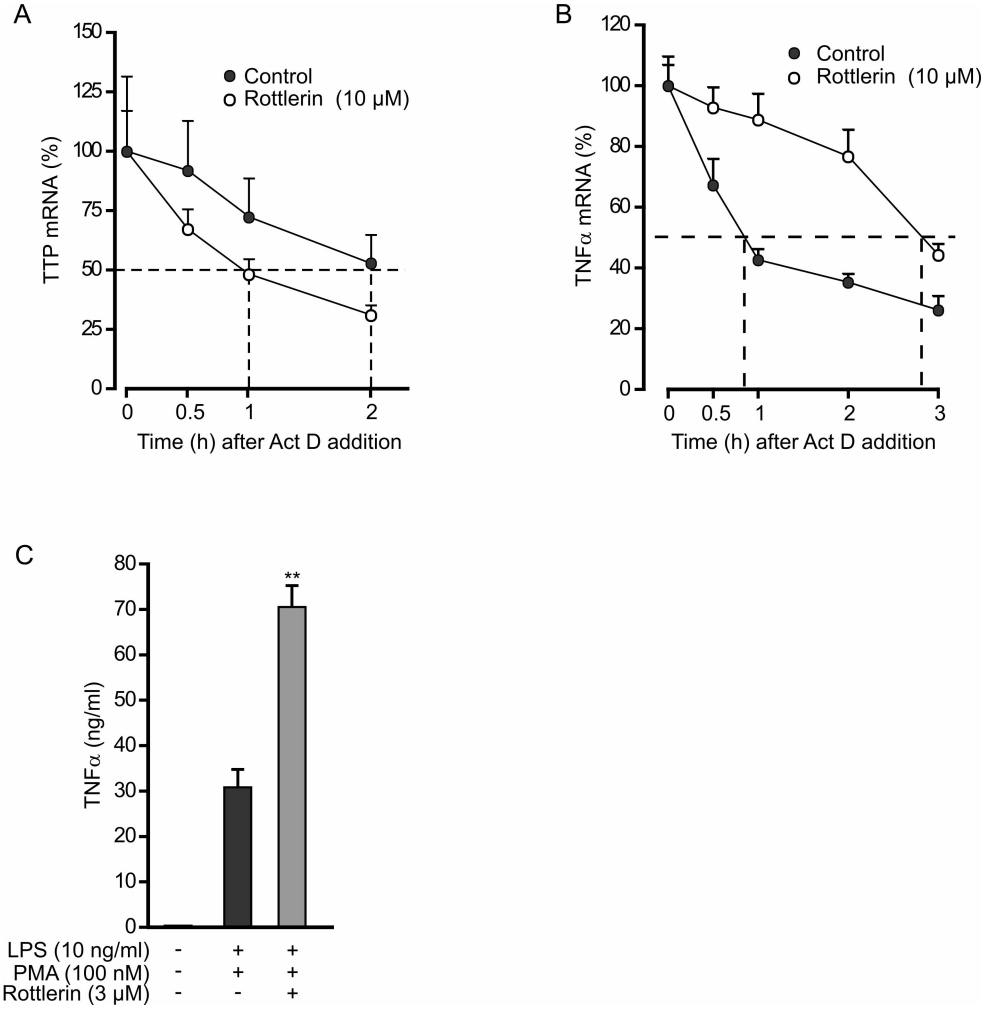

Figure 17. *The effect of rottlerin on (A) TTP mRNA decay (B) TNFα mRNA decay (C) and on TNFα production. Cells were treated with rottlerin for 30 min before LPS and PMA were added to the culture medium. After 4 h (A) and 9 h (B) incubation, actinomycin D (1 µg/ml) was added to stop the transcription. Incubations were terminated at the indicated time points after actinomycin D and extracted total RNA was subjected to RT-PCR (n=3, mean + SEM). (C) After 48 h incubation, the effect of rottlerin on LPS and PMA –induced TNFα production was measured by ELISA (n=4, + SEM). (Reprinted with permission from Leppänen et al. 2010, Eur J Pharmacol 628:220-225 © Elsevier Ltd, modified).*

5. The anti-inflammatory effects of rottlerin *in vivo* (IV)

An intradermal injection of carrageenan has been reported to cause a local inflammatory response in the mouse and rat. This response is partly mediated by increased NO production (Salvemini et al. 1996). Studies with NOS inhibitors have shown that iNOS is responsible for the production of NO in this rodent model (Handy and Moore 1998).

In the present study, PKCδ inhibitor rottlerin had significant anti-inflammatory properties in LPS- or cytokine-stimulated murine macrophages and fibroblasts.

Therefore the anti-inflammatory properties of rottlerin were also studied *in vivo*, by using the carrageenan-induced paw inflammation model in the mouse. Intradermal injection of carrageenan caused a marked inflammatory oedema and an increase in the volume of the mouse paw (net oedema ~100 µl 3 h after the carrageenan injection). Intraperitoneal administration of iNOS inhibitor L-NIL (50 mg/kg) reduced the carrageenan-induced inflammatory paw oedema by over 50%. The PKCδ inhibitor rottlerin (10 mg/kg) had a very similar effect as L-NIL, i.e. it reduced carrageenan-induced paw oedema by 54% (Figure 18). These results indicated that the novel isoenzyme PKCδ participates in the regulation of the production of the inflammatory response also *in vivo*.

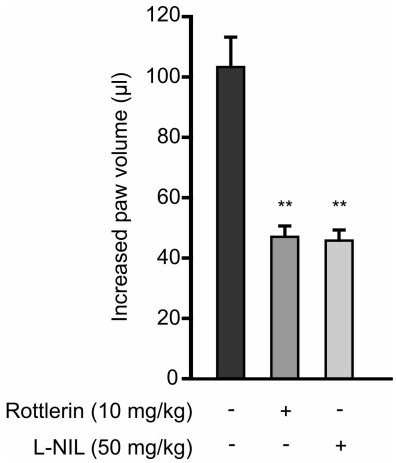

Figure 18. *Effects of PKCδ inhibitor rottlerin on carrageenan-induced mouse paw oedema. L-NIL and rottlerin were administered i.p. 2 h before carrageenan was injected intradermally. Paw oedema was measured before and 3 hours after carrageenan injection. Oedema is expressed as the difference between the carrageenan injected paw and the control paw injected with the vehicle only (n=6, mean + SEM), **p<0.01.*

6. Summary of the results

In the present study, the effects of PKC isoenzymes α, βI, βII, and δ on the expression of iNOS and TTP were studied. The roles of different isoenzymes were investigated by using a battery of PKC inhibitors with different selectivity profiles, by downregulating PKC isoenzymes with PMA, and by downregulating PKCδ with siRNA.

The results reveal, that both the classical isoenzyme PKCβ and the novel isoenzyme PKCδ do participate in the regulation of iNOS and TTP expression in murine inflammatory cells, but the underlying mechanisms seem to be somewhat different between these isoenzymes. However, this study did not evaluate the interplay between the two isoenzymes. The main results obtained in this study are summarized in Table 10.

Table 10. *Summary of the results*

Effects of inhibition of PKC isoenzymes on the iNOS pathway							
	NO production	iNOS expression	iNOS mRNA	iNOS mRNA decay	Activation of		
					IRF1	NF-κB	STAT1
PKCβ	↓	↓	↓	–	nd	–	↓
PKCδ	↓	↓	↓	–	↓	–	nd

Effects of inhibition of PKC isoenzymes on the TTP pathway							
	TTP protein expression	TTP mRNA expression	TTP mRNA decay	Activation of			
				AP-2	EGR1	NF-κB	Sp1
PKCβII	↓	↓	–	↓	↑	–	–
PKCδ	↓	↓	↑	–	–	–	–

nd = not determined, ↓ = decrease, ↑ = increase, - = no effect

Discussion

1. Methodology

In these studies, immortalized cell lines (murine J774 macrophages and murine L929 fibroblasts) were used to study whether the PKC isoenzymes are involved in the expression of iNOS and TTP. Culturing conditions of the cell lines were standardized and variation in the conditions was kept to a minimum. Immortalized cell lines provide a stable and non-varying research material where the repeatability is excellent and the experiments are comparable. However, the immortalization of cells may cause them to lose some of the properties of primary cells and thus they do not represent primary cells as such. Even though cell lines lack the individual variation that the use of experimental animals may cause, they also lack the signals that the environment i.e. surrounding cells, provide in a living organism.

NO produced by the cells was assessed as nitrite accumulating in the cell culture medium and measured by the method of Griess (Green et al. 1982). Nitrite is a stable metabolite of NO in aqueous solution, whereas in blood, the main metabolite of NO is nitrate (due to the action of haemoglobin). The standards of the nitrite measurement were diluted into the complete cell culture medium of the cell line in question in order to eliminate the possibility of the presence of nitrite and other interfering substances in the culture medium. Inhibitors of iNOS were used to ensure that the nitrite production observed was due to NO produced by the iNOS pathway.

Standard molecular and cellular biology methods were used to determine protein (Western blot) and mRNA (quantitative RT-PCR) expression. In order to control the amount of protein loaded in Western blot gels, the protein concentration in each sample was measured by the Coomassie blue method (Bradford 1976) and equal amounts of protein were loaded for every sample. In addition, loading controls, i.e.

actin for whole cell samples and lamin A/C for nuclear samples, were used to further control the amount of protein. Protein expression was detected with the use of specific antibodies. The expression of mRNA was detected by quantitative real-time RT-PCR. GAPDH was used as housekeeping gene and the levels of the mRNA of interest were normalized against it. The expression of GAPDH is usually not affected by different treatments, however, certain limitations should be kept in mind (Bustin 2000).

The effects of PKC inhibitors on the degradation of iNOS or TTP mRNA were studied by the actinomycin D assay. The rate of mRNA decay can be detected by measuring the levels of the target mRNAs at different time intervals after the transcription has been inhibited with actinomycin D. This method is widely used, but it has its own disadvantages. Actinomycin D has been suggested to affect the degradation of some mRNAs (Chen et al. 1995, Seiser et al. 1995, Dixon et al. 2000), and our group has previously shown that actinomycin D may also stabilize iNOS mRNA (Lahti et al. 2006). These points have to be kept in mind when interpreting the results. In addition, actinomycin D may inhibit the synthesis of a factor involved in the expression of the gene of interest and thus cause false interpretations of the results.

The activation of transcription factors was studied by three different methods. The activations of the transcription factors for TTP and STAT1 for iNOS were studied by detecting their translocation from cell cytosol to the nuclei, which is a required step in their activation cascade. However, this method does not directly measure the transcription factor mediated transcription. Activation of NF-κB was studied also by EMSA, which provides information about the translocation of the transcription factor to the nuclei, and about its DNA binding activity. On the other hand, EMSA does not detect phosphorylation and other modifications that may alter the activity of transcription factors. Therefore the effects of the PKCδ inhibitor rottlerin on NF-κB mediated transcription were studied also in a cell line stably transfected with a luciferase reporter gene construct in which luciferase expression was under the control of a NF-κB responsive promoter.

The effects of different PKC isoenzymes on iNOS and TTP expression were studied by using PKC isoenzyme selective inhibitors, by downregulating PKC isoenzymes by PMA, and by downregulating PKCδ by siRNA. PKC inhibitors were

used as pharmacological tools to evaluate the effects of different isoenzymes. The inhibitors used possessed varying isoenzyme selectivity profiles and the use of several inhibitors helped to pinpoint on which isoenzymes might be involved in the regulation of the expression of iNOS and TTP. However, the inhibitors are not totally selective, they may also affect the activity of other kinases. Philip Cohen and his coworkers have studied the selectivity of kinase inhibitors in a panel of nearly one hundred protein kinases. They have studied the selectivity of some cPKC inhibitors and reported that e.g. bisindoylmaleimides may affect also other kinases (Davies et al. 2000, Bain et al. 2007). In order to strengthen the results obtained with cPKC inhibitors, phorbol esters were used as a tool to activate and downregulate cPKC. Although PKC is probably the most widely studied target of phorbol esters, it has been shown that phorbol esters have also some other targets (Kazanietz et al. 2000). Nonetheless, the use of selective inhibitors and phorbol esters together provided convincing evidence that cPKC isoenzymes do participate in the regulation of iNOS and TTP expression.

The selectivity of rottlerin has been evaluated also in large protein kinase panels, and it has been found to inhibit some other kinases in addition to PKCδ (Davies et al. 2000, Bain et al. 2007). It has also been suggested to act as a mitochondrial uncoupler and thus evoke cellular effects that are independent from its direct effects of PKCδ activity (Soltoff 2007). In the present study, PKCδ specific siRNA was used as another means to investigate the role of PKCδ in the regulation of iNOS and TTP. siRNA was shown to downregulate the expression of PKCδ by over 80% in the cell lines used, and it had similar effects on iNOS and TTP expression as rottlerin. When rottlerin was used in cells where PKCδ had been downregulated with siRNA, the effects of rottlerin were abolished. Therefore it was concluded that the effects seen with rottlerin on the expression of iNOS and TTP are most likely mediated by PKCδ.

The use of siRNA has disadvantages as well. The transfection of cells with siRNA oligos may have some effect on the responses studied and therefore a non-targeting siRNA was used as a control. siRNA may also have off-target effects, which must be kept in mind when interpreting the results. The use of knockout animals may have provided a better research background to study the effects of individual PKC isoenzymes. However, knockout animals tend to develop

compensatory signalling systems, especially in the case of PKC, where there seems to be some functional redundancy among the various isoenzymes. In summary, it seems that the determination of the role of individual PKC isoenzymes could be most effective when the results from different research methods are combined, as was attempted in the present study.

2. Regulation of NO production and iNOS expression by cPKC isoenzymes

The role of cPKC isoenzymes α, βI, βII, and γ was studied on the regulation of NO production and on iNOS expression in LPS-stimulated murine J774 macrophages. With the exception of PKCγ, all of the other cPKCs were expressed in J774 macrophages. By using PKC inhibitors with different selectivity profiles, it was shown that PKCβ, but not PKCα, takes part in the regulation of LPS-induced NO production and iNOS expression. These experiments revealed a novel finding, i.e. inhibition of PKCβ, either by a selective inhibitor or downregulation of PKCβ by PMA, leads to reduced activation of transcription factor STAT1 and in this way it mediates its effects on iNOS expression.

The classical isoenzymes PKCα, PKCβI, and PKCβII are ubiquitously expressed (Kubo et al. 1987, Nakashima 2002), whereas the expression of PKCγ is largely restricted to the brain and spinal cord (Saito and Shirai 2002). The regulation of cell signalling events by PKC is known to be isoenzyme specific and furthermore, the effects of a single isoenzyme can also be cell type and tissue specific (Tan and Parker 2003). PKCβ has been shown to play a role in LPS signalling and in LPS-induced changes in mouse peritoneal macrophages (Shinji et al. 1994). PKCα and PKCβ have also been shown to participate in the regulation of iNOS expression in RAW 264.7 macrophages (Chen et al. 1998b, St-Denis et al. 1998), and in J774 macrophages (Fujihara et al. 1994). However, no detailed mechanisms accounting for this regulation have been investigated in any of the previous studies. In contrast to our findings, Fujihara et al. reported that PKCβII was the only classical isoenzyme expressed in J774 macrophages.

The role of classical isoenzymes in the regulation of NO production and iNOS expression was studied by using four PKC inhibitors with different selectivity profiles. RO318220 (inhibits β, γ, and ε) (Davis et al. 1992, Wilkinson et al. 1993), GÖ6976 (inhibits all cPKCs) (Martiny-Baron et al. 1993), and LY333531 (inhibits βI and βII) (Jirousek et al. 1996) reduced LPS-induced NO production and iNOS expression in a dose dependent manner, whereas HBDDE (inhibits α and γ) (Kashiwada et al. 1994) had no effect on NO production. The selectivity profiles of the inhibitors in question suggested that cPKC isoenzymes are involved in iNOS regulation and that PKCβ is most likely the isoenzyme responsible for this regulation.

Since the selectivity of the inhibitors has been indicated to be somewhat questionable (Davies et al. 2000, Bain et al. 2007), the effects of cPKCs were studied also by other means. Phorbol esters, such as PMA and PDD, are known activators of cPKCs. They are also known to cause downregulation of cPKCs when used for prolonged periods of time (Huang et al. 1989, Chen 1993). In this study PKCα, PKCβI, and PKCβII were shown to be activated and downregulated by PMA. Under the conditions where PKC isoenzymes were shown to be downregulated, the production of NO and the expression of iNOS were reduced, i.e. similar to the results obtained with the PKC inhibitors. Activation of cPKC isoenzymes by phorbol esters led to an enhancement in NO production and iNOS expression. These results further support the role of cPKC isoenzymes in the regulation of iNOS expression.

The reducing effect of PKC inhibitors RO318220 and GÖ6976 on iNOS mRNA expression was seen already at the early time points after stimulating the cells by the addition of LPS. This suggested that the mechanism by which cPKCs regulate iNOS expression occurs at the transcriptional level. This hypothesis was further supported by the results from the actinomycin D assay i.e. cPKCs did not alter the decay of iNOS mRNA. Surprisingly, cPKC inhibitors had no effect on the activation of NF-κB which is a critical transcription factor for iNOS (Lowenstein et al. 1993, Xie et al. 1994), even though PKCβ has been linked to the NF-κB signalling in PKCβ knockout studies (Saijo et al. 2002). In addition to NF-κB (Xie et al. 1994), STAT1 has also been identified as an important transcription factor for iNOS (Gao et al. 1997). The role of STAT1 in LPS-induced iNOS expression in murine macrophages

has been investigated also by our group (Sareila et al. 2008). Indeed, RO318220, GÖ6876, and LY333531, as well as downregulation of cPKCs by PMA, inhibited the activation of STAT1 when this was measured as the translocation of STAT1α to the nuclei. The typical activation of the JAK-STAT pathway begins with the activation of type II interferon receptor, which triggers the formation of JAK heterodimers. This leads to phosphorylation of STATs, which dimerize and translocate to the nucleus to activate gene transcription. These results indicate that the regulation of LPS-induced iNOS expression by cPKCs is NF-κB independent and likely to be mediated through the activation of the transcription factor STAT1. In addition, PKCβ seems to be the isoenzyme responsible for this regulation.

3. Regulation of NO production and iNOS expression by PKCδ. Anti-inflammatory effects of rottlerin.

In this study, the role of a novel isoenzyme PKCδ was studied in the regulation of NO production and iNOS expression. By using the PKCδ inhibitor rottlerin and PKCδ specific siRNA, it was shown that this novel isoenzyme participates in the regulation of NO production and iNOS expression in murine macrophages and fibroblasts. The regulation by PKCδ was shown to be mediated at least partly through the activation of transcription factor IRF1. In addition, anti-inflammatory effects of rottlerin were demonstrated *in vivo*.

PKCδ is known to play an important role in the regulation of cellular responses in immunity (Perletti and Terrian 2006). However, less is known about the role of PKCδ in the regulation of inflammatory genes. Our results are supported by the previous studies where PKCδ was reported to regulate the expression of iNOS in pancreatic β-cells by stabilizing iNOS mRNA (Carpenter et al. 2001) and in RAW 264.7 macrophages through the activation of NF-κB (Chen et al. 1998b).

The PKCδ inhibitor rottlerin was shown to reduce LPS-induced NO production and iNOS expression in J774 macrophages and cytokine-induced NO production and iNOS expression in L929 fibroblasts. However, since the selectivity of rottlerin against PKCδ has been questioned (Davies et al. 2000, Bain et al. 2007, Soltoff 2007), PKCδ specific siRNA was used to confirm the results obtained with rottlerin.

In both cell lines, PKCδ siRNA downregulated the expression of PKCδ by over 80% and reduced NO production and iNOS expression in a manner similar to rottlerin. More importantly, rottlerin had no effect on NO production or iNOS expression when used under conditions where PKCδ was downregulated by siRNA. These results suggest that the effects seen with rottlerin are most likely mediated through PKCδ.

In contrast to the results obtained with cPKC inhibitors, PKCδ siRNA or rottlerin had no effect on iNOS mRNA expression when measured at the early time points (< 6 h after stimulating the cells by adding LPS), but significantly reduced iNOS mRNA expression at later time points (> 6 h after stimulating the cells by adding LPS). This is in line with the results from the study by Carpenter et al. (2001) i.e. the effect of PKCδ on iNOS mRNA expression was observed at 12 h after the stimulation of the cells with IL-1β. However, in contrast to the results by Carpenter et al. no effect on the decay of iNOS mRNA was seen in this study when measured by actinomycin D assay. Consistent with the iNOS mRNA data, PKCδ inhibition by rottlerin did not affect the activation of transcription factor NF-κB.

IRF1 has been shown to be an important transcription factor for iNOS, and macrophages from IRF1 knockout mice show markedly reduced LPS and IFNγ -induced iNOS mRNA expression. In contrast to NF-κB, IRF1 is considered to be a later phase transcription factor (Kamijo et al. 1994, Fujimura et al. 1997). In the present study, inhibition of PKCδ by rottlerin and PKCδ downregulation by siRNA reduced also the LPS-induced expression of IRF1 mRNA when measured at the 4 h time point, indicating that IRF1 might mediate the effects of PKCδ on iNOS expression. In addition, downregulation of PKCδ by siRNA reduced the production of another IRF1 responsive inflammatory mediator, IL-6 (Faggioli et al. 1997). These findings are supported by the report by Momose et al. (2000), that in murine macrophages PMA could synergistically increase IFNγ-induced iNOS expression, this being mediated through IRF1.

In the present study, the anti-inflammatory effects of the PKCδ inhibitor rottlerin were also studied *in vivo* in a carrageenan-induced inflammatory model. The paw oedema induced by carrageenan administration has been reported to be attributable to a local inflammatory response which is partly mediated by increased NO production (Salvemini et al. 1996, Handy and Moore 1998). Similarly to its effects

in the cell lines, rottlerin was demonstrated to possess anti-inflammatory effects also *in vivo*. The effects of rottlerin were similar to the effects of the iNOS inhibitor L-NIL, suggesting that the anti-inflammatory effect of rottlerin on carrageenan-induced paw inflammation may be, at least partly, mediated by its effects on NO.

4. Transcriptional regulation of TTP by PKCβII

The present study also examined the role of cPKC isoenzymes on TTP expression. The results indicate that cPKCs, especially PKCβII, upregulate TTP expression in activated macrophages, this being mediated at least partly through the activation of the transcription factor AP-2.

The expression of TTP in murine J774 macrophages was induced by treatment with LPS, and it was further enhanced by addition of PMA. Pretreatment of cells with a higher concentration of PMA before stimulation with LPS or LPS and PMA, was shown to downregulate cPKC isoenzymes. It also abolished the enhancing effect of the combination of LPS and PMA on TTP expression as compared to TTP expression obtained with LPS alone. This pretreatment did not affect LPS-induced TTP expression levels. The results suggested that cPKC isoenzymes might be involved in the regulation of LPS and PMA –induced expression of TTP. Inhibitors of cPKCs were used to further evaluate the role of classical isoenzymes on TTP expression. Since CGP53353 (inhibitor of βII) (Chalfant et al. 1996), as well as other compounds that inhibit PKCβ, reduced the TTP protein and mRNA expression induced by LPS and PMA, it is concluded that PKCβII is the main isoenzyme responsible for the regulation of the expression of TTP.

Little is known about the transcription factors that enhance the transcription of TTP mRNA. However, analysis of sequence elements in TTP promoter and intron regions have revealed binding sites for AP-2, EGR1, NF-κB, and Sp1 (DuBois et al. 1990, Lai et al. 1995, Lai et al. 1998). The present study investigated the effects of PKCβII on the nuclear translocation of these transcription factors. Inhibition of PKCβII by CGP53353 had no effect on the translocation of EGR1, NF-κB or Sp1, but it reduced that of AP-2. This effect on AP-2 activation was also seen with downregulation of PKC due to PMA pretreatment. These results suggest that the

effect of PKCβII on LPS and PMA –induced TTP expression is mediated, at least partly, through transcription factor AP-2. Subsequently to this study, our group has observed that β2-agonists and other cAMP-elevating agents can increase TTP mRNA and protein expression, an effect also associated with the activation of AP-2 (Jalonen et al. 2007).

TTP is a factor that regulates the stability of mRNAs of certain inflammatory genes. It binds to the AREs within mRNA and causes destabilization of the mRNA (Lai et al. 2000). TTP may be considered primarily as an anti-inflammatory factor. The majority of TTP's known targets are proinflammatory mediators whose mRNA is destabilized by TTP (e.g. TNFα, GM-CSF, IL-2, and IFNγ) (Carballo et al. 1998, Carballo et al. 2000, Ogilvie et al. 2005, Ogilvie et al. 2009). In addition, TTP knockout mice were shown to develop a severe inflammatory syndrome including arthritis and autoimmunity (Taylor et al. 1996a). Thus, it seems that by upregulating the expression of TTP, PKCβII takes part in the inhibition of the stability of mRNAs of inflammatory genes, serving as a possible anti-inflammatory feed-back mechanism to limit the inflammatory reaction.

5. Post-transcriptional regulation of TTP by PKCδ

Study III focused on the role of novel isoenzyme PKCδ on the regulation of TTP expression in activated macrophages. It was shown that downregulation of PKCδ by siRNA and inhibition of PKCδ by rottlerin reduced TTP expression by enhancing the degradation of TTP mRNA.

Most of the studies concerning the regulation of TTP expression have focused on the effects of MAP kinases (Mahtani et al. 2001, Tchen et al. 2004, Brook et al. 2006). However, a few reports have indicated that phorbol esters and possibly PKC may also regulate TTP expression (Lai et al. 1990, Inuzuka et al. 1999, Murata et al. 2000a). The role of cPKC isoenzymes in the regulation of TTP expression was examined in study II. Since the novel isoenzyme PKCδ is also PMA responsive in J774 macrophages, its role in the regulation of TTP expression was also evaluated.

PKCδ downregulation with siRNA reduced TTP protein and mRNA expression. Similar results were obtained with the PKCδ inhibitor rottlerin. When the effects of

rottlerin were studied in cells in which PKCδ had been downregulated by siRNA, the effects of rottlerin were abolished, suggesting that the effects seen with rottlerin are mainly mediated through PKCδ. Rottlerin had no effect on the activation of transcription factors AP-2, EGR1, NF-κB, or Sp1. Instead, rottlerin was shown to destabilize TTP mRNA in an mRNA degradation assay. Previously, p38 MAPK has been observed to alter the stability of LPS-induced TTP mRNA in RAW 264.7 macrophages (Tchen et al. 2004). The functional relevance of rottlerin's effect on TTP mRNA was evaluated in a preliminary study. Since TTP has been shown to regulate the stability of TNFα mRNA (Carballo et al. 1998), the effect of rottlerin on the decay of TNFα mRNA was studied. It was found that rottlerin reduced the decay of TNFα mRNA under conditions where the inhibitory effect of rottlerin on TTP protein expression was also present. In addition, rottlerin enhanced LPS and PMA –induced TNFα production, suggesting that the changes seen in TTP expression levels after rottlerin may have functional significance.

These results are the first evidence that inhibition of novel isoenzyme PKCδ can impair the expression of TTP in activated macrophages. It seems that PKCδ is involved in the upregulation of TTP expression via a mechanism related to the stabilization of TTP mRNA, but the specific molecular mechanisms responsible for the effects of PKCδ on TTP mRNA stability remain to be clarified. Thus, the regulation of TTP mRNA stability by PKCδ may serve as a feed-back loop to limit the inflammatory reaction. However, the overall effect of PKCδ in inflammation, at least in acute inflammation, is likely to be proinflammatory as evidenced by the anti-inflammatory effect of rottlerin in the carrageenan-induced paw inflammation as shown in study IV.

6. PKCs as anti-inflammatory drug targets

In the present study, the role of PKC isoenzymes, especially PKCβ and PKCδ, in the regulation of the expression of inflammatory genes iNOS and TTP was examined. PKCβ has been claimed to be an important factor in B cell receptor (BCR) mediated functions (Leitges et al. 1996) and in NF-κB signalling (Saijo et al. 2002) based on studies with PKCβ knockout animals. PKCβ has also been proposed

to take part in LPS signalling and LPS-induced macrophage functions (Shinji et al. 1994).

Studies with PKCδ knockout animals have revealed that this isoenzyme can regulate the proliferation of B cells and the formation of self reactive B cells (Leitges et al. 2001a, Mecklenbräuker et al. 2002, Miyamoto et al. 2002). PKCδ has also been reported to modulate the expression of iNOS in pancreatic β-cells by stabilizing iNOS mRNA (Carpenter et al. 2001) and in RAW 264.7 macrophages through the activation of NF-κB (Chen et al. 1998b). However, little is known about the effects of PKCβ and PKCδ in the regulation of inflammatory genes, especially TTP.

In the present study, inhibition of PKCβ and PKCδ reduced the expression of iNOS and production of NO under inflammatory conditions. Both PKC isoenzymes, β and δ, seemed to regulate transcription factors known to be important for iNOS. In addition, the PKCδ inhibitor rottlerin reduced carrageenan-induced paw inflammation in mice. In preliminary studies by our group, an inhibitor of classical PKCs GÖ6976 and PKCδ inhibitor rottlerin reduced cytokine-induced NO production and iNOS expression also in A549 human lung epithelial cells. Since the transcription factors involved in the effects of PKC on murine iNOS (STAT1 and IRF1) are also known to be important for human iNOS (Kleinert et al. 2003, Korhonen et al. 2005), it would be interesting to investigate whether the effects of PKCs are mediated through the same mechanisms in human cells as in murine cells. Compounds that inhibit the expression or activation of iNOS have been demonstrated to have anti-inflammatory properties in various forms of experimentally-induced inflammation. The results from the present study suggest that inhibition of PKC isoenzymes β and δ could possess anti-inflammatory properties in diseases which are complicated by increased iNOS expression and NO production.

In inflammation, also the factors that limit the inflammatory process, such as TTP, are activated. In the present study, the expression of TTP was found to be induced by LPS and PMA, and PMA was used as an example of a compound that can activate PKC. The present results suggest that PKCβII and PKCδ could also regulate the inflammatory response by upregulating the expression of TTP. TTP has been shown to stabilize human iNOS mRNA through an interaction with KSRP

(Fechir et al. 2005, Linker et al. 2005), but less is known about the role of TTP as a regulator of the stability of murine iNOS mRNA. In the present study, PKC isoenzymes did not affect the stability of iNOS mRNA, however, this does not exclude the possibility that TTP could in part mediate the effects of PKC isoenzymes on iNOS expression.

Overall, PKC isoenzymes, especially PKCβ and PKCδ, are interesting targets for anti-inflammatory drug development. In addition to their known effects on B cell functions, they seem to take part in the regulation of inflammatory genes. However, the cell type specificity of PKC functions needs to be borne in mind.

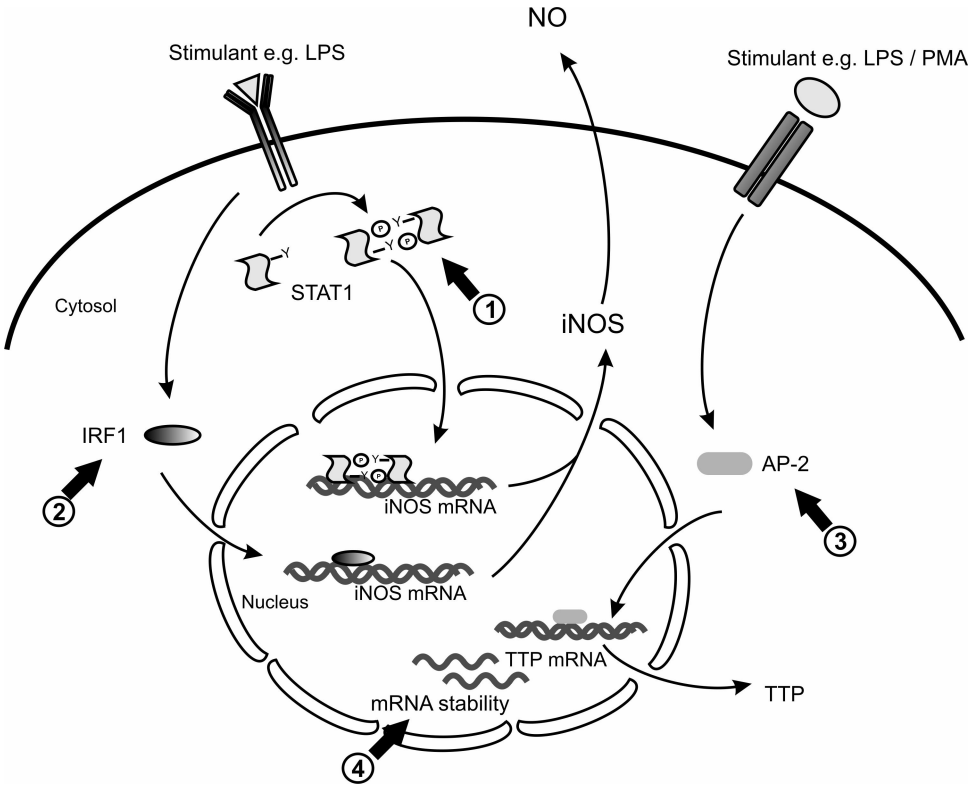

Figure 19. *Summary of the effects of PKCβ and PKCδ on the expression of iNOS and TTP.*
1. *Inhibition of PKCβ attenuated the activation of transcription factor STAT1 and expression of iNOS.* ***2.*** *Inhibition of PKCδ reduced the expression of transcription factor IRF1 and the expression of iNOS.* ***3.*** *Inhibition of PKCβII attenuated the activation of transcription factor AP-2 and the expression of TTP.* ***4.*** *Inhibition of PKCδ destabilized TTP mRNA and thus reduced TTP expression.*

Summary and conclusions

The aim of the present study was to evaluate the role of classical PKC isoenzymes and novel isoenzyme PKCδ in the regulation of inflammatory genes, with iNOS and TTP as examples. The main objectives of the study were to determine which isoenzymes take part in the regulation of iNOS and TTP expression and to investigate the underlying mechanisms of this regulation. The major findings and conclusions are as follows:

1. Classical PKC isoenzymes take part in the regulation of LPS-induced NO production and iNOS expression in macrophages. The main isoenzyme responsible for the regulation seems to be PKCβ. The effects of PKCβ on LPS-induced NO production and iNOS expression are mediated mainly at the level of transcription, probably by affecting the activity of transcription factor STAT1.
2. Classical PKC isoenzymes, most probably PKCβII, regulate the expression of TTP in macrophages and this seems to be mediated, at least partly, through the activation of transcription factor AP-2.
3. The novel isoenzyme PKCδ participates in the regulation of NO production and iNOS expression in activated macrophages and fibroblasts. PKCδ does not regulate the early transcriptional mechanisms related to iNOS expression, but enhances the expression of IRF1. This may explain its effect on iNOS expression.
4. PKCδ regulates TTP protein and mRNA expression in macrophages by affecting TTP mRNA decay.
5. The PKCδ inhibitor rottlerin possesses anti-inflammatory effects also *in vivo* as shown in a carrageenan-induced inflammatory model in mice.

The present study provides novel information about the role of PKC isoenzymes in the regulation of inflammatory genes. Compounds, like PKC inhibitors, that

downregulate the activation or expression of iNOS possess anti-inflammatory properties and may be beneficial in the treatment of inflammatory diseases. TTP expression is also enhanced in inflammation as a regulatory mechanism. It destabilizes the mRNAs of inflammatory genes and this way downregulates their expression. PKC was found to enhance TTP expression which may serve as a feed-back loop to downregulate the inflammatory reaction. These results add our understanding about the inflammatory process and this information can be utilized in the development of novel anti-inflammatory drugs.

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Inhibition of classical PKC isoenzymes downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages

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1 Proinflammatory cytokines and bacterial products trigger inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in inflammatory and tissue cells. In inflammation, NO acts as an important mediator having both proinflammatory and destructive effects.

2 Protein kinase C (PKC) is a family of serine–threonine protein kinase isoenzymes involved in signal transduction pathways related to inflammatory responses. The aim of the present study was to investigate the role of classical PKC (cPKC) isoenzymes in the regulation of iNOS expression and NO production in murine J774 macrophages and the mechanisms involved.

3 RO318220 (inhibits PKCβ, PKCγ and PKCε), GÖ6976 (inhibits cPKC isoenzymes PKCα and PKCβ) and LY333531 (inhibits PKCβ) reduced lipopolysaccharide (LPS)-induced NO production and iNOS expression in a dose-dependent manner as did 6 h pretreatment with 1 μM phorbol 12-myristate 13-acetate (PMA) (which was shown to downregulate PKC expression).

4 PKC inhibitors also reduced LPS-induced iNOS mRNA levels, but they did not affect the half-life of iNOS mRNA. PKC inhibitors did not alter LPS-induced activation of NF-κB as measured by electrophoretic mobility shift assay.

5 All PKC inhibitors used and pretreatment with 1 μM PMA inhibited signal transducer and activator of transcription 1 (STAT1) activation as measured by the translocation of STAT1α from the cytosol to the nucleus by Western blot. In addition, inhibition of STAT1 activation by AG-490, an inhibitor of JAK-2, also reduced NO production.

6 These results suggest that cPKC isoenzymes, especially PKCβ, mediate the upregulation of iNOS expression and NO production in activated macrophages in an NF-κB-independent manner, possibly through the activation of transcription factor STAT1.
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Abbreviations: EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; STAT1, signal transducer and activator of transcription 1

Introduction

Nitric oxide (NO) acts as a signalling molecule in, for example, cardiovascular and neuronal systems. In inflammation, NO is an important mediator having both proinflammatory and destructive effects (Moilanen *et al*., 1999; Korhonen *et al*., 2005). High amount of NO is produced by inducible nitric oxide synthase (iNOS) for prolonged time as a response to bacterial products, such as lipopolysaccharide (LPS), and to proinflammatory cytokines (MacMicking *et al*., 1997; Vallance & Leiper, 2002). NO production in activated macrophages is primarily regulated at the level of iNOS expression (Kleinert *et al*., 2003; Korhonen *et al*., 2005).

The protein kinase C (PKC) pathway represents a major signal transduction system in inflammation (Spitaler & Cantrell, 2004). Different tissues seem to have their own characteristic patterns of PKC isoenzyme expression and function (Way *et al*., 2000). The mammalian PKC family comprises of serine–threonine protein kinase isoenzymes, which are divided into three classes based on their structure and ability to bind cofactors (Newton, 2001). The classical PKC (cPKC) isoenzymes (α, γ and the splice variants βI and βII) are activated by diacylglycerol (DAG), Ca^{2+} and phosphatidylserine. These isoenzymes are targets of the tumor-promoting phorbol ester PMA (phorbol 12-myristate 13-acetate, also called TPA), a surrogate of DAG. The novel (nPKC) isoenzymes (δ, ε, η and θ) are Ca^{2+}-independent and activated by DAG and phosphatidylserine. The third group, atypical PKC (aPKC) isoenzymes (ζ and ι/λ), are Ca^{2+}- and DAG-independent kinases. In contrast to the classical and novel isoenzymes, aPKCs do not respond to phorbol esters (Newton, 2001; Spitaler & Cantrell, 2004). In addition, PKCμ

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and PKCν are sometimes regarded to form a fourth class of PKC isoenzymes (Newton, 2001).

A role for PKC has been identified in inflammatory diseases, cancer and heart disease, and PKC inhibitors are under development to treat these diseases (Bowling *et al*., 1999; Chen *et al*., 2001; Goekjian & Jirousek, 2001; Newton, 2001; Tan & Parker, 2003; Aksoy *et al*., 2004). Several lines of evidence suggest that cPKC isoenzymes (Fujihara *et al*., 1994; St-Denis *et al*., 1998; Giroux & Descoteaux, 2000; Molina-Holgado *et al*., 2000; Foey & Brennan, 2004), PKCδ (Chen *et al*., 1998a; Tepperman *et al*., 2000; Carpenter *et al*., 2001), PKCη (Chen *et al*., 1998b; Pham *et al*., 2003) and PKCε (Castrillo *et al*., 2001; Kang *et al*., 2001) are involved in the LPS- and cytokine-induced expression of inflammatory genes including iNOS.

The aim of the present study was to investigate the role of cPKC isoenzymes in the regulation of iNOS expression and NO production in activated macrophages and the mechanisms involved. The results suggest that cPKC isoenzymes, probably PKCβ, mediate the upregulation of iNOS expression and NO production in activated macrophages in an NF-κB-independent manner, possibly through the activation of transcription factor signal transducer and activator of transcription 1 (STAT1).

Methods

Materials

Reagents were purchased as follows: RO318220, phorbol 12,13-didecanoate (PDD) and LY333531 were from Alexis Biochemicals (Lausen, Switzerland); GÖ6976, HBDDE and recombinant PKCγ were from Calbiochem (La Jolla, CA, U.S.A.); LPS (*Escherichia coli* 0111:B4, product number L-4391) was from Sigma Chemical Co. (St Louis, MO, U.S.A.); mouse monoclonal PKCα antibody, rabbit polyclonal iNOS, PKCβI, PKCβII, PKCγ and STAT1α antibodies and goat anti-rabbit HRP-conjugated polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and goat anti-mouse HRP-conjugated antibody was from Pierce Biotechnology (Rockford, IL, U.S.A.). All other reagents were from Sigma Chemical Co.

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, U.K.) and harvested with trypsin-EDTA (Gibco). Cells were seeded on 24-well plates for nitrite measurements and RT–PCR, on six-well plates for Western blot analysis and on 10 cm dishes for translocation studies, preparation of nuclear extracts and electrophoretic mobility shift assay. Cells were grown to confluence prior to the experiments. Toxicity of the tested compounds was ruled out by measuring cell viability using Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN, U.S.A.) according to manufacturer's instructions.

Nitrite assays

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 Griess reaction (Green *et al*., 1982).

Preparation of cell lysates for Western blotting

At indicated time points, 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 EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 μg ml^{-1} leupeptin, 50 μg ml^{-1} aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged (13,400 × *g*, 4°C, 10 min), supernatants were collected and stored in SDS sample buffer in −20°C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford, 1976).

Preparation of cytosolic and particulate fractions for PKC Western blotting

At indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold buffer A (20 mM Tris-base, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin and 1.25 mM NaF). After incubation for 15 min on ice, lysates were centrifuged at 100,000 × *g* for 1 h at 4°C, supernatants were collected and marked as the cytosolic fraction. Pellets were resuspended in cold lysis buffer B (20 mM Tris-base, pH 7.4, 10 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 1.25 mM NaF and 10 μM *n*-octyl-β-D-glucopyranoside). After incubation for 2 h on ice, lysates were centrifuged at 100,000 × *g* for 1 h at 4°C, supernatants were collected and marked as the particulate fraction. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford, 1976).

Preparation of nuclear extracts for electrophoretic mobility shift assay (EMSA) and STAT1α Western blotting

At indicated time points, 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 phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 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, 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 phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 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.

Protein contents of the nuclear extracts were measured by the Coomassie blue method (Bradford, 1976).

Western blotting

Prior to Western blotting, proteins were boiled for 10 min with SDS sample buffer and 20 μg of protein was used per lane on 8% (iNOS, STAT1α) or 10% (PKC) SDS–polyacrylamide gel and transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences, U.K., Ltd, Little Chalfont, Buckinghamshire, U.K.). After transfer, the membrane was blocked in TBS-T (20 mM Tris-base, pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4°C overnight. The membrane was washed with TBS-T and incubated with the secondary antibody in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using Super Signal® West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). Super Signal® West Dura and Femto (Pierce) were used for the detection of PKC isoenzymes.

Electrophoretic mobility shift assay

EMSA was performed as described previously (Lahti *et al*., 2002). Briefly, transcription factor consensus oligonucleotides for NF-κB (Promega, Madison, WI, U.S.A.) were 5′-^{32}P-end-labeled with DNA 5′-End Labeling Kit (Roche Diagnostics, Indianapolis, IN, U.S.A.). For binding reactions, 5 μg of nuclear extract was incubated in 20 μl of total reaction volume containing 0.1 mg ml^{-1} (poly)dI–dC, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl and 10% glycerol for 20 min in room temperature. ^{32}P-labeled oligonucleotide probe (0.2 ng) was added and the reaction mixture was incubated for 10 min. Protein–DNA complexes were separated from DNA probe by electrophoresis on a native 4% polyacrylamide gel. The gel was dried and autoradiographed using intensifying screen at −70°C. The quantitation of densities of specific bands was carried out using FluorChem™ software version 3.1.

RNA extraction and quantitative RT–PCR

Cell homogenization, RNA extraction, reverse transcription of RNA to cDNA and PCR reactions were performed as described previously (Lahti *et al*., 2003), with the exception that in the reverse transcription reaction, the amount of total RNA reverse transcribed was 100 ng and cDNA used in PCR corresponded to approximately 2.5 ng of total RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control gene.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2. Total RNA was isolated from LPS-stimulated J774 macrophages and reverse transcribed. Standard curves for GAPDH and iNOS were created using dilution series of cDNA corresponding to approximately 1 pg to 10 ng of total RNA in PCR. The threshold cycle values obtained were plotted against dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated using the standard curve. The relative amount of gene transcript present was calculated and normalized by dividing the calculated value of iNOS by the GAPDH value in each sample.

Statistics

Results are expressed as mean ± standard error of mean (s.e.m.). Statistical significance of the results was calculated by the analysis of variances supported by Dunnett adjusted significance levels. Differences were considered significant at $P<0.05$.

Results

Effects of PKC inhibitors on LPS-induced NO production and iNOS protein expression

Bacterial endotoxin LPS induced iNOS protein expression and NO production in J774 macrophages. To determine whether PKC activation participated in the upregulation of NO production by LPS, we measured NO production in the presence of PKC inhibitors. RO318220, an inhibitor of PKC isoenzymes β, γ and ε (Davis *et al*., 1992; Wilkinson *et al*., 1993), and GÖ6976, a selective inhibitor of cPKC isoenzymes (Martiny-Baron *et al*., 1993), both inhibited LPS-induced NO production in a dose-dependent manner (Figure 1a and b). Exposure to increasing concentration of RO318220 resulted in a 34% (0.3 μM) and 74% (1 μM) inhibition of NO production during 24 h incubation. Exposure to GÖ6976 resulted in 59% (0.3 μM) inhibition of NO production and larger doses (1 and 3 μM) inhibited NO production almost completely (91 and 95%). Since the results with GÖ6976 suggest that the effect of PKC on NO production could be mediated by the cPKC isoenzymes, we studied the effects of LY333531, a selective inhibitor of PKCβ (Jirousek *et al*., 1996), and HBDDE, an inhibitor of PKC isoenzymes α and γ (Kashiwada *et al*., 1994), on LPS-induced NO production. LY333531 inhibited NO production in a dose-dependent manner (Figure 1c), but HBDDE did not have any effect on NO production when used up to 100 μM concentrations (at concentrations higher than 100 μM HBDDE started to be toxic to J774 macrophages).

In further studies, we investigated the effects of PKC inhibitors on iNOS expression by Western blot. Cells cultured in the absence of LPS did not contain detectable amounts of iNOS protein. Exposure to LPS enhanced iNOS protein expression markedly. RO318220 (1–3 μM), GÖ6976 (0.1–1 μM) and LY333531 (2.5–7.5 μM) inhibited LPS-induced iNOS expression in a dose-dependent manner (Figure 2a–c).

PKC isoenzyme expression in J774 macrophages and the effect of PMA on PKC isoenzyme translocation

Western blot with antibodies specific for cPKC isoenzymes (α, βI, βII and γ) were carried out. Resting J774 cells expressed three cPKC isoenzymes α, βI and βII, but PKCγ was not found (Figure 3). In the further studies, cells were treated with a PKC activator PMA (100 nM), and after 10 min incubation, all three isoenzymes were activated as measured by isoenzyme translocation from the cytosol to the membrane (Figure 3). In

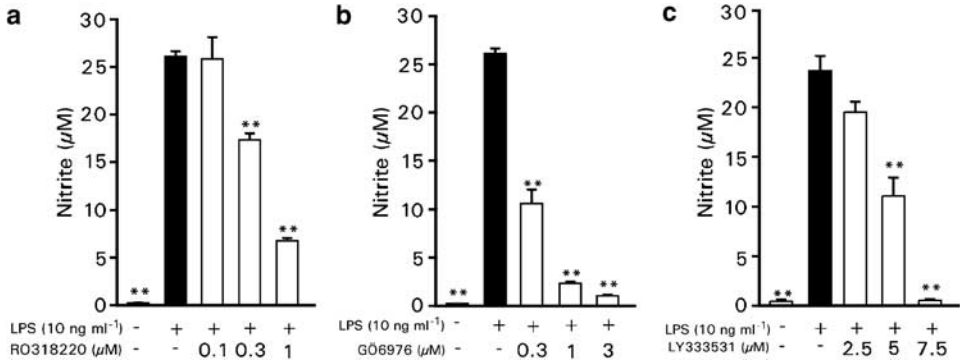

Figure 1 Effects of PKC inhibitors on LPS-induced NO production in J774 cells. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of RO318220 (a), GÖ6976 (b) or LY333531 (c). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n = 6$). $^{**}P < 0.01$ as compared with cells treated with LPS only.

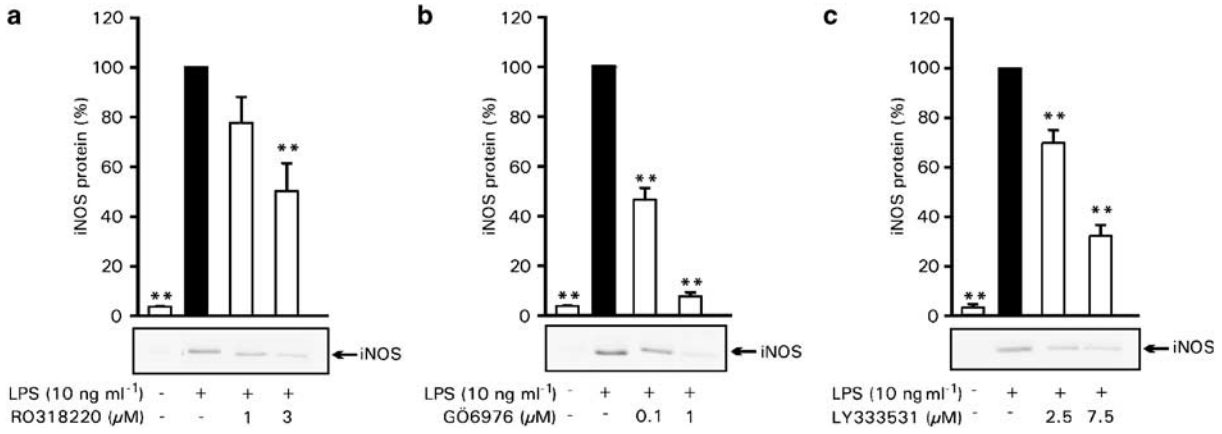

Figure 2 Effects of PKC inhibitors on LPS-induced iNOS protein expression in J774 cells. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of RO318220 (a), GÖ6976 (b) or LY333531 (c). After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n = 3$). $^{**}P < 0.01$ as compared with cells treated with LPS only.

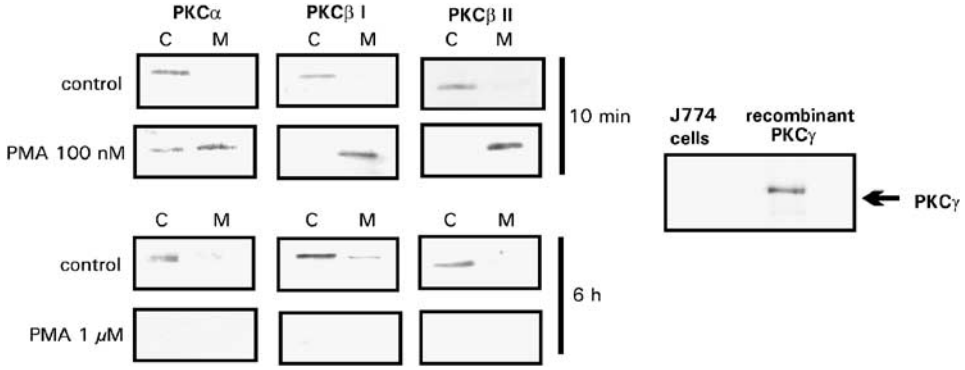

Figure 3 cPKC expression in J774 macrophages and the effects of PMA on PKC isoenzyme translocation. J774 cells were treated with 100 nM PMA or 1 μM PMA as indicated for 10 min or 6 h, respectively. Subsequent to preparation of cell lysates, the expression of individual PKC isoenzymes was assessed by immunoblotting with isoenzyme specific antibodies as outlined in the Methods section. Each experiment is a representative of three others with similar results. C = cytosolic fraction; M = membrane fraction. The expression of PKCγ in resting J774 macrophages was tested by Western blotting using recombinant human PKCγ as a positive control.

addition, incubation with a high concentration of PMA (1 μM) for 6 h resulted in the downregulation of all three PKC isoenzymes (Figure 3). Prolonged exposure to higher concentrations of phorbol esters, such as PMA, is known to cause almost complete downregulation of cPKCs and nPKCs, presumably as a result of proteolysis and it can be used as another means to downregulate PKC activity (Huang *et al*., 1989; Liu & Heckman, 1998).

Effects of phorbol esters on LPS-induced NO production and iNOS protein expression

To further determine the participation of PKC in LPS-induced NO production and iNOS expression, we measured the effects of PMA on NO production and iNOS protein expression. When PMA was used at concentrations (100 nM) that activate PKC (Figure 3), it enhanced LPS-induced NO production and iNOS protein expression as shown in Figure 4a and b. Another phorbol ester, PDD, also enhanced iNOS protein expression, when it was used at 100 nM concentration (Figure 4b).

When the cells were pretreated with 1 μM PMA for 6 h before LPS addition (which was shown to downregulate PKC expression, see Figure 3), both LPS-induced NO production and iNOS protein expression were inhibited similarly to the effects of PKC inhibitors (Figure 5a and b). In addition, 6 h pretreatment with PDD (1 μM) had a similar suppressive effect on iNOS expression as 1 μM PMA (Figure 5b). These results

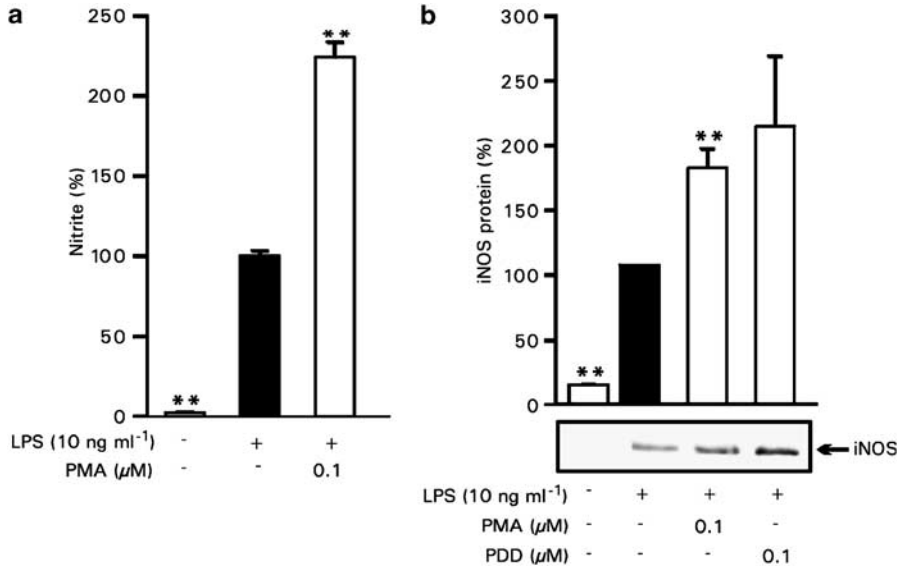

Figure 4 Activation of PKC by phorbol esters induces iNOS protein expression and NO production in J774 cells. (a) J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with PMA (100 nM) or vehicle (DMSO). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n = 6$). (b) J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with PMA (100 nM), PDD (100 nM) or vehicle. After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n = 3$). $^{**}P < 0.01$ as compared with cells treated with LPS.

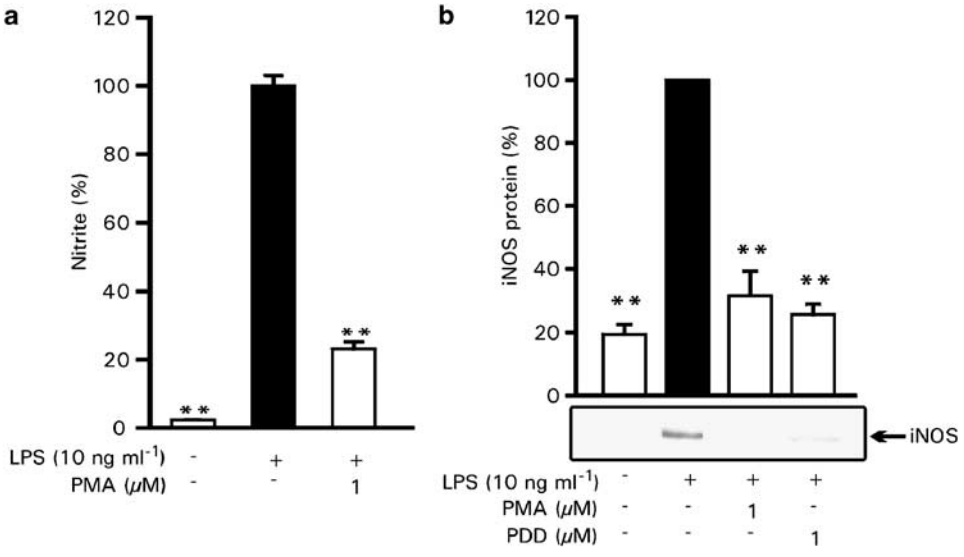

Figure 5 PKC downregulation by 6 h pretreatment with phorbol esters inhibits iNOS protein expression and NO production in J774 cells. (a) J774 cells were pretreated with PMA (1 μM) or vehicle for 6 h before stimulation by LPS (10 ng ml^{-1}). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n = 6$). (b) J774 cells were pretreated with PMA (1 μM), PDD (1 μM) or vehicle for 6 h before stimulation by LPS (10 ng ml^{-1}). After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n = 3$). $^{**}P < 0.01$ as compared with cells treated with LPS.

further suggest that PKC is involved in the signalling mechanisms mediating LPS-induced iNOS expression and NO production.

Effects of PKC inhibitors on iNOS mRNA expression

We used RT–PCR to investigate the effects of RO318220 and GÖ6976 on LPS-induced iNOS mRNA expression. In J774 macrophages, LPS-induced transient iNOS mRNA expression which had a 2 h lag phase and peaked at 6 h after the addition of LPS. We chose our time points 2 h before and after the 6 h peak to investigate the effects of PKC inhibitors on iNOS mRNA expression. At all measured time points, 4, 6 and 8 h after LPS induction, the iNOS mRNA levels were reduced in both RO318220- and GÖ6976-treated cells (Figure 6a). To determine whether PKC inhibitors reduce the half-life of iNOS mRNA, the cells were treated with LPS and the tested drugs, and after 6 h, transcription inhibitor actinomycin D ($0.1\,\mu g\,ml^{-1}$) was added into the culture. Cells were then further incubated for 0, 2, 4, 6 or 8 h before total RNA was extracted. As shown in Figure 6b, neither of the PKC inhibitors seemed to have any effect on iNOS mRNA half-life, nor did 100 nM PMA or pretreatment with 1 μM PMA (Figure 6c). These results suggest that the effect of cPKC isoenzymes on LPS-induced iNOS protein expression is mediated at the level of iNOS induction rather than at the level of post-transcriptional events.

Effects of PKC inhibitors on transcription factors NF-κB and STAT1

To evaluate whether the effect of PKC inhibitors on iNOS mRNA expression levels could be a consequence of their effects on transcription factors, we measured the effects of RO318220 and GÖ6976 on the activation of NF-κB and STAT1, which are essential transcription factors for LPS-induced iNOS expression. The activation of NF-κB was measured by EMSA. RO318220 or GÖ6976 had no effect on NF-κB activation or binding activity (Figure 7). In contrast, when we investigated the effects of PKC inhibitors on STAT1 activation, as measured by the translocation of STAT1α from the cytosol to the nuclei by Western blot, both RO318220 and GÖ6976 inhibited STAT1α translocation (Figure 8a). In addition, the PKCβ-selective inhibitor LY333531 (5 μM), as well as pretreatment for 6 h with 1 μM PMA inhibited STAT1α translocation to the nuclei (Figure 8b and c). These data suggest that the effects of cPKC isoenzymes on LPS-induced iNOS protein expression are NF-κB-independent, but may well be mediated through the activation of transcription factor STAT1.

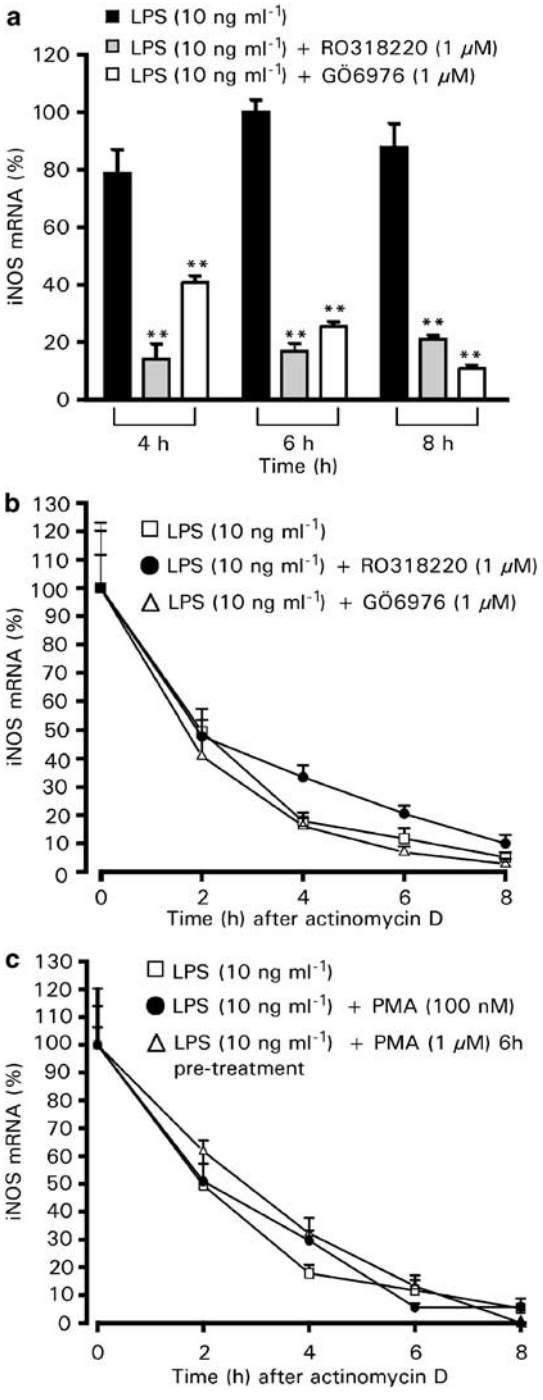

Figure 6 Effects of PKC inhibitors on iNOS mRNA expression and stability in J774 cells. (a) J774 cells were stimulated by LPS ($10\,ng\,ml^{-1}$) and treated with RO318220 (1 μM) or GÖ6976 (1 μM) for 4, 6 or 8 h. At indicated time points, the incubations were terminated and extracted total RNA was subjected to RT–PCR. iNOS mRNA levels were normalized against GAPDH mRNA. (b) Effect of PKC inhibitors on iNOS mRNA degradation. Cells were stimulated by LPS ($10\,ng\,ml^{-1}$) and treated with RO318220 (1 μM) or GÖ6976 (1 μM) for 6 h before the addition of actinomycin D ($0.1\,\mu g\,ml^{-1}$) to inhibit transcription. Incubations were terminated at indicated time points after actinomycin D and extracted total RNA was subjected to RT–PCR. iNOS mRNA levels were normalized against GAPDH mRNA. (c) Effect of PMA on iNOS mRNA degradation. Cells were treated with LPS ($10\,ng\,ml^{-1}$), with LPS ($10\,ng\,ml^{-1}$) and PMA (100 nM) to activate PKC or pretreated with PMA (1 μM) for 6 h to downregulate PKC before the addition of LPS ($10\,ng\,ml^{-1}$). After 6 h incubation with LPS, actinomycin D ($0.1\,\mu g\,ml^{-1}$) was added to inhibit transcription. Incubations were terminated at indicated time points after actinomycin D and extracted total RNA was subjected to RT–PCR. iNOS mRNA levels were normalized against GAPDH mRNA. Values are mean ± s.e.m. ($n=3$). $^{**}P<0.01$ as compared with cells treated with LPS only.

Effects of JAK-2 inhibitor AG-490 on LPS-induced NO production and STAT1 activation

To further investigate the role of STAT1 on LPS-induced NO production, we used JAK-2 inhibitor AG-490. JAK-2 (Janus kinase-2) is an upstream kinase of STAT1 and inhibition of JAK-2 leads to the inhibition of STAT1 (Shuai & Liu, 2003). AG-490 inhibited LPS-induced NO production in a dose-dependent manner (Figure 9a). Same concentrations that downregulated LPS-induced NO production also inhibited LPS-induced STAT1 activation, as measured by the translocation of STAT1α from the cytosol to the nuclei by Western blot (Figure 9b). These results further suggest that the effects of cPKC isoenzymes on iNOS expression and NO production could be mediated through the activation of STAT1.

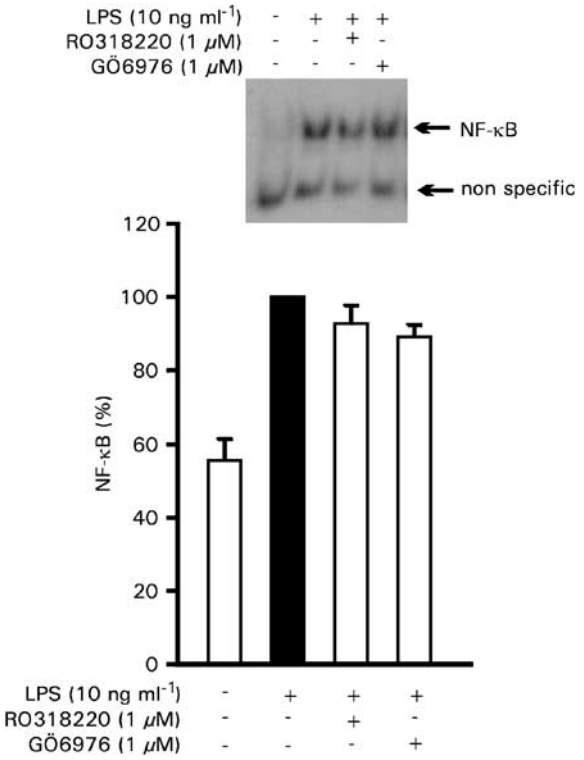

Figure 7 Effect of PKC inhibitors on NF-κB activity. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with RO318220 (1 μM) or GÖ6976 (1 μM) for 30 min before the preparation of nuclear extracts. NF-κB DNA binding activity was analyzed by EMSA. Densities of specific bands were quantified as described under the Methods section. Values are mean ± s.e.m. ($n=3$).

Discussion

In the present study, we show that inhibition of classical isoenzymes, especially PKCβ, inhibits LPS-induced iNOS expression and NO production in activated J774 macrophages, and that this effect is possibly mediated through the inhibition of transcription factor STAT1.

Distribution of PKC isoenzymes is cell type- and tissue-specific. PKCα, βI, βII, δ, ε and ζ seem to be ubiquitous isoenzymes, and are found in most tissues (Liu & Heckman, 1998). Classical isoenzyme PKCγ is largely restricted to the central nervous system and spinal cord (Liu & Heckman, 1998; Way *et al*., 2000). In the present study, we focused on cPKCs and found that PKCα, PKCβI and PKCβII are expressed in macrophage cultures used. PKC regulates various inflammatory functions in an isoenzyme-specific manner (Tan & Parker, 2003). The regulation of cell signalling events by single PKC isoenzymes have also been shown to differ between cell types (Paul *et al*., 1997).

In the present study, four PKC inhibitors with different PKC isoenzyme profiles were used to study the role of PKC and its classical isoenzymes in LPS-induced iNOS protein expression and NO production in macrophages. PKC inhibitors RO318220, GÖ6976 and LY333531 inhibited LPS-induced iNOS expression and NO production in a dose-dependent manner. RO318220 is a PKC inhibitor, which inhibits isoenzymes β, γ and ε (Davis *et al*., 1992; Wilkinson

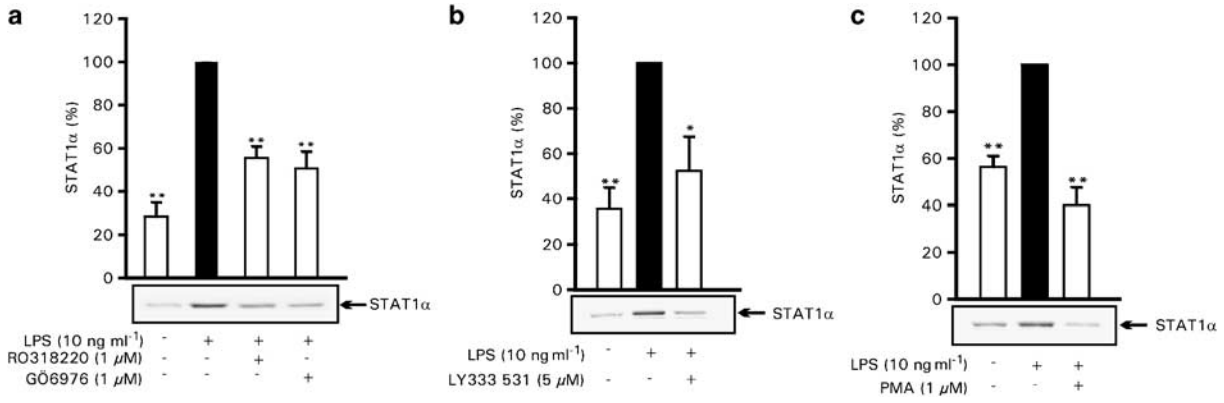

Figure 8 The effect of PKC inhibitors and PMA pretreatment on STAT1α translocation. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with RO318220 (1 μM), GÖ6976 (1 μM) (a) or LY333531 (5 μM) (b) for 6 h before the preparation of nuclear extracts. STAT1α translocation to the nuclei was determined by Western blotting using specific antibody against STAT1α. (c) Cells were pretreated with PMA (1 μM) or vehicle (DMSO) for 6 h before stimulation with LPS (10 ng ml^{-1}). Cells were further incubated for 6 h before the nuclear extracts were prepared. STAT1α translocation to the nuclei was determined by Western blotting using specific antibody against STAT1α. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n=3$). *$P<0.05$, **$P<0.01$ as compared with cells treated with LPS only.

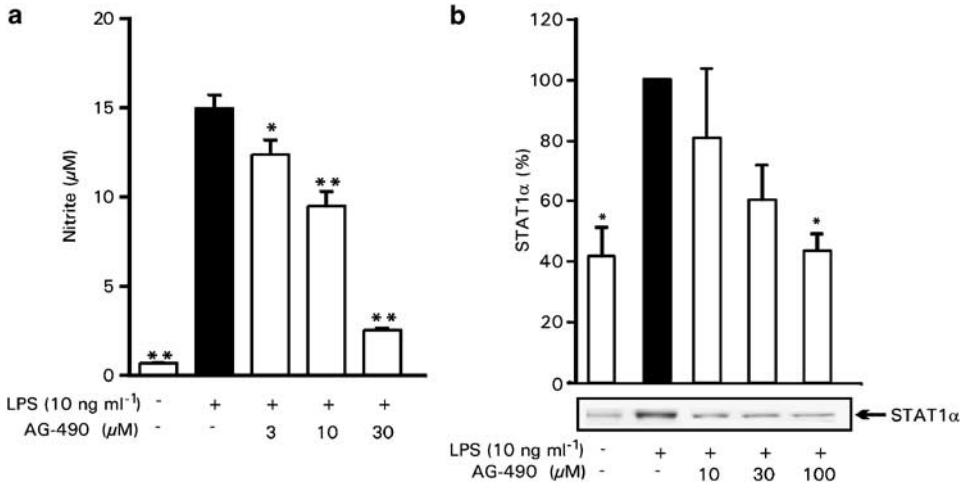

Figure 9 Effect of JAK-2 inhibitor AG-490 on LPS-induced NO production in J774 cells. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of AG-490 (a). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n=6$). (b) The effect of JAK-2 inhibitor AG-490 on STAT1α nuclear translocation. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of AG-490 for 4 h before the preparation of nuclear extracts. STAT1α translocation to the nucleus was determined by Western blotting using specific antibody against STAT1α. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n=3$). *$P<0.05$, **$P<0.01$ as compared with cells treated with LPS only.

et al., 1993), GÖ6976 is more selective to cPKC isoenzymes (Martiny-Baron *et al*., 1993) and LY333531 is a selective inhibitor of PKCβ (Jirousek *et al*., 1996). HBDDE, which is a relatively selective inhibitor of PKCα and PKCγ (Kashiwada *et al*., 1994), did not have any effect on LPS-induced NO production. These results suggest that PKC, probably its isoenzymes PKCβI and PKCβII, mediate the LPS-induced upregulation of iNOS expression and NO production in macrophages.

The results may be complicated by the fact that RO318220 and GÖ6976 have been reported to inhibit some other kinases in addition to PKC (Davies *et al*., 2000). Therefore, we also studied the effects of phorbol esters PMA and PDD on LPS-induced iNOS expression and NO production. Phorbol esters are known to activate cPKC isoenzymes (Castagna *et al*., 1982), whereas a longer pretreatment with a higher concentration of phorbol esters have been shown to result in the downregulation of cPKCs, presumably due to proteolysis (Huang *et al*., 1989; Liu & Heckman, 1998). The bidirectional effect of PMA and PDD on cPKCs was seen also in the present study. PMA and PDD at 100 nM concentration enhanced cPKC activation, while 6 h pretreatment with 1 μM concentration of PMA or PDD suppressed cPKC expression. When used at cPKC-activating concentrations, PMA and PDD enhanced iNOS expression and NO production. In contrast, cPKC downregulation due to 6 h pretreatment with PMA or PDD (1 μM) resulted in the suppression of iNOS expression and NO production, similarly as inhibition of cPKCs by pharmacological means. These results further support the role of cPKC isoenzymes in the regulation of iNOS expression and NO production in macrophages.

To determine the mechanisms by which the regulation by PKC is mediated, we studied the effects of PKC inhibitors RO318220 and GÖ6976, and PMA on LPS-induced iNOS mRNA expression and mRNA stability. Our results show that inhibition of cPKCs does not effect the stability of LPS-induced iNOS mRNA. The effect of PKC isoenzymes is rather at the level of iNOS transcription, since PKC inhibitors decreased the expression of iNOS mRNA already at the early time points after addition of LPS.

NF-κB and STAT1 appear to be important transcription factors for the enhanced iNOS gene expression in macrophages exposed to LPS (Lowenstein *et al*., 1993; Chartrain *et al*., 1994; Xie *et al*., 1994; Gao *et al*., 1998; Jacobs & Ignarro, 2001). In the present study, all three PKC inhibitors used (RO318220, GÖ6976 and LY333531) inhibited STAT1 activation as measured by translocation of the transcription factor from the cytosol to nuclei as did pretreatment with 1 μM PMA. In contrast, none of the treatments did inhibit NF-κB activation as measured by EMSA. These results suggest that the regulation of LPS-induced iNOS protein expression by PKC is NF-κB-independent and is most likely mediated through the activation of transcription factor STAT1.

RO318220 and GÖ6976 have earlier been reported to inhibit LPS and IFN-γ-induced NO production in macrophages (Paul *et al*., 1997; Chen, B.C. *et al*., 1998; Chen *et al*., 1998a). In addition, PKCδ and PKCη have been suggested to regulate NO production and iNOS expression in activated macrophages and some other cell types (Chen *et al*., 1998a, b; Carpenter *et al*., 2001; Banan *et al*., 2003; Pham *et al*., 2003). The present study extends the earlier data by providing a cellular mechanism for the inhibitory effects of RO318220 and GÖ6976 on LPS-induced iNOS expression and NO production in macrophages. Our results show that inhibition of cPKC isoenzymes results in the suppression of STAT1 activation, which may well explain the inhibitory effect on iNOS expression and NO production. In addition, we were able to show that LY333531, a selective inhibitor of PKCβ, also suppressed STAT1 activation and iNOS expression, supporting the role of PKCβ in the regulation of iNOS expression in activated macrophages.

In conclusion, the present results show that inhibition of cPKC isoenzymes, especially PKCβ, inhibits the LPS-induced activation of transcription factor STAT1, iNOS expression

and NO production in macrophages. The results suggest that inhibition of cPKC isoenzymes provides a way to prevent iNOS protein expression and NO production in inflammation, offering a novel target for the development of anti-inflammatory drugs.

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Inflammation Research

Inhibition of protein kinase C βII downregulates tristetraprolin expression in activated macrophages

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Abstract. *Objective and design:* Tristetraprolin (TTP) is a 3'-UTR-binding protein known to destabilize mRNAs of TNFα and some other cytokines and to act as an anti-inflammatory factor. The aim of this study was to investigate the role of classical protein kinase C isoenzymes (cPKC) in the regulation of TTP expression in activated macrophages.
Materials and methods: The expression of TTP in J774 macrophages was induced by a combination of LPS and phorbol myristate acetate (PMA). The effects of cPKC inhibitors and the effects of cPKC activation and downregulation by PMA on TTP protein and mRNA expression were determined by Western blotting and quantitative RT-PCR, respectively. Also, the effect of PKCβII inhibitor CGP53353 on the activation of transcription factors AP-2, NF-κB, EGR1 and Sp1 was assessed.
Results: cPKC inhibitors RO318220, GÖ6976, LY333531 and CGP53353 inhibited LPS and PMA –induced expression of TTP protein and mRNA. Similar effects were obtained when cPKC isoenzymes were downregulated by PMA. In addition, CGP53353 decreased the activation of transcription factor AP-2.
Conclusions: The results suggest that cPKCs, most likely PKCβII, upregulate TTP expression in activated macrophages. This regulation is possibly mediated through the activation of transcription factor AP-2, and serves as an additional mechanism how PKCβ regulates the inflammatory process.

Keywords: tristetraprolin – PKC – AP-2 – macrophages

Introduction

Tristetraprolin (TTP, also known as TIS11, Nup475, Zfp36 or G0S24) is a member of a small family of tandem CCCH zinc finger proteins. TTP was originally described as a gene that was induced rapidly and transiently by the stimulation of fibroblasts, and it is now known to be a factor that can bind to AU-rich elements within 3'-UTR regions of mRNA resulting in deadenylation and destabilization of mRNA and eventually to decreased production of the target protein. TTP expression can be induced by serum, insulin, tumor promoting phorbol esters and inflammatory stimuli, such as bacterial products [1–3]. mRNAs of inflammatory genes, e.g. tumor necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), cyclooxygenase 2 (COX-2), interleukin-2 (IL-2), interleukin-3 (IL-3) and interleukin-6 (IL-6) have been reported to be destabilized by TTP [4–9]. In contrast to the preceding inflammatory genes, the degradation of human inducible nitric oxide synthase (iNOS) mRNA has been reported to be inhibited by TTP by an indirect mechanism [10]. TTP deficient mice develop a profound inflammatory syndrome with erosive arthritis, autoimmunity and myeloid hyperplasia and this has been reported to be mainly due to excessive production of TNF-α [4, 11]. Altogether, TTP seems to have a role as an anti-inflammatory or arthritis suppressor gene.

The function of TTP has been studied extensively. However, less is known about the regulation of the expression of TTP itself. Mitogen-activated protein kinases (MAPK) p38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1 and 2 (ERK1/2), and MAPK-activated protein kinase 2 (MAPKAPK2 or MK2) have been reported to regulate expression and subcellular localization of TTP [12–17]. In addition, TTP itself has been reported to regulate its own mRNA expression [18, 19]. Tumor promoter phorbol myristate acetate (PMA) has been shown to increase TTP mRNA levels or enhance TTP promoter activity in astrocytes and hepatoma cells [20, 21]. However, the signalling pathways responsible for PMA-mediated TTP induction are not known in detail.

One of the cellular targets of PMA is protein kinase C (PKC). PKC is a family of serine-threonine protein kinase isoenzymes and represents one of the major signal transduction systems in inflammation [22, 23]. Differences in the

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structure of the isoenzymes and substrate requirements have led to the division of the PKC isoenzymes into three groups: classical (cPKC), novel (nPKC) and atypical (aPKC) isoenzymes. Classical isoenzymes (α, γ and the splice variants βI and βII) are activated by phosphatidylserine, Ca^{2+} and diacylglycerol (DAG). These isoenzymes are also targets of tumor promoting phorbol esters, such as PMA, a surrogate of DAG. Novel isoenzymes (δ, ε, η and θ) are activated by phosphatidylserine, DAG and phorbol esters, but not by Ca^{2+}. The third group of PKC isoenzymes, atypical isoenzymes (ζ and ι/λ), are activated by phosphatidylserine only [24, 25].

cPKC isoenzymes may regulate the expression of inflammatory genes by regulating the activation of transcription factors NF-κB and AP-1 [26, 27]. PKC may also be involved in the regulation of mRNA stability of inflammatory genes if it regulates the expression of TTP. Because the expression of TTP is induced by PMA, we hypothesized that PKC is involved in the regulation of the expression of TTP. In that case PKC could have a dual effect on the regulation of inflammatory genes i.e. regulating the activity of transcription factors important for inflammatory genes and regulating the stability of mRNA of inflammatory genes.

The aim of the present study was to investigate the hypothesis that cPKC isoenzymes α, βI or βII regulate TTP expression in activated macrophages. The results suggest that cPKC isoenzymes, probably PKCβII, are involved in the upregulation of TTP expression in activated macrophages and that this regulation is mediated through the activation of transcription factor AP-2.

Materials and Methods

Materials

Reagents were purchased as follows: RO318220 and LY333531 were from Alexis Biochemicals (Lausen, Switzerland) and GÖ6976 was from Calbiochem (La Jolla, CA, USA). All other reagents were from Sigma Chemical Co (St. Louis, MO, USA).

Cell Culture

J774 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 ºC in 5 % CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Cambrex BioScience, Verviers, Belgium) supplemented with 10 % heat-inactivated foetal bovine serum (Cambrex BioScience), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Gibco, Paisley, UK) and harvested with trypsin-EDTA (Gibco). Cells were seeded on 24-well plates for RT-PCR and on 6-well plates for Western blot analysis and cells were then grown for 72 h to confluence prior to the experiments. Toxicity of the tested compounds was ruled out by measuring cell viability using XTT test (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions.

Preparation of Cell Lysates

Cells were incubated with the tested compounds for 30 min prior to the addition of lipopolysaccharide (LPS; 10 ng/ml) or LPS (10 ng/ml) and PMA (100 nM). At indicated time points, 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 EDTA, 50 mM NaCl, 1 % Triton X-100, 0.5 mM phenylmethylsulfonyl 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 for 15 min on ice, cell lysates were centrifuged (13 400 x *g*, 4 ºC, 10 min), supernatants were collected and stored in SDS sample buffer in –20 ºC. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [28].

Preparation of Nuclear Extracts

J774 cells were seeded on 10-cm dishes and grown for 72 h to confluence before the experiments. Cells were incubated with the tested compounds for 30 min prior to the addition of LPS (10 ng/ml) or the combination of LPS (10 ng/ml) and PMA (100 nM). Thereafter, at indicated time points, 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 phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA). After incubation for 10 min on ice, cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 ºC, 21 000 x *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 $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 25 µg/ml 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 ºC, 21 000 x *g* for 2 min. Protein contents of the nuclear extracts were measured by the Coomassie blue method [28].

Western Blotting

Prior to Western blotting, proteins were boiled for 10 min with SDS sample buffer and 30 µg of protein was used per lane on 10 % (for AP-2, EGR1, NF-κB and Sp1 Western blotting) or 12 % (for TTP Western blotting) SDS-polyacrylamide gel and transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences UK, Ltd, Little Chalfont, Buckinghamshire, UK). After transfer the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1 % Tween-20) containing 5 % non-fat dry milk or 5 % BSA for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 ºC overnight. The membrane was washed with TBS/T and incubated with the secondary antibody in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using Super Signal® West Pico and Dura chemiluminescent substrate (Pierce, Rockford, USA) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). Mouse monoclonal Sp1 antibody, rabbit polyclonal actin, lamin A/C, and AP-2 antibodies and goat anti-rabbit polyclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and goat anti-mouse antibody was from Pierce Biotechnology. NF-κB p65 and EGR1 antibodies were from Cell Signaling Technology (Danvers, MA, USA). The mouse TTP antibody was a kind gift from Dr Perry Blackshear (NIEHS, Research Triangle Park, NC, USA).

PKC translocation studies

The activation and downregulation of PKC isoenzymes was studied by determining their translocation from cell cytosol to cell membrane by Western blotting. Cytosolic and membrane fractions were prepared as previously described [29]. For Western blotting 20 µg of protein was used per lane on 10 % SDS-polyacrylamide gel and transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences UK). After transfer the membrane was blocked in TBS/T containing 5 % non-fat dry milk for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 ºC overnight. The membrane was

washed with TBS/T and incubated with the secondary antibody in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using Super Signal® West Dura and Femto (Pierce) chemiluminescent substrate and FluorChem™ 8800 imaging system (Alpha Innotech Corporation). Mouse monoclonal PKCα antibody and rabbit polyclonal PKCβI and PKCβII antibodies were from Santa Cruz Biotechnology, Inc.

RNA Extraction and Quantitative Real-Time PCR (RT-PCR)

Cell homogenization, RNA extraction, reverse-transcription of RNA to cDNA and PCR reactions were performed as previously described [29]. The mouse TTP primers and probe were designed using Primer Express® Software (Applied Biosystems, Foster City, CA, USA) and were as follows: 5'-CTCAGAAAGCGGGCGTTGT-3', 5'-GATTGGCTTGGCGAAGTTCA-3' (forward and reverse mouse TTP primer respectively, both 300 nM) and 5'-CCAAGTGCCAGTTTGCTCACGGC-3' (mouse TTP probe, containing 6-FAM (6-carboxy-fluoroscein) as 5'-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3'-quencer, 200 nM). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control gene [30].

Statistics

Results are expressed as mean ± standard error of mean (S.E.M.). Statistical significance of the results was calculated by analysis of variances supported by Dunnett multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

The Expression of TTP is Induced by LPS or Combination of LPS and PMA in J774 Macrophages

Resting J774 macrophages expressed very low levels of TTP protein. To study the induction of TTP after different stimuli we treated the cells with bacterial endotoxin LPS (10 ng/ml), phorbol ester PMA (100 nM) or with their combination (Fig. 1a) and determined the expression of TTP protein by Western blotting. PMA alone had a minor effect on TTP protein expression in J774 macrophages, whereas LPS clearly induced TTP protein expression and this was further enhanced by addition of PMA. Following treatment with LPS alone or with LPS and PMA, the expression of TTP protein peaked at 9 hours (Fig. 1b).

Similar expression pattern was seen when TTP mRNA was measured by quantitative real-time RT-PCR. PMA alone was not sufficient to induce significant TTP mRNA expression. LPS alone or in combination with PMA induced TTP mRNA expression. As found in TTP protein expression, treatment with a combination of LPS and PMA further enhanced TTP mRNA expression as compared to treatment with LPS alone (see Fig. 3b and 4b below).

Activation of Classical PKC Isoenzymes Enhances the Expression of TTP Protein and mRNA

We have previously shown that classical PKC isoenzymes PKCα, PKCβI and PKCβII are expressed in J774 macrophages and that they are responsive to PMA treatment [29].

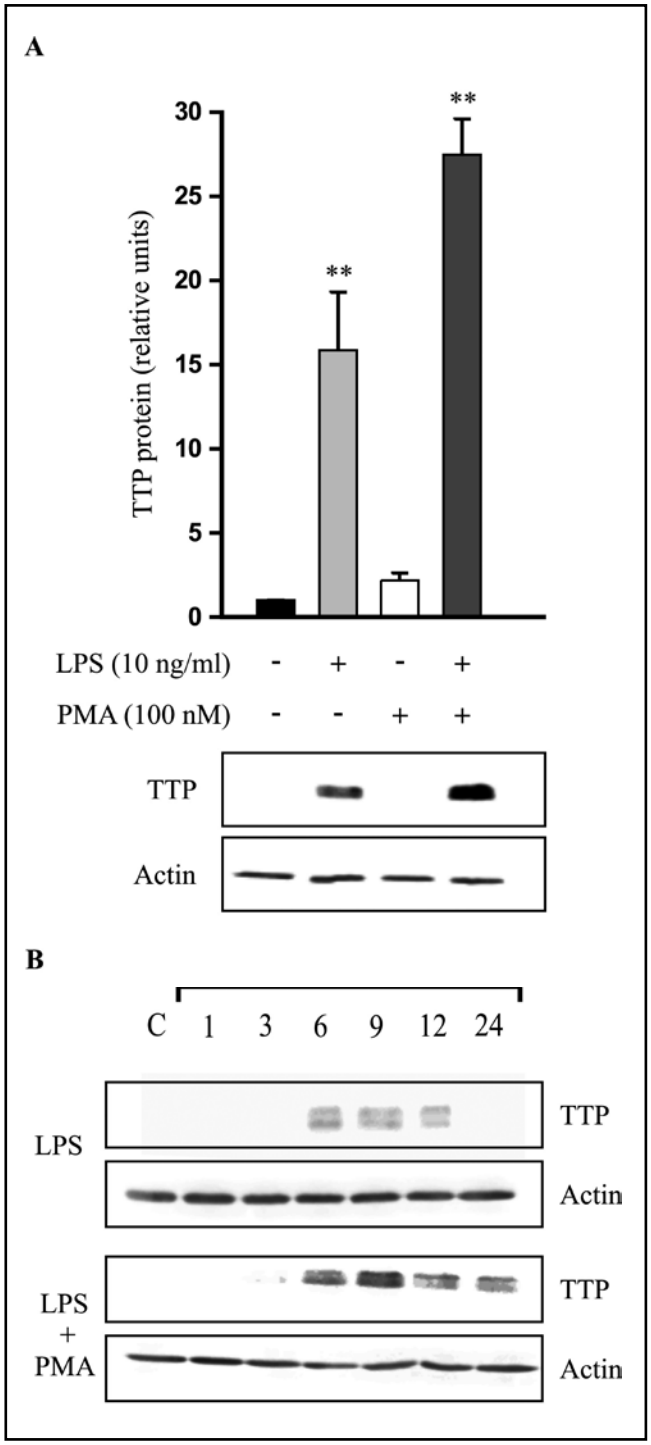

Fig. 1. Effect of LPS and PMA on TTP expression in J774 murine macrophages. A: J774 cells were stimulated with LPS (10 ng/ml), PMA (100 nM) or the combination of LPS and PMA for 9 h. Incubations were terminated and immunoblots were run using antibody against TTP. Chemiluminescent signal was quantified as described under *Materials and Methods*. Values are mean ± S.E.M. (n = 3). **$P < 0.01$ as compared to unstimulated cells. B: J774 cells were stimulated with LPS (10 ng/ml) alone or with the combination of LPS (10 ng/ml) and PMA (100 nM) for 1, 3, 6, 9, 12 or 24 h. At indicated time points, incubations were terminated and immunoblots were run using antibody against TTP. Actin was detected as a loading control. Chemiluminescent signal was quantified as described under *Materials and Methods*. Each experiment is a representative of three others with similar results.

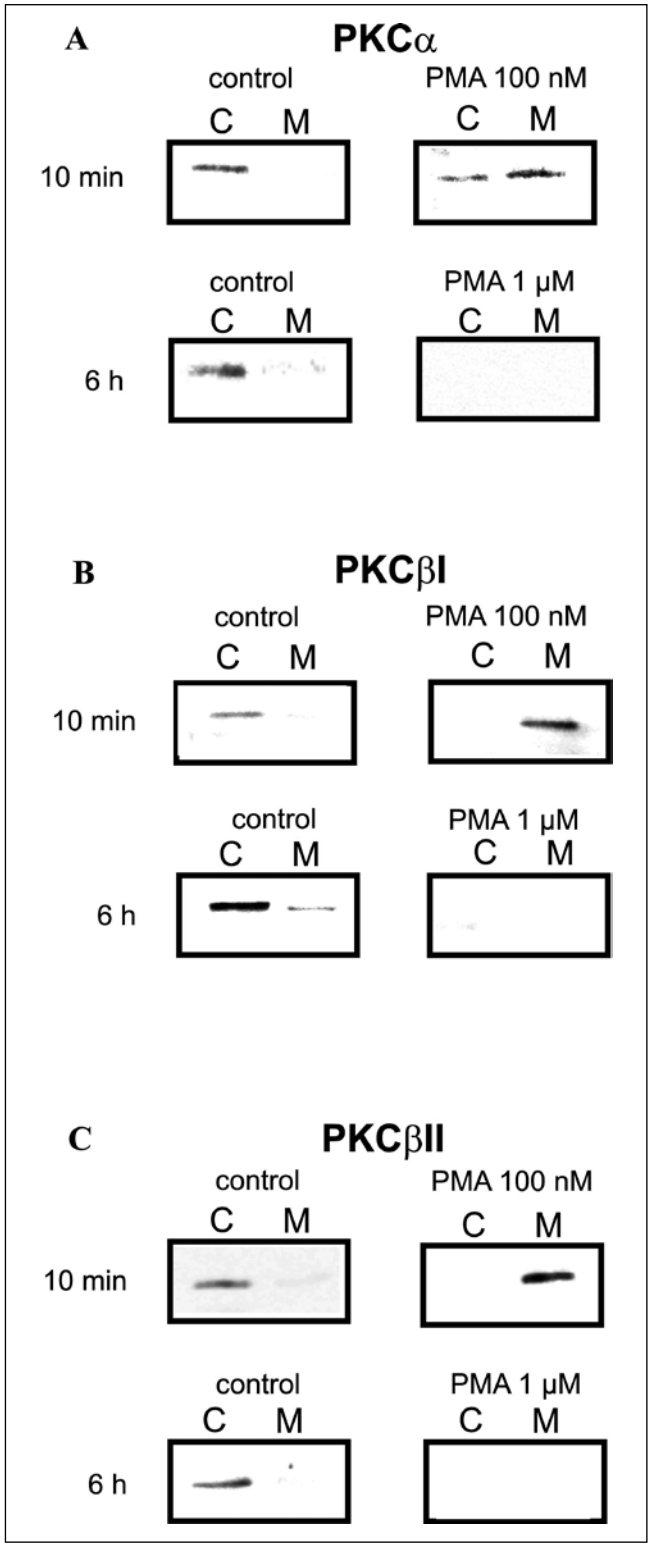

Fig. 2. Activation and downregulation of cPKC isoenzymes after PMA treatment in J774 macrophages. Cells were treated with 100 nM PMA for 10 min or with 1 μM PMA for 6 h. The incubations were terminated at indicated time points and cytosolic and membrane fractions were prepared as described in *Materials and Methods.* The expression of PKC isoenzymes α (A), βI (B) and βII (C) was assessed by Western blotting using isoenzyme specific antibodies. Each experiment is a representative of three others with similar results. C = cytosolic fraction, M = membrane fraction.

Phorbol esters, such as PMA, are known activators of PKC isoenzymes. However, when cells are exposed to higher concentrations of phorbol esters for prolonged times, an almost complete downregulation of PKC isoenzymes can be detected as a result of proteolysis [31, 32]. This dual effect of phorbol esters on cPKC activity was seen in J774 macrophages

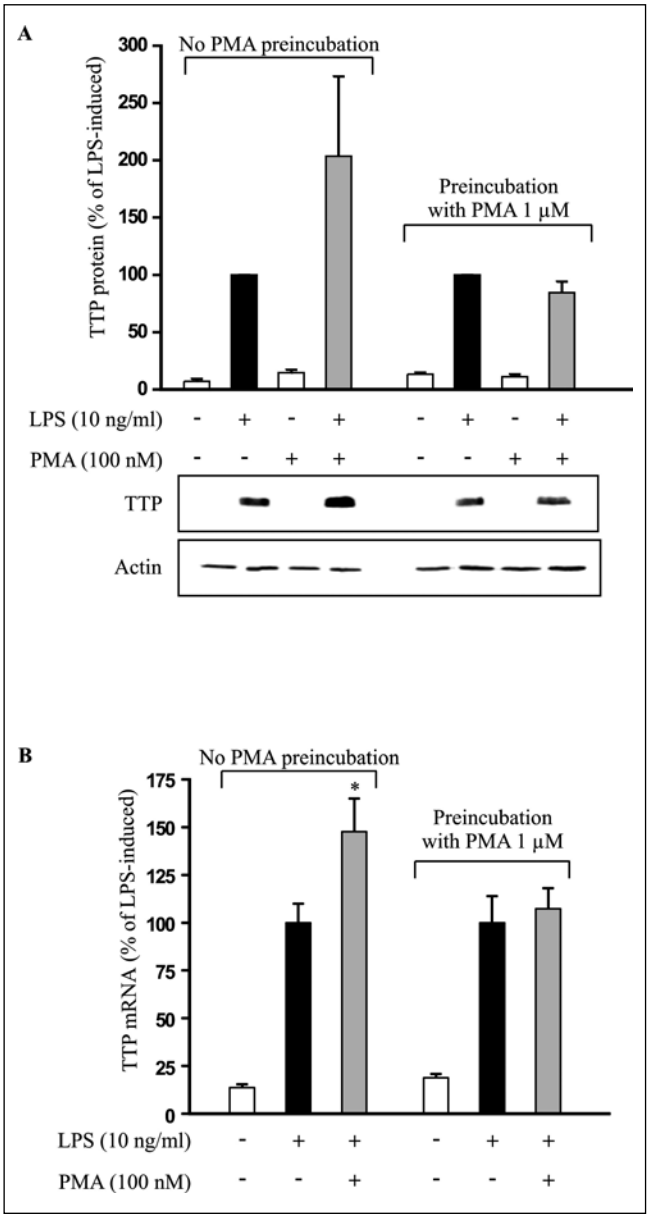

Fig. 3. The effects of PKC activation and downregulation by PMA on TTP protein and TTP mRNA expression in J774 macrophages. J774 cells were treated with vehicle (= no preincubation) or with 1 μM PMA for 24 h before the addition of LPS (10 ng/ml), PMA (100 nM) or the combination of LPS (10 ng/ml) and PMA (100 nM). A: After 9 h, incubations were terminated and immunoblots were run using antibody against TTP. Actin was detected as a loading control. Chemiluminescent signal was quantified as described under *Materials and Methods*. Values are mean ± S.E.M. (n = 3). B: After 6 h the incubations were terminated and extracted total RNA was subjected to real time PCR. TTP mRNA levels were normalized against GAPDH mRNA. Values are mean ± S.E.M. (n=3). *P <0.05 as compared to cells treated with LPS only.

(Fig 2a–c): when cells were treated with 100 nM PMA for 10 min all three PKC isoenzymes (α, βI and βII) were activated as measured by their translocation from cytosol to the membrane fraction by Western blotting and when the cells were incubated with 1 μM PMA for 6 h all three PKC isoenzymes were downregulated.

Since PMA (100 nM), when used together with LPS (10 ng/ml), induced both TTP protein and mRNA expression, we wanted to study if classical PKC isoenzymes are involved in the regulation of TTP expression. First we studied the effects of PKC activation and downregulation by PMA on TTP protein expression by Western blotting (Fig. 3a). Cells were preincubated for 24 h with vehicle or 1 μM PMA to downregulate cPKC expression. Thereafter LPS (10 ng/ml), PMA (100 nM) or their combination was added into the culture, and cells were harvested for TTP determination after 9 h incubation. The treatment with the combination of LPS and PMA enhanced TTP protein expression as compared to treatment with LPS alone. Downregulation of cPKC isoenzymes by 24 h preincubation with 1 μM PMA did not alter the TTP expression induced by LPS alone, but abolished the enhancing effect of 100 nM PMA on LPS-induced TTP protein expression.

We also studied the effects of PKC activation and downregulation by PMA on TTP mRNA expression (Fig. 3b). As in TTP protein studies, treatment with the combination of LPS and PMA enhanced TTP mRNA expression as compared to treatment with LPS alone. Preincubation for 24 h with 1 μM PMA abolished the enhancing effect of PMA (100 nM) on LPS-induced TTP mRNA expression as measured by quantitative RT-PCR. Similarly to the protein data, the 24 h preincubation with 1 μM PMA did not affect the TTP mRNA levels induced by LPS alone. These results suggest that the activation of PKC isoenzymes α, βI and / or βII are likely to take part in the regulation of LPS + PMA-induced TTP protein and TTP mRNA expression.

PKC Inhibitors Downregulated TTP Protein and mRNA Expression

To further determine whether PKC activation is involved in the regulation of TTP expression we studied the expression of TTP protein and TTP mRNA in the presence of PKC inhibitors. First we studied the effects of two known PKC inhibitors, RO318220, an inhibitor of PKC isoenzymes α, β, γ and ε, and GÖ6976, a selective inhibitor of classical PKC isoenzymes [33,34]. PKC inhibitors were added to the cell culture 30 min prior to the addition of the combination of LPS (10 ng/ml) and PMA (100 nM). Both inhibitors inhibited LPS + PMA-induced TTP protein expression (Fig. 4a) as measured by Western blotting. In addition, when studied at the concentration of 1 μM, both PKC inhibitors reduced the expression of TTP mRNA (Fig. 4b) as measured by RT-PCR.

Selective PKCβ Inhibitors Downregulated the Expression of TTP Protein and mRNA

In further studies we wanted to determine which of the three classical PKC isoenzymes expressed in J774 macrophages

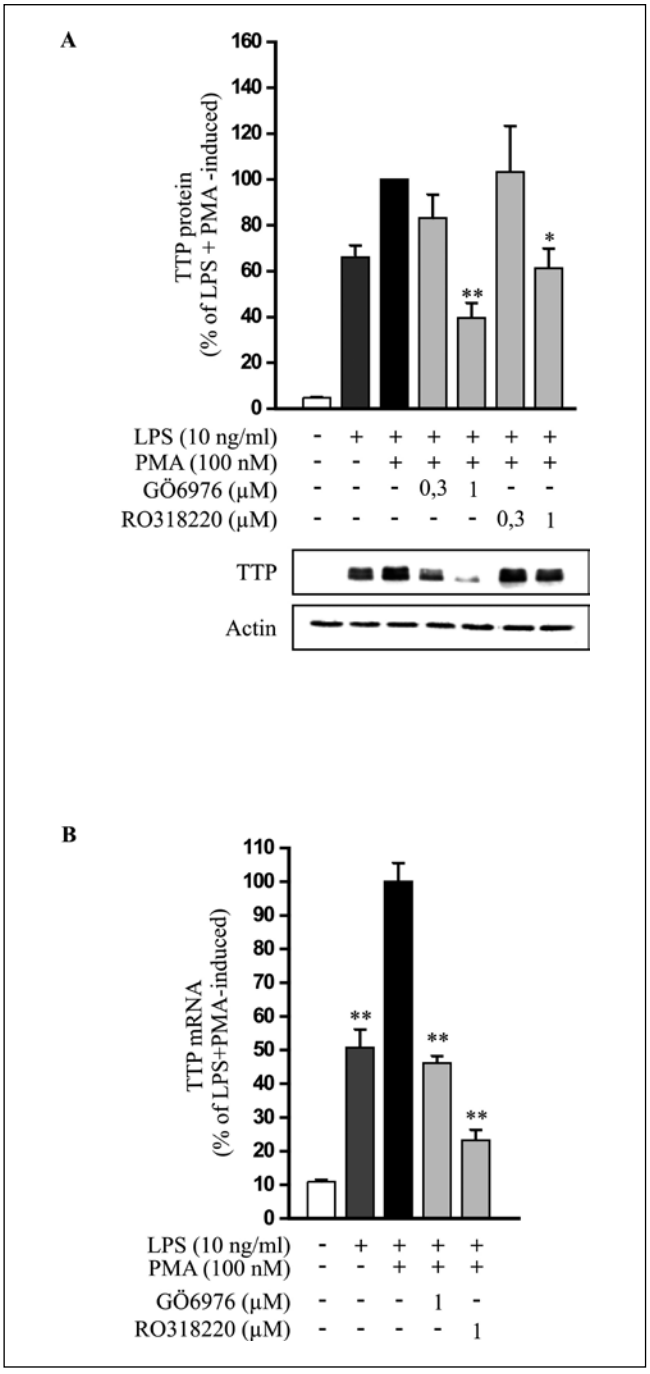

Fig. 4. Effects of PKC inhibitors on LPS + PMA -induced TTP protein and TTP mRNA expression in J774 cells. A: J774 cells were treated with increasing concentrations of RO318220, GÖ6976 or vehicle (DMSO) for 30 min before the addition of combination of LPS (10 ng/ml) and PMA (100 nM) or cells were treated with LPS (10 ng/ml) alone. After 9 h, incubations were terminated and immunoblots were run using antibody against TTP. Actin was detected as a loading control. Chemiluminescent signal was quantified as described under *Materials and Methods*. Values are mean ± S.E.M. (n = 3). B: J774 cells were treated with RO318220 (1 μM), GÖ6976 (1 μM) or vehicle (DMSO) for 30 min before the addition of combination of LPS (10 ng/ml) and PMA (100 nM) or cells were treated with LPS (10 ng/ml) alone. After 6 h the incubations were terminated and extracted total RNA was subjected to real time PCR. TTP mRNA levels were normalized against GAPDH mRNA. Values are mean ± S.E.M. (n = 3). *P <0.05, **P <0.01 as compared to cells treated with LPS + PMA.

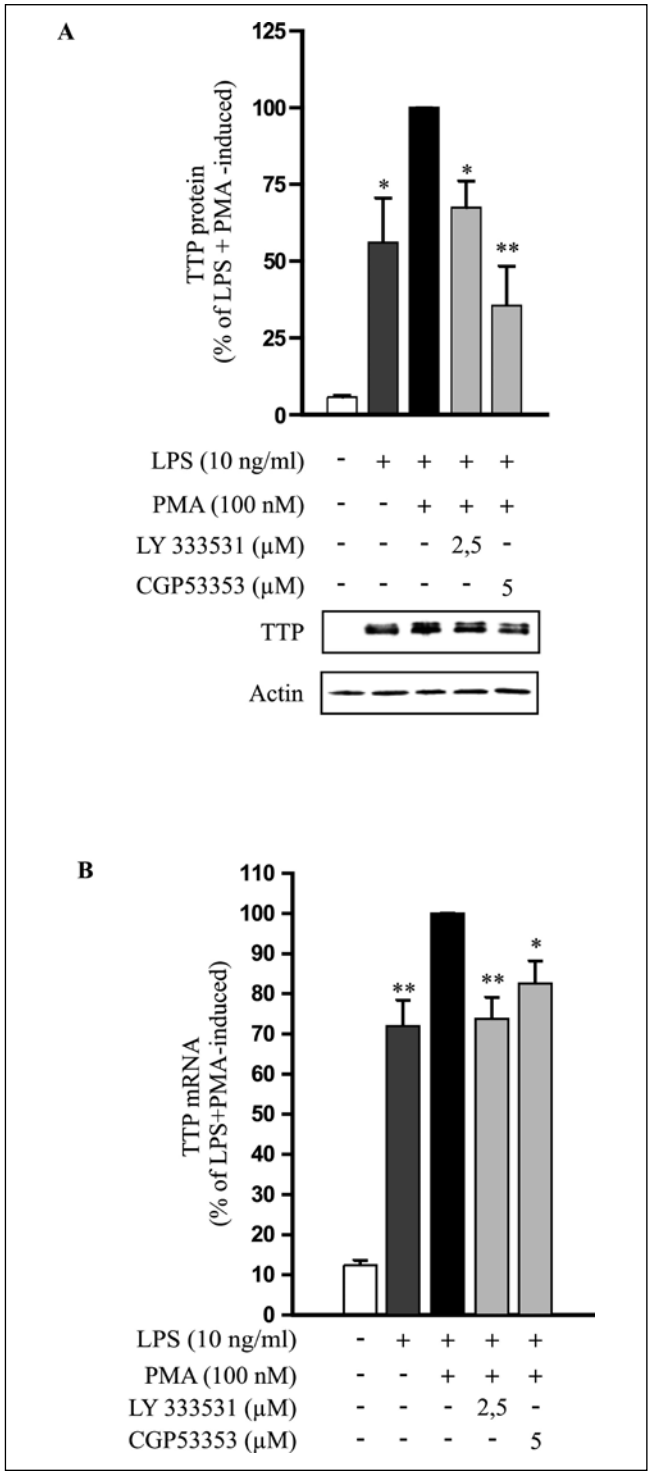

Fig. 5. Effects of PKCβ inhibitors on LPS + PMA -induced TTP protein and TTP mRNA expression in J774 cells. J774 cells treated with LY333531 (2.5 μM), CGP53353 (5 μM) or vehicle (DMSO) for 30 min before the addition of combination of LPS (10 ng/ml) and PMA (100 nM) or cells were treated with LPS (10 ng/ml) alone. A: After 9 h, incubations were terminated and immunoblots were run using antibody against TTP. Actin was detected as a loading control. Chemiluminescent signal was quantified as described under *Materials and Methods*. Values are mean ± S.E.M. (n = 3). B: After 6 h, incubations were terminated and extracted total RNA was subjected to real time PCR. TTP mRNA levels were normalized against GAPDH mRNA. Values are mean ± S.E.M. (n=9). *P <0.05, **P <0.01 as compared to cells treated with LPS + PMA.

(α, βI and βII) are involved in the regulation of LPS + PMA-induced TTP expression. Both of the PKC inhibitors used, RO318220 and GÖ6976, are reported to inhibit PKCα, PKCβI, PKCβII and PKCγ. Since PKCγ is not expressed in the cell line used [29], and an inhibitor of PKCα, HBDDE [35], did not inhibit LPS + PMA-induced TTP mRNA expression (data not shown), we concentrated our study on the effects of PKCβ isoenzymes. We investigated the effects of two PKCβ inhibitors with different selectivity. LY333531 is an inhibitor of both PKCβI and PKCβII [36] and CGP53353 is a selective PKCβII inhibitor [37]. LY333531 (2.5 μM) and CGP53353 (5 μM) were added to the cell culture 30 min prior to the addition of the combination of LPS (10 ng/ml) and PMA (100 nM). Both PKCβ inhibitors reduced LPS + PMA-induced TTP protein and TTP mRNA expression (Fig. 5 a–b). Since both inhibitors are reported to inhibit PKCβII, we continued our studies with PKCβII selective inhibitor CGP53353.

CGP53353 did not Alter TTP mRNA Half-life as Measured by RT-PCR

In order to study the mechanisms involved in the regulation of LPS + PMA-induced expression by PKC, we studied the effects of CGP53353 on the half-life of TTP mRNA by actin-

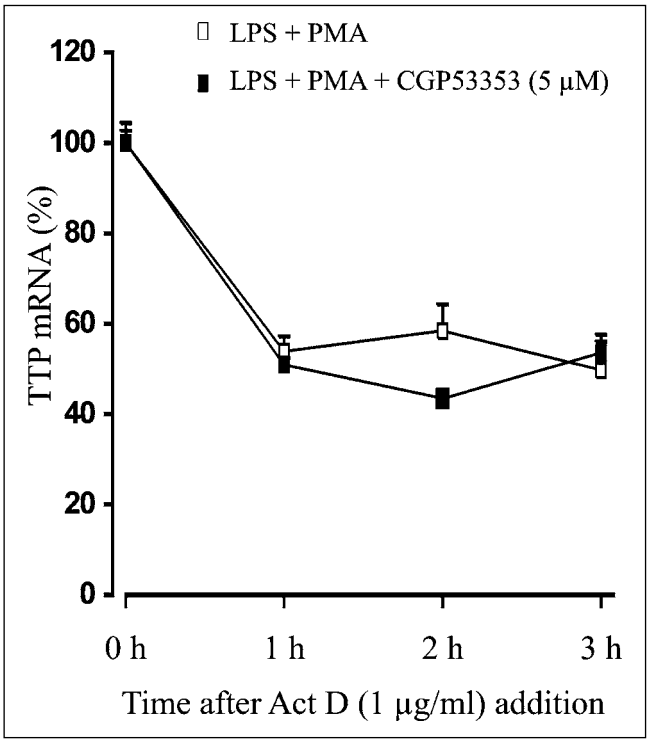

Fig. 6. The effect of PKCβII inhibitor CGP53353 on TTP mRNA degradation. Cells were treated with CGP53353 (5 μM) or vehicle (DMSO) for 30 min prior to the addition of LPS (10 ng/ml) and PMA (100 nM). After 4 h actinomycin D (1 μg/ml) was added to the cell culture. Incubations were terminated at indicated time points after actinomycin D and extracted total RNA was subjected to real time PCR. TTP mRNA levels were normalized against GAPDH mRNA. Values are mean ± S.E.M. (n = 3).

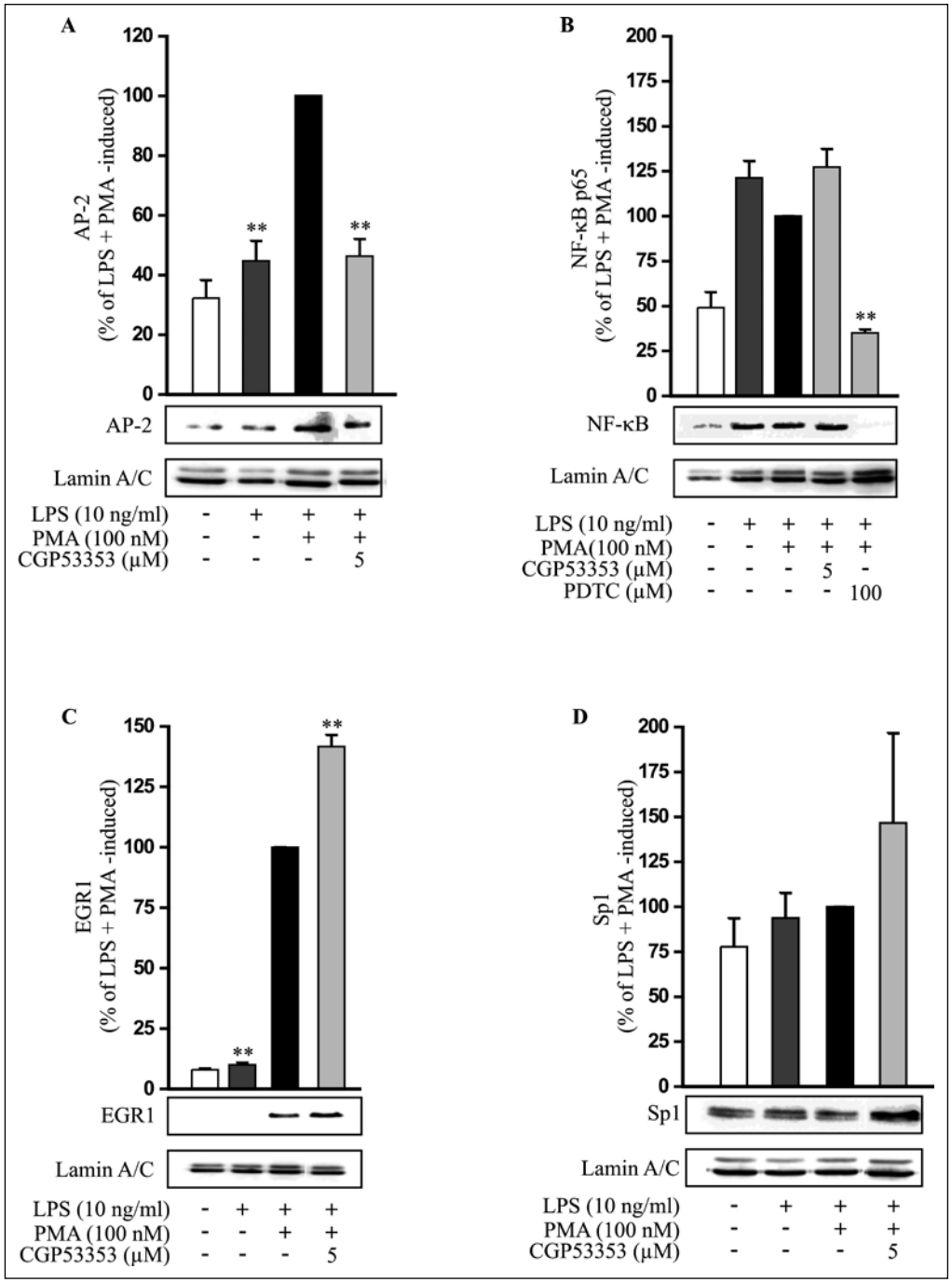

Fig. 7. The effect of PKCβII inhibitor CGP53353 on nuclear translocation of transcription factors AP-2 (A), NF-κB p65 (B), EGR1 (C), and Sp1 (D). J774 cells were treated with 5 μM CGP53353 for 30 min prior to the stimulation with the combination of LPS (10 ng/ml) and PMA (100 nM) or cells were stimulated with LPS (10 ng/ml) alone. After 30 min (A) or 1 h (B-D) the incubations were terminated and nuclear extracts were prepared. Transcription factor translocation to the nuclei was determined by Western blotting using specific antibodies. Lamin A/C was detected as a loading control. Chemiluminescent signal was quantified as described under *Materials and Methods*. PDTC 100 μM was used as a inhibitor of NF-κB. Values are mean ± S.E.M. (n = 3). **P <0.01 as compared to cells treated with LPS + PMA.

omycin D assay (Fig. 6). CGP53353 (5 μM) was added to the cell culture 30 min prior to the addition of the combination of LPS (10 ng/ml) and PMA (100 nM). After 4 h incubation, transcription inhibitor actinomycin D (1 μg/ml) was added into the cell culture. Cells were then further incubated for 0, 1, 2 or 3 h before total RNA was extracted. As shown in Figure 6, CGP53353 had no effect on TTP mRNA half-life. These results suggest that the suppressive effect of PKCβ inhibitors on LPS + PMA-induced TTP mRNA levels is mediated at the level of TTP transcription and not at the level of TTP mRNA stability.

CGP53353 Inhibited the Activation of Transcription Factor AP-2 as Measured by Its Nuclear Translocation by Western Blotting

To study the possibility that the effects of CGP53353 on TTP expression are mediated through its effects on transcription factors, we measured the activation of NF-κB, AP-2, EGR1 and Sp1 after CGP53353 treatment. These transcription factors have been reported to have at least one binding site in the TTP gene promoter or intron region [2,38,39]. CGP53353 (5 μM) was added to the cell culture 30 min prior to the

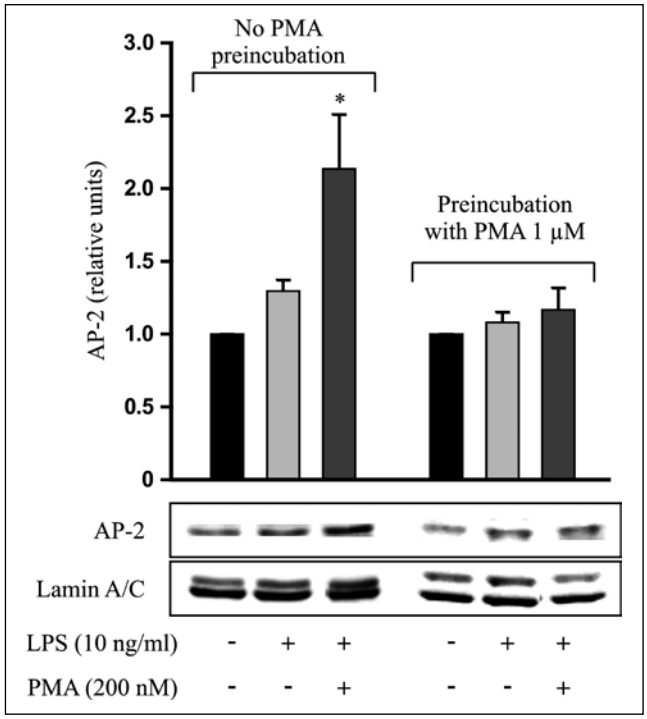

Fig. 8. The effect of PKC downregulation by PMA preincubation on nuclear translocation of transcription factor AP-2. J774 cells were treated with vehicle (= no preincubation) or with 1 μM PMA for 16 h before the addition of LPS (10 ng/ml) or the combination of LPS (10 ng/ml) and PMA (200 nM). After 30 min the incubations were terminated and nuclear extracts were prepared. AP-2 translocation to the nuclei was determined by Western blotting. Lamin A/C was detected as a loading control. Chemiluminescent signal was quantified as described under *Materials and Methods*. Values are mean ± S.E.M. (n = 3–4). **P <0.01 as compared to unstimulated cells.

addition of the combination of LPS (10 ng/ml) and PMA (100 nM). After 30 min (AP-2) or 1 h (NF-κB p65, EGR1 and Sp1) incubation with LPS + PMA cytosolic and nuclear proteins were extracted and the activation of transcription factors was measured as their translocation from the cytosol to the nuclei by Western blot. CGP53353 reduced the activation of AP-2 (Fig. 7a), but it had no effect on the activation of NF-κB, EGR1 and Sp1 (Fig. 7b–d).

To further confirm the role of cPKC in the regulation of activation of AP-2, we studied the LPS + PMA-induced activation of AP-2 also in cells in which cPKC isoenzymes had been downregulated by preincubation with PMA. First, cells were preincubated for 16 h with vehicle or 1 μM PMA to downregulate cPKC expression. Thereafter LPS (10 ng/ml) or LPS together with PMA (200 nM) was added into the culture, and after 30 min, cytosolic and nuclear proteins were extracted and the activation of AP-2 was measured as its translocation from the cytosol to the nuclei by Western blot. The treatment with the combination of LPS and PMA enhanced nuclear translocation of AP-2 in control cells. However, downregulation of cPKCs by preincubation with PMA totally abolished the LPS + PMA-induced activation of AP-2 (Fig 8).

This data suggest that the effects of classical PKC isoenzymes, especially PKCβII, on LPS + PMA –induced expression of TTP may be mediated, at least in part, through the activation of transcription factor AP-2 in J774 macrophages.

Discussion

In the present study we have shown that inhibition of classical PKC isoenzymes downregulates the expression of TTP in activated macrophages and that the downregulation is mainly due to the inhibition of PKCβII. In addition, we have presented a possible mechanism for the regulation of TTP expression by PKCβII. Our data suggest that the regulation may be mediated, at least partly, through activation of transcription factor AP-2 by PKCβII.

PKC is known to regulate cellular functions in an isoenzyme-specific manner [22]. PKC isoenzymes exhibit different patterns of tissue expression, PKCα, βI, βII, δ, ε and ζ being the most ubiquitous isoenzymes [32]. In addition, differences in subcellular localization as well as in activator and substrate requirements imply to functional diversity among isoenzymes. We have previously studied the expression of cPKC isoenzymes in murine J774 macrophages, and shown that PKCα, βI and βII, but not PKCγ, are expressed in these cells [29]. Classical isoenzymes PKCβI and PKCβII are generated by alternative splicing from a single gene locus and they are expressed as major PKC isoforms in a variety of tissues [40].

cPKCs may upregulate inflammatory genes by activating inflammatory transcription factors NF-κB and AP-1 [22, 26, 27]. The present results suggest also an inhibitory effect of mRNA stability by upregulating TTP and thus serving as a possible anti-inflammatory feed-back mechanism.

TTP is a factor that binds to AU-rich elements within certain mRNAs and causes destabilization of those mRNAs. AU-rich elements (AREs) are critical *cis*-acting elements in the 3'UTRs of many cytokine and transcription factor mRNAs and they are targets for *trans*-acting proteins that regulate mRNA stability and translation. At least 14 distinct proteins have been identified as ARE binding proteins. However, only few of them have been shown to regulate mRNA stability [41]. These include AU-rich element RNA-binding protein 1 (AUF1), HuR and TTP [4, 42, 43]. TTP was originally described as an immediately activated early gene that was induced in response to stimuli such as insulin and other growth factors, activators of innate immunity, and phorbol esters [1–3]. Today, TTP is the best-understood member of a small family of tandem CCCH zinc-finger proteins, the other members being TIS11b (also known as ZFP36L1, cMG1, ERF1, BRF1 and Berg36) and TIS11d (also known as ZFP36L2, ERF2 and BRF2) [44]. TTP has been shown to regulate the stability of mRNAs of inflammatory genes TNF-α, GM-CSF, IL-2, IL-3, IL-6, COX-2 and iNOS [4–10]. In addition, Jalonen et al. [45] have recently provided data suggesting that IL-12, MIP2 (a homologue to human IL-8) and MIP3α are novel inflammatory cytokine targets for TTP-mediated mRNA decay.

Today, the role of TTP in inflammation has become more evident. Mice deficient in TTP appear normal at birth, but in a few months time they develop a severe inflammatory syndrome including polyarticular arthritis, myeloid hyperplasia, autoimmunity and cachexia [4, 11]. Repeated injections of

a specific antibody against mouse TNF-α completely prevented the development of TTP deficiency phenotype, indicating that this severe inflammatory syndrome is mainly due to excessive production of TNF-α, which for one part leads to excessive production of GM-CSF and other inflammatory factors [44, 46]. This has made TTP a potential target for the development of new anti-inflammatory drugs. Overall, in the treatment of inflammatory diseases, shutting down inflammatory genes, which are already activated, is a more reasonable target of drug treatment compared to trying to affect the early events in the transcriptional regulation of the inflammatory factors. The experimental data so far suggest that factors that enhance TTP expression are likely to have anti-inflammatory effects through reduced expression of inflammatory genes.

In the present study, we investigated the role of classical PKC isoenzymes in the regulation of TTP expression in J774 macrophages. In our previous study [29] we showed which cPKC isoenzymes are expressed in J774 cells. In this study, we demonstrated the conditions where cPKC isoenzymes are activated and downregulated after PMA treatment and used this knowledge to investigate their role in the regulation of TTP expression. Treatment of J774 macrophages with a combination of LPS and PMA enhanced TTP protein and mRNA expression and the effect was greater than that caused by LPS alone. Downregulation of cPKCs by a preincubation with a higher concentration of PMA abolished the effect of LPS + PMA –treatment indicating that cPKCs take part in the regulation of TTP expression. However, PMA preincubation did not alter the levels of TTP expression when the cells were treated with LPS alone.

The role of different cPKC isoenzymes in the regulation of TTP expression was studied using PKC inhibitors with distinct isoenzyme selectivity. RO318220 has been reported to inhibit PKC isoenzymes α, β, γ and ε [34] and GÖ6976 all classical isoenzymes [33]. More selective inhibitors were LY333531, inhibitor of PKCβI and βII [36], and CGP53353, inhibitor of PKCβII [37]. All four inhibitors reduced LPS + PMA –induced TTP protein and mRNA expression, while HBDDE, an inhibitor of PKCα [35], had no inhibiting effect on TTP mRNA expression. These results indicate that cPKCs, most likely PKCβII, take part in the regulation of TTP expression in murine macrophages. In general, the selectivity of kinase inhibitors is not always very clear. Both RO318220 and GÖ6976 have been reported to inhibit also mitogen and stress activated protein kinase 1 (MSK1) [47], which has been reported to take part in the regulation of TTP expression [16]. Although our results with cells in which classical PKCs had been downregulated by PMA preincubation support our finding with PKC inhibitors, the role of MSK1 can not be ruled out. Furthermore, PKCβ inhibitors seemed to have a slightly bigger effect on TTP protein than TTP mRNA levels. This may signify that PKCβ isoenzymes take part also in the regulation of TTP protein stability. This possibility can not be excluded based on current data but requires further studies.

In order to determine the mechanisms by which cPKCs regulate TTP expression we first investigated the degradation of TTP mRNA. Our results show that PKCβII inhibitor CGP53353 did not affect the stability of TTP mRNA as measured by actinomycin D assay, indicating that the effect of PKCβII on TTP expression is rather at the level of TTP transcription.

Binding sites for NF-κB, Sp1, EGR1 and AP-2 are found in the TTP gene promoter or intron [2, 38, 39, 48]. Therefore we studied the effects of PKCβII inhibitor on the activation of those four transcription factors. PKCβII inhibitor CGP53353 had no effect on the activation of NF-κB, Sp1 or EGR1 but it reduced the activation of transcription factor AP-2 as determined by nuclear translocation of AP-2 by Western blotting. In general, activation of AP-2 transcription factor family is involved in the regulation of cell growth, differentiation and inflammation associated genes [49]. AP-2 is known to be induced by e.g. cytokines, phorbol esters and cyclic AMP (cAMP) [49–51] and it has been shown to be involved in PKC signaling pathways [52–57].

The expression of TTP has not been studied as intensively as the functions of TTP in cellular systems. However, MAP kinases p38, JNK, ERK1/2 and MK2 as well as cAMP-enhancing compounds and TTP itself have been reported to regulate the expression of TTP, either at the level of subcellular localization, transcription, mRNA stability or protein degradation [12–14, 16–19, 58–60]. Recently, dexamethasone as well as cinnamon extract, cinnamon polyphenols and green tea were reported to modulate TTP expression [15, 61, 62]. Our current results extend the previous data by providing evidence that classical PKC isoenzymes, most probably PKCβII, upregulate TTP expression in activated macrophages and that the regulation may be, at least partly, mediated through the activation of AP-2 transcription factor.

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List of abbrevations: AP-2: activator protein-2, EGR1: early growth response gene-1, LPS: lipopolysaccharide, NF-κB: nuclear factor kappa B, PKC: protein kinase C, PMA: phorbol 12-myristate 13-acetate, Sp1: specificity protein 1, TNF-α: tumor necrosis factor α, TTP: tristetraprolin

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Immunopharmacology and Inflammation

Inhibition of protein kinase Cδ reduces tristetraprolin expression by destabilizing its mRNA in activated macrophages

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ABSTRACT

Tristetraprolin (TTP) binds to AU-rich elements within the mRNAs of several inflammatory genes and causes destabilization of the target mRNAs. The protein kinase C (PKC) pathway represents a major signalling system in inflammation and PKCδ is one of the key isoenzymes in the regulation of inflammatory processes. In the present study, we investigated the role of PKCδ in the regulation of the expression of tristetraprolin in activated macrophages by using the PKCδ inhibitor, rottlerin, and by downregulating PKCδ expression by using PKCδ siRNA. TTP protein and mRNA expression were measured by Western blotting and quantitative RT-PCR, respectively. TTP and TNFα mRNA decays were studied by the actinomycin D assay. In addition, we measured nuclear translocation of transcription factors believed to be important for TTP transcription, i.e. NF-κB, AP-2, SP1 and EGR1. Downregulation of PKCδ by siRNA decreased significantly TTP expression in activated macrophages. Rottlerin also decreased TTP expression in wild type cells but not in cells in which PKCδ had been downregulated by siRNA. Rottlerin decreased TTP mRNA half-life as measured by actinomycin D assay but it did not affect the nuclear translocation of transcription factors NF-κB, Sp1, AP-2 or EGR1 (which have been shown to be involved in TTP transcription). In addition, rottlerin reduced the decay of TNFα mRNA, which is an important target of TTP. The results suggest that PKCδ up-regulates the expression of TTP by stabilizing its mRNA which may serve as a feed-back loop to regulate the inflammatory response.

1. Introduction

Tristetraprolin (TTP) was found more than 15 years ago as an early response gene which was induced in response to growth factors, inflammatory stimuli and phorbol esters (DuBois et al., 1990; Lai et al., 1990; Varnum et al., 1989). TTP is known to bind to AU-rich elements within the mRNAs of its target genes, leading to destabilization of the mRNA and reduced production of the protein (Blackshear, 2002). In a knock-out mouse model, the lack of TTP has been shown to increase the stability of tumour necrosis factor-α (TNFα) (Carballo et al., 1998; Lai et al., 1999), granulocyte-macrophage colony-stimulating factor (Carballo et al., 2000), interleukin-2 (Ogilvie et al., 2005), and interferon-γ (Ogilvie et al., 2009) mRNAs, and the production of those proinflammatory cytokines. TTP has also been reported to regulate the expression of inflammatory genes cyclooxygenase 2, interleukin-3, interleukin-6, inducible nitric oxide synthase, interleukin-12, macrophage inflammatory protein-2 (MIP2, a homologue to human interleukin-8), and macrophage inflammatory protein-3α (Stoecklin et al., 2000; Sawaoka et al., 2003; Fechir et al., 2005; Jalonen et al., 2006). TTP knock-out mice appear normal at birth, but within a few months develop a characteristic phenotype that includes loss of body weight and body fat, severe polyarticular erosive arthritis and myeloid hyperplasia. This has been reported to be a consequence of excessive production of TNFα, since treatment of TTP-deficient mice with neutralizing antibodies to TNFα could prevent the development of this phenotype (Taylor et al., 1996). TTP can be considered as an anti-inflammatory or arthritis suppressive gene.

Protein kinase C (PKC) is a family of serine/threonine kinases that play a crucial role in cellular signal transduction. Members of PKC family are divided into three groups based on their structure and cofactor requirements. Conventional isoenzymes (α, βI, βII and γ) require calcium, diacylglycerol and phosphatidylserine for activation. Novel isoenzymes (δ, ε, η and θ) are calcium independent, but require diacylglycerol and phosphatidylserine for activation. The third group, atypical isoenzymes (ζ and ι/λ), are independent of both calcium and diacylglycerol for activation (Hofmann, 2004; Spitaler and Cantrell, 2004). PKC isoenzymes regulate various inflammatory responses and they may be considered as key signalling mediators in inflammation (Lee et al., 2008).

PKCδ was first cloned from rat brain cDNA library and was biochemically characterized in the late eighties by Ono et al. (1987). PKCδ is the most extensively studied isoenzyme of the novel PKC

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group. It is ubiquitously expressed in various cells and tissues and it has been shown to participate in the regulation of cell growth and differentiation, apoptosis and immune function (Lee et al., 2008; Kikkawa et al., 2002; Steinberg, 2004). The objective of the present study was to investigate the role of PKCδ in the regulation of the expression of TTP in activated macrophages. That was based on the previous findings that phorbol esters (which, in addition to their other effects, also activate PKCδ) enhance the expression of TTP (Varnum et al., 1989; Leppänen et al., 2008). Our results suggest that PKCδ is involved in the regulation of TTP expression by influencing the stability of TTP mRNA.

2. Materials and methods

2.1. Materials

Reagents were purchased as follows: mouse monoclonal Sp1 antibody, rabbit polyclonal β-actin, lamin A/C, PKCδ and AP-2 antibodies and goat anti-rabbit HRP-conjugated polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and goat anti-mouse HRP-conjugated antibody was from Pierce Biotechnology (Rockford, IL, USA). NF-κB p65 and EGR1 antibodies were from Cell Signalling Technology (Danvers, MA, USA). PKCδ siRNA, non-targeting control siRNA and DharmaFECT 4 transfection reagent were from Dharmacon (Lafayette, CO, USA). All other reagents were from Sigma Chemical Co (St. Louis, MO, USA). The mouse TTP antibody was a kind gift from Dr Perry Blackshear (NIEHS, Research Triangle Park, NC, USA). Rottlerin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and the purity was ≥85% by HPLC according to the manufacturer. Rottlerin was dissolved in DMSO to obtain 10 mM stock solution which was stored at −20 °C. It was further dissolved in culture medium with a ratio of 1:1000 immediately prior to every experiment. The DMSO concentration in cell culture experiments was adjusted to 0.1% in controls and in all rottlerin concentrations used, and it was tested not to affect TTP expression.

2.2. Cell culture

J774 macrophages (American Type Culture Collection, VA, USA) 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 foetal bovine serum (Cambrex BioScience), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Gibco, Paisley, UK) and harvested with trypsin-EDTA (Gibco). Cells were seeded on 24-well plates for RT-PCR and on 6-well plates for Western blot analysis and cells were then grown for 72 h to confluence prior to the experiments. The toxicity of rottlerin was evaluated by measuring cell viability using the XTT test (Roche Diagnostics, Mannheim, Germany) and by the Trypan blue method.

2.3. Preparation of cell lysates for TTP protein expression studies

Cells were incubated with rottlerin for 30 min prior to the addition of lipopolysaccharide (LPS, 10 ng/ml) and phorbol 12-myristate 13-acetate (PMA, 100 nM). At the indicated time points, 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 ethylenediamine tetraacetate (EDTA), 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodiumorthovanadate (Na_2VO_4), 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM sodium fluoride (NaF), 2 mM sodium pyrophosphate and 10 μM n-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, the cell lysates were centrifuged (13 400×*g*, 4 °C, 10 min), supernatants were collected and stored in SDS sample buffer at −20 °C. An aliquot of the supernatant was used to determine the protein concentration with the Coomassie blue method (Bradford, 1976).

2.4. Preparation of nuclear extracts for transcription factor translocation studies

J774 cells were seeded on 10-cm dishes and grown for 72 h to confluence before the experiments. Cells were incubated with rottlerin for 30 min prior to the addition of LPS (10 ng/ml) and PMA (100 nM). Thereafter, at the indicated time points, cells were rapidly washed with ice-cold PBS and nuclear extracts were prepared as described previously (Sareila et al., 2006). The protein contents of the nuclear extracts were measured by the Coomassie blue method (Bradford, 1976).

2.5. Preparation of soluble and particulate fractions for PKCδ translocation studies

At the indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold buffer A (20 mM Tris-base, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.5 mM PMSF, 2 mM Na_2VO_4, 10 mg/ml leupeptin, 25 mg/ml aprotinin and 1.25 mM NaF). After incubation for 15 min on ice, the lysates were centrifuged at 100 000×*g* for 1 h at 4 °C, supernatants were collected and marked as the soluble fraction. Pellets were resuspended in cold lysis buffer B (20 mM Tris-base, pH 7.4, 10 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5 mM PMSF, 2 mM Na_2VO_4, 10 mg/ml leupeptin, 25 mg/ml aprotinin, 1.25 mM NaF and 10 mM n-octyl-β-D-glucopyranoside). After incubation for 2 h on ice, the lysates were centrifuged at 100 000×*g* for 1 h at 4 °C, supernatants were collected and marked as the particulate fraction. An aliquot of the supernatant was used to determine the protein concentration by the Coomassie blue method (Bradford, 1976).

2.6. Western blotting

Prior to Western blotting, proteins were boiled for 10 min with SDS sample buffer and 20 μg (PKCδ studies) or 30 μg (TTP and transcription factor studies) of protein was loaded per lane on 10% or 12% SDS-polyacrylamide gel and transferred to the Hybond ECL™ nitrocellulose membrane (Amersham Biosciences UK, Ltd, 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% non-fat dry milk or 5% bovine serum albumin for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 °C overnight. The membrane was washed with TBS/T and incubated with the secondary antibody in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using Super Signal® West Pico and Dura chemiluminescent substrates (Pierce, Rockford, USA) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA).

2.7. RNA extraction and quantitative real-time PCR (RT-PCR)

Cell homogenization, RNA extraction, reverse-transcription of RNA to cDNA and PCR reactions were performed as previously described (Salonen et al., 2006). Primers and probes (Table 1) for TTP, TNFα and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, used as a control gene) were designed using Primer Express® Software (Applied Biosystems, Foster City, CA, USA) and supplied by Metabion (Martinsried, Germany).

2.8. Downregulation of PKCδ expression by siRNA

PKCδ expression was downregulated using Dharmacon ON-TARGET plus siRNA oligos. J774 macrophages were grown to ~80% confluence and transfected with PKCδ siRNA or non-targeting control siRNA using DharmaFECT 4 transfection reagent according to the manufacturer's instructions (Dharmacon). Cells were incubated for

Table 1
Primer and probe sequences.

Gene	Oligonucleotide	Sequence 5′→3′
Murine TTP	Forward primer	CTCAGAAAGCGGGCGTTGT
	Reverse primer	GATTGGCTTGGCGAAGTTCA
	Probe	CCAAGTGCCAGTTTGCTCACGGC
Murine TNFα	Forward primer	AATGGCCTCCCTCTCATCAGTT
	Reverse primer	TCCTCCACTTGGTGGTTTGC
	Probe	CTCAAAATTCGAGTGACAAGCCTGTAGCCC
Murine GAPDH	Forward primer	GCATGGCCTTCCGTGTTC
	Reverse primer	GATGTCATCATACTTGGCAGGTTT
	Probe	TCGTGGATCTGACGTGCCGCC

6 h with siRNA duplexes and the transfection reagent. Subsequently, the medium was replaced with fresh culture medium, and cells were further incubated for 42 h before the extraction of proteins. PKCδ protein levels were determined by Western blotting.

When the effect of the downregulation of PKCδ by siRNA on the expression of TTP was determined, cells were transfected as described above. After 42 h incubation with fresh culture medium, cells were incubated with rottlerin for 30 min prior to the addition of LPS (10 ng/ml) and PMA (100 nM). Incubations were terminated after 9 h and proteins were extracted. TTP protein levels were determined by Western blotting.

2.9. Statistics

Results are expressed as mean + standard error of mean (S.E.M.). Statistical significance of the results was calculated by analysis of variances supplemented with Dunnett multiple comparisons test. Differences were considered significant at $P<0.05$.

3. Results

3.1. PKCδ is expressed in J774 macrophages, and activated by PMA

PKCδ was expressed in resting J774 macrophages. When the cells were incubated with a known activator of PKC, phorbol ester PMA (100 nM) for 10 min, PKCδ was activated. This was detected as translocation of the enzyme from the cytosol to the membrane fraction (Fig. 1).

3.2. Rottlerin downregulates TTP protein and mRNA expression

Rottlerin (which inhibits PKCδ; Gschwendt et al., 1994) was added into the culture medium 30 min prior to activating the cells with LPS (10 ng/ml) and PMA (100 nM), and TTP protein and mRNA were measured by Western blotting and quantitative RT-PCR, respectively. As shown in Fig. 2A, rottlerin inhibited TTP protein expression in a dose-dependent manner. Rottlerin (10 μM) also decreased TTP mRNA expression (Fig. 2B).

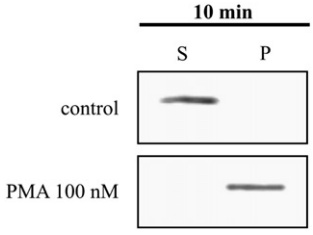

Fig. 1. Expression of PKCδ in J774 macrophages, and its activation after treatment with the phorbol ester, PMA. Cells were incubated in the absence (control) or in the presence of 100 nM PMA for 10 min and then cytosolic and membrane fractions were prepared. PKCδ levels were assessed by Western blotting using an isoenzyme specific antibody. The panel shown is representative of the three others with similar results. S = soluble fraction, P = particulate fraction.

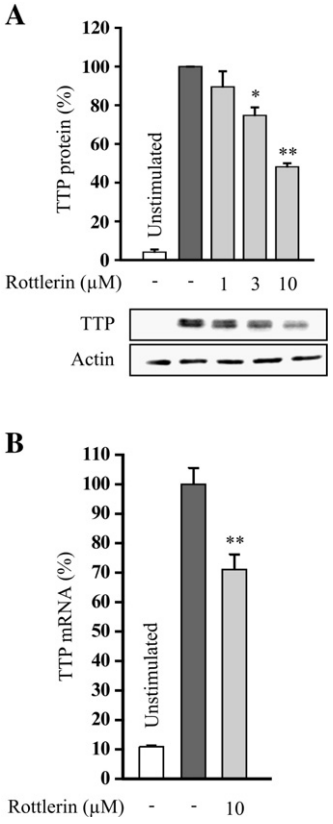

Fig. 2. Effects of rottlerin on TTP protein and mRNA expression in J774 cells. A. Cells were treated with increasing concentrations of rottlerin for 30 min before the cells were activated by adding LPS (10 ng/ml) and PMA (100 nM). After 9 h, the incubations were terminated and immunoblots were run using an antibody against TTP. Actin was detected as a loading control. Values are mean + S.E.M. ($n=4$). *$P<0.05$, **$P<0.01$ as compared with cells treated with LPS + PMA. B. Cells were treated with rottlerin (10 μM) or vehicle (DMSO) for 30 min before the addition of the stimuli. After 6 h, the incubations were terminated and extracted total RNA was subjected to real-time PCR. TTP mRNA levels were normalized against GAPDH mRNA. Open column represents unstimulated cells, and dark grey column represents LPS + PMA treated cells in the absence of rottlerin. Values are mean + S.E.M. ($n=3$). **$P<0.01$ as compared with cells treated with LPS + PMA.

3.3. Downregulation of PKCδ by siRNA reduces TTP expression

Cells were treated with siRNA targeting PKCδ, and there was a significant reduction in PKCδ expression (Fig. 3A). Under these conditions, TTP expression was clearly lower than in cells treated with the negative control siRNA (which did not alter PKCδ expression) (Fig. 3B). Moreover, when cells were treated with PKCδ targeting siRNA, rottlerin had no effect on TTP expression. These results indicate that inhibition and downregulation of PKCδ reduce TTP expression in activated macrophages and that the effects of rottlerin are mediated through inhibition of PKCδ. In the subsequent studies, we investigated the mechanisms by which rottlerin inhibits TTP expression.

3.4. Rottlerin does not affect the nuclear translocation of transcription factors NF-κB, Sp1, AP-2 and EGR1

Transcription factors nuclear factor kappa B (NF-κB), specificity protein 1 (Sp1), activator protein-2 (AP-2) and early growth response gene-1 (EGR1) have been shown to have at least one binding site on the TTP gene promoter or intron region and are considered to be important for TTP transcription (DuBois et al., 1990; Lai et al., 1995; Lai et al., 1998). Activation of these transcription factors was studied by measuring their translocation from the cell cytosol to its nucleus by Western blot. Rottlerin (10 μM) was added to the cell culture 30 min

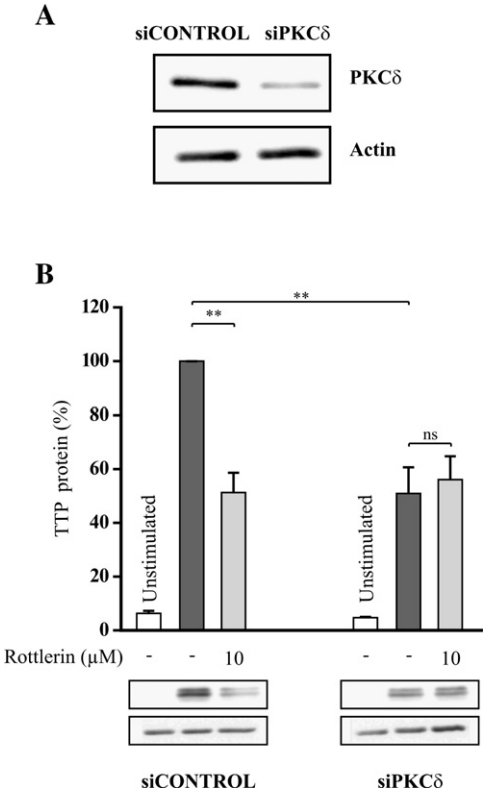

Fig. 3. Downregulation of PKCδ by siRNA and its effect on TTP expression. J774 cells were transiently transfected with siRNAs using DharmaFECT 4 transfection reagent. Treatment with non-targeting siRNA (siCONTROL) was used as control. A. Protein extracts were prepared and PKCδ expression was determined using Western blotting. The gels shown are representative of the five others with similar results. B. Cells were treated with rottlerin (10 μM) for 30 min before the cells were activated by adding a combination of LPS (10 ng/ml) and PMA (100 nM). After 9 h, the incubations were terminated and immunoblots were run using an antibody against TTP. Actin was detected as a loading control. Open column represents unstimulated cells, and dark grey column represents LPS + PMA treated cells in the absence of rottlerin. Values are mean + S.E.M. ($n = 6$), $^{**}P < 0.01$.

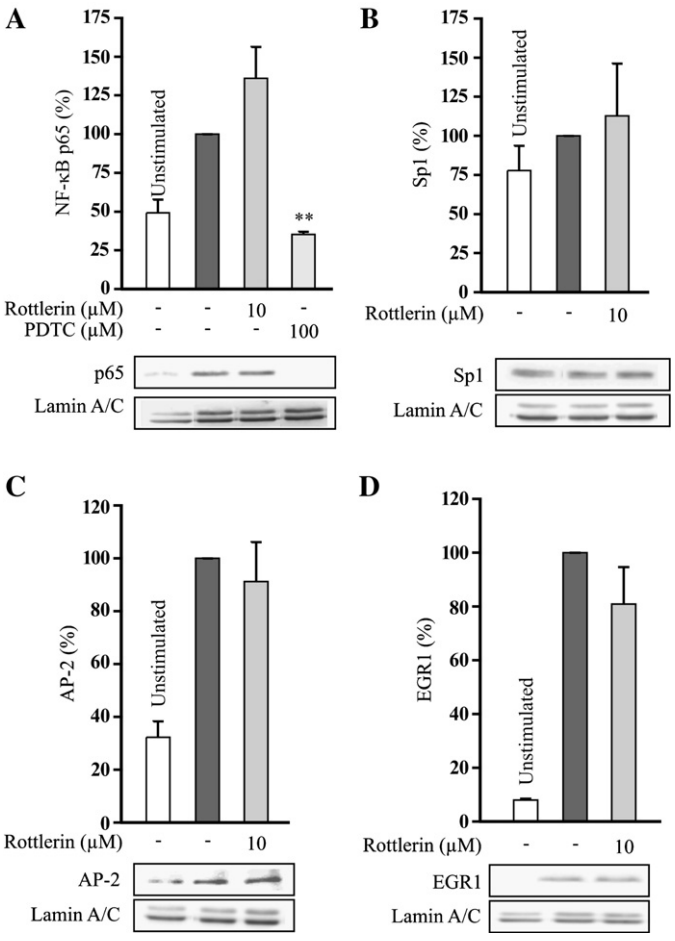

Fig. 4. The effect of rottlerin on nuclear translocation of transcription factors NF-κB p65 (A), Sp1 (B), AP-2 (C), and EGR1 (D). J774 cells were treated with 10 μM rottlerin for 30 min prior to the stimulation with LPS (10 ng/ml) and PMA (100 nM). After 30 min (C) or 1 h (A, B, D), the incubations were terminated and nuclear extracts were prepared. Transcription factor levels in nuclear extracts were determined by Western blotting using specific antibodies. Lamin A/C was used as a loading control. Open column represents unstimulated cells, and dark grey column represents LPS + PMA treated cells in the absence of rottlerin. PDTC 100 μM was used as a control inhibitor of NF-κB. Values are mean + S.E.M. ($n = 3$–4). $^{**}P < 0.01$ as compared with cells treated with LPS + PMA.

prior to the cells being activated by a combination of LPS and PMA. After 30 min (AP-2) or 1 h (NF-κB p65, Sp1 and EGR1) incubation, the transcription factors present in the nuclear extracts were measured. Nuclear levels of NF-κB (p65), AP-2 and EGR1 were increased in the stimulated cells, but rottlerin had no effect on the nuclear translocation of any of the four transcription factors studied (Fig. 4A–D).

3.5. Rottlerin decreases TTP mRNA half-life

In the next experiments, we investigated the effects of rottlerin on TTP mRNA decay by means of the actinomycin D assay. Rottlerin (10 μM) was added to the cell culture 30 min prior to the cells being activated with LPS and PMA. After 4 h incubation, actinomycin D (1 μg/ml), an inhibitor of transcription, was added to the cell culture. The cells were then further incubated for 0.5, 1 or 2 h before total RNA was extracted. Real-time RT-PCR analysis showed that when transcription was blocked with actinomycin D, the TTP mRNA levels were decreasing faster in rottlerin treated cells (Fig. 5). The half-life of TTP mRNA was reduced from 2 to 1 h by rottlerin.

3.6. Rottlerin affects the stability of TNFα mRNA

In macrophages derived from TTP-deficient mice, lack of TTP has been shown to increase the half-life of TNFα mRNA (Carballo et al.,

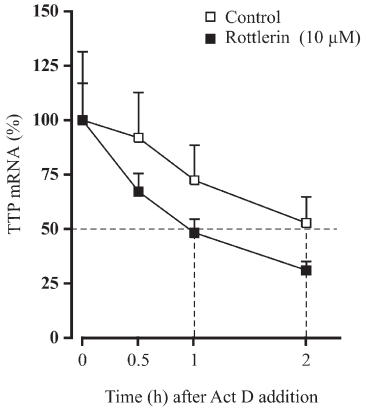

Fig. 5. The effect of rottlerin on TTP mRNA decay. Cells were treated with rottlerin (10 μM) for 30 min before the cells were activated by adding a combination of LPS (10 ng/ml) and PMA (100 nM). After 4 h, actinomycin D (1 μg/ml) was added into the cell culture to stop transcription. Incubations were terminated at the indicated time points after actinomycin D and extracted total RNA was subjected to real-time PCR. TTP mRNA levels were normalized against GAPDH mRNA. Open and black symbols represent LPS + PMA treated cells in the absence or in the presence of rottlerin, respectively. Values are mean + S.E.M. ($n = 3$).

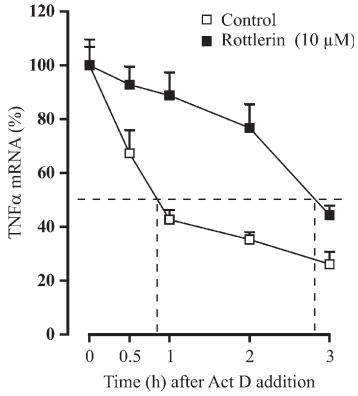

Fig. 6. The effect of rottlerin on TNFα mRNA decay. J774 cells were treated with rottlerin (10 μM) for 30 min before the cells were activated by adding a combination of LPS (10 ng/ml) and PMA (100 nM). After 9 h incubation, actinomycin D (1 μg/ml) was added into the cell culture to stop transcription. Incubations were terminated at the indicated time points after actinomycin D and extracted total RNA was subjected to real-time PCR. TNFα mRNA levels were normalized against GAPDH mRNA. Open and black symbols represent LPS + PMA treated cells in the absence or in the presence of rottlerin, respectively. Values are mean + S.E.M. ($n = 3$).

1998; Lai et al., 1999). The aim was to investigate if the downregulation of TTP expression by rottlerin would be sufficient to alter TNFα mRNA decay. Rottlerin (10 μM) was added to the cell culture 30 min prior to stimuli, and after 9 h incubation (when rottlerin evoked a clear reduction in TTP protein levels), actinomycin D (1 μg/ml), an inhibitor of transcription, was added to the cell culture. Cells were then further incubated for 0.5, 1, 2, or 3 h before extraction of total RNA. Analysis of TNFα mRNA showed that when transcription was blocked with actinomycin D, the decay of TNFα mRNA was slower in rottlerin treated cells (Fig. 6). The half-life of TNFα mRNA was increased by 2 h in cells treated with rottlerin, indicating that the downregulation of TTP by rottlerin may be functionally significant.

4. Discussion

In the current study we were able to show that downregulation of PKCδ by siRNA and inhibition of PKCδ by rottlerin reduced TTP expression in activated macrophages. Rottlerin was demonstrated to enhance the decay of TTP mRNA and to stabilize TNFα mRNA.

TTP is recognized as an important factor in inflammation and immunity. It is known to mediate its functions by altering the stability of mRNAs of several transiently expressed inflammatory genes. Studies on the regulation of TTP expression have focused mainly on the effects of mitogen-activated protein kinases (MAPKs) (Mahtani et al., 2001; Brook et al., 2006; Hitti et al., 2006). Very little is known about the possible role of other protein kinases, including PKC. However, phorbol esters are known to enhance TTP production (Varnum et al., 1989) and though they have many effects, they also activate classical and novel PKC isoenzymes (Spitaler and Cantrell, 2004). This suggests that the PKC pathways could be involved in the regulation of TTP expression. In our earlier study, classical PKC isoenzymes were shown to take part in the regulation of TTP expression and PKCβII seemed to up-regulate TTP expression by affecting the activity of transcription factor AP-2 (Leppänen et al., 2008). The present study extends the previous data by providing evidence, for the first time, that another PKC isoenzyme, i.e. PKCδ (belonging to novel PKCs), is likely to up-regulate TTP expression by stabilizing its mRNA. These two studies together indicate that PKC is involved in the regulation of TTP expression, but the underlying mechanisms of regulation seem to be different in these two PKC isoenzymes.

The PKC family, including PKCδ, is a major signalling system in inflammation. The role of PKCδ in inflammation and immunity was confirmed when PKCδ knock-out mice were developed independently by two groups (Leitges et al., 2001; Miyamoto et al., 2002). The PKCδ deficient mice showed increased proliferation of B lymphocytes and were prone to suffer autoimmune diseases (Miyamoto et al., 2002). Mecklenbräuker et al. (2002) reported that a deficiency in PKCδ resulted in the maturation and differentiation of self-reactive B-cells and proposed a role for PKCδ in the production of immunologic tolerance.

Rottlerin, also known as mallotoxin, is a natural compound isolated from *Mallotus philippinensis*. Rottlerin was first found to be an inhibitor of PKCδ in the study of Gschwendt et al. (1994). They investigated the effects of rottlerin on PKCδ in a kinase assay using both an enzyme isolated from porcine spleen and a recombinant enzyme from baculovirus-infected Sf9 insect cells, and found IC_{50} values of 3 and 6 μM, respectively. Higher concentrations of rottlerin inhibited also other PKC isoenzymes, e.g. $IC_{50} = 42$ μM for PKCβ. In the study of Keenan et al. (1997), the selectivity of rottlerin towards PKCδ was demonstrated in a rapid *in vivo* assay using individual PKC isoenzymes expressed in fission yeast *Schizosaccharomyces pombe*. However, Davies et al. (2000) assayed a wide range of kinases and noted, that rottlerin inhibited also other kinases i.e. MAPKAP-K2, PRAK, PDK1 and PKA. In the current study, PKCδ was found to be expressed in J774 macrophages, and it was activated by PMA. Downregulation of PKCδ by siRNA reduced TTP expression as did rottlerin treatment. Moreover, rottlerin had no effect on TTP expression in cells in which PKCδ had been downregulated by siRNA. Therefore it seems likely that the effects of rottlerin on TTP expression found in the present experiments were mediated through inhibition of PKCδ.

In the next series of experiments, the mechanisms involved in regulation of TTP expression by rottlerin were investigated in more detail. Rottlerin had no significant effect on the nuclear translocation (activation) of NF-κB, Sp1, AP-2 or EGR1, which are transcription factors known to participate in TTP expression or to have a binding site on the TTP gene intron or promoter region (DuBois et al., 1990; Lai et al., 1995; Lai et al., 1998). In contrast, when the effect of rottlerin on TTP mRNA stability was investigated using an mRNA degradation assay, we observed a more rapid TTP mRNA decay in rottlerin treated cells. However, the specific molecular mechanisms responsible for the PKCδ-mediated destabilization of TTP mRNA remain to be clarified.

We also made a preliminary attempt to evaluate if the inhibitory effect of rottlerin on TTP expression might have functional effects on TTP target genes. TTP-deficient mice are known to have elevated levels of TNFα due to increased TNFα mRNA stability (Carballo et al., 1998; Lai et al., 1999). In the present study, we found that rottlerin reduced the TTP protein levels significantly when measured after 9 h incubation, and under these conditions TNFα mRNA decay was also reduced in rottlerin treated cells. This suggests that the changes seen in TTP protein levels after rottlerin may have functional significance.

In summary, these results provide evidence that inhibition and downregulation of PKCδ inhibit the expression of TTP in activated macrophages and indicate that PKCδ is involved in the up-regulation of TTP expression via a mechanism related to the stabilization of TTP mRNA which, in turn, may serve as a feed-back loop to downregulate the inflammatory reaction.

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