



OUTI SAREILA

Pharmacological Regulation of Inducible Nitric Oxide
Synthase Expression

Effects of Janus kinase inhibitors,
orazipone and simendans



ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on May 2nd, 2008, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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www.uta.fi/taju
<http://granum.uta.fi>

Cover design by
Juha Siro

Acta Universitatis Tamperensis 1306
ISBN 978-951-44-7290-9 (print)
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 713
ISBN 978-951-44-7291-6 (pdf)
ISSN 1456-954X
<http://acta.uta.fi>

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2008

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List of original communications

The thesis is based on the following original publications referred to in the text by their Roman numerals (I–IV). In addition, some unpublished results are presented.

- I Outi Sareila, Riku Korhonen, Outi Kärpäniemi, Riina Nieminen, Hannu Kankaanranta and Eeva Moilanen (2006) JAK inhibitors AG-490 and WHI-P154 decrease IFN- γ -induced iNOS expression and NO production in macrophages. *Mediators Inflamm* 2006(2) 16161.
- II Outi Sareila, Riku Korhonen, Outi Kärpäniemi, Riina Nieminen, Hannu Kankaanranta and Eeva Moilanen (2008) Janus kinase 3 inhibitor WHI-P154 in macrophages activated by bacterial endotoxin: Differential effects on the expression of iNOS, COX-2 and TNF- α . *Int Immunopharmacol* 8:100–108.
- III Outi Sareila, Mari Hämäläinen, Erkki Nissinen, Hannu Kankaanranta and Eeva Moilanen (2008) Orazipone inhibits activation of inflammatory transcription factors nuclear factor-kappa B and signal transducer and activator of transcription 1 and decreases inducible nitric-oxide synthase expression and nitric oxide production in response to inflammatory stimuli. *J Pharmacol Exp Ther* 324:858–866.
- IV Outi Sareila, Riku Korhonen, Hanna Auvinen, Mari Hämäläinen, Erkki Nissinen, Hannu Kankaanranta and Eeva Moilanen. Anti-inflammatory properties of simendans: Levo- and dextrosimendan decrease nitric oxide production by inhibiting inducible nitric oxide synthase promoter activity. (submitted for publication)

Abbreviations

AG-490	α -cyano-(3,4-dihydroxy)-N-benzylcinnamide (JAK2 inhibitor)
AP	activator protein
AUF	ARE/poly(U)-binding/degradation factor
BH ₄	tetrahydrobiopterin
cAMP	3'-5'-cyclic adenosine monophosphate
C/EBP	CCAAT-enhancer box binding protein
CM	cytokine mixture containing IFN γ , TNF- α and IL-1 β
COX-2	cyclo-oxygenase 2
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	guanylate cyclase
GAS	gamma activated site
GSH	glutathione
HIF	hypoxia induced factor
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K _{ATP}	ATP-dependent kalium (channels)
KLF	krüppel like factor
LPS	lipopolysaccharide

LUC	luciferase
MAPK	mitogen-activated protein kinase
NAC	<i>N</i> -acetyl-L-cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor- κ B
NO	nitric oxide
OR-1384	3-[4-(methylsulfonyl)benzylidene]pentane-2,4-dione (orazipone)
OR-1958	3-[3-chloro-4-(methylsulfonyl)benzylidene]pentane-2,4-dione (orazipone analog)
OR-2149	3-[4-(methylsulfonyl)benzyl]pentane-2,4-dione (the non-thiol modulating control compound for orazipone)
OR-1560	simendan analog
PDE	phosphodiesterase
PDTC	pyrrolidine dithiocarbamate (NF- κ B inhibitor)
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator activated receptor
PTB	polypyrimidine tract-binding protein
RT-PCR	reverse transcriptase/real time PCR
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
TLR	Toll-like receptor
TNF- α	tumor necrosis factor alpha
USF	upstream stimulatory factor
3'-UTR	3'-untranslated region of mRNA
WHI-P154	4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (JAK3 inhibitor)
WHI-P131	4-(4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (JAK3 inhibitor)

Abstract

Inflammation is a defence mechanism by which an organism reacts to pathogens and tissue damage. Activated inflammatory cells produce signalling molecules that modulate the inflammatory response and the progress of inflammation. The inflammatory response is usually beneficial and required for survival, but when inappropriately focused or regulated, or excessive or prolonged, the inflammatory reaction may be harmful to the host. This is the case in septic shock and in chronic inflammatory diseases like arthritis.

One of the genes that is activated in inflammation is inducible nitric oxide synthase (iNOS). iNOS is an enzyme that catalyzes a reaction where nitric oxide is produced. Nitric oxide is a signalling molecule that modulates immune response and inflammation, regulates the tonus of the blood vessels and acts as a neurotransmitter. Even though nitric oxide has many positive effects, it has detrimental effects in chronic inflammation, where it is overproduced. Nitric oxide is involved in the pathophysiology of various inflammatory diseases including septic shock, rheumatoid arthritis, osteoarthritis and asthma. Compounds that inhibit iNOS expression or activity have been found to possess anti-inflammatory properties in various forms of experimentally-induced inflammation.

In the present study, the pharmacological regulation of iNOS expression and nitric oxide production was investigated in activated macrophages. Janus kinase (JAK2 and JAK3) inhibitors, orazipone and simendans were found to inhibit iNOS expression and nitric oxide production. JAK2 inhibitor AG-490 and JAK3 inhibitor WHI-P154 decreased the activation of signal transducer and activator of transcription (STAT) 1, which may explain their inhibitory effects on iNOS expression. Orazipone inhibited the activation of NF- κ B, one important transcription factor for iNOS expression. Orazipone also inhibited the activation of STAT1. The results suggest that the thiol modulating property of orazipone is

involved in the mechanism by which orazipone inhibits iNOS expression. Simendans did not affect the activation or the DNA binding activities of STAT1 or NF- κ B, but they inhibited NF- κ B-dependent transcription. iNOS mRNA decay was not affected by simendans but they decreased iNOS mRNA expression by inhibiting iNOS promoter activity.

In the present study, the pharmacological compounds used were found to have anti-inflammatory properties. The molecular mechanisms behind their effects were described. The information can be utilized in the development of novel anti-inflammatory drugs.

Tiivistelmä

Tulehdus on puolustusmekanismi, jolla elimistö reagoi taudinaiheuttajiin ja kudosaaurioon. Aktivoituneet tulehdussolut tuottavat välittäjäaineita, jotka säätelevät tulehdusreaktiota ja tulehduksen etenemistä. Tulehdusreaktio on yleensä hyödyllinen ja tärkeä eliön selviämisen kannalta, mutta jos se kohdentuu väärin tai jos reaktio on liiallinen, pitkittynyt tai väärin säädelty, tulehdusreaktiosta voi olla haittaa elimistölle. Tällainen tilanne on kyseessä septisessä sokissa ja kroonisissa tulehdussairauksissa kuten nivelreumassa.

Yksi tulehduksessa aktivoituvista geneeistä on indusoituva typpioksidisyntaasi (iNOS). Typpioksidisyntaasi on entsyymi, jonka katalysoimassa reaktiossa syntyy typpioksidia. Typpioksidi on signaalinvälittäjämolekyyli, joka säätelee immuunivastetta ja tulehdusreaktiota, osallistuu verisuonten tonuksen ja verenpaineen säätelyyn sekä toimii hermovälittäjäaineena. Vaikka typpioksidilla on monia positiivisia vaikutuksia, kroonisessa tulehduksessa typpioksidia tuotetaan ylimäärin ja sillä on haitallisia vaikutuksia. Typpioksidi liittyy mm. septisen sokin, nivelreuman, nivelrikon ja astman patogeneesiin. iNOS:n ilmentymistä tai aktiivisuutta estävillä yhdisteillä on todettu olevan tulehdusta vaimentavia vaikutuksia monissa tulehdustautien kokeellisissa malleissa.

Tässä tutkimuksessa selvitettiin iNOS:n ilmentymisen ja typpioksidin tuoton farmakologista säätelyä aktivoituissa makrofaageissa. Tutkimuksessa havaittiin sekä Janus kinaasien (JAK2 ja JAK3) estäjien että oratsiponin ja simendaanien estävän iNOS:n ilmentymistä ja typpioksidin tuottoa. JAK2 estäjä AG-490 ja JAK3 estäjä WHI-P154 vähensivät STAT1:n (signal transducer and activator of transcription 1) aktivoitumista, mikä voi selittää niiden typpioksidin tuottoa vähentävän vaikutuksen. Oratsiponi esti yhden iNOS:lle tärkeän transkriptiotekijän, NF- κ B:n, aktivoitumista. Oratsiponi esti lisäksi STAT1-transkriptiotekijän aktivoitumista. Tulosten perusteella voidaan päätellä, että oratsiponin toimiminen tiolimodulaattorina on ainakin yksi mekanismeista, joilla se vähentää

iNOS:n ilmentymistä. Simendaanit eivät vaikuttaneet STAT1:n tai NF- κ B:n aktivoitumiseen tai kykyyn sitoutua DNA:han, mutta ne estivät NF- κ B-välitteistä transkriptiota. Simendaanit eivät myöskään vaikuttaneet iNOS mRNA:n hajoamiseen, mutta ne vähensivät iNOS mRNA:n ilmentymistä estämällä iNOS-promoottorin aktiivisuutta.

Tutkimuksessa havaittiin tutkituilla lääkeaineilla olevan tulehdusta vaimentavia ominaisuuksia ja saatiin selville ilmiön taustalla olevia molekulaarisia mekanismeja. Tietoa voidaan käyttää hyväksi kehitettäessä uusia tulehdusta vaimentavia lääkeaineita.

Introduction

Inflammation is a defence mechanism by which an organism reacts to tissue damage. Inflammatory cells are activated by stimuli like pathogens, allergens, irritants or physical injury. Extracellular stimuli are recognized by specific receptors on cell surface. These receptors transduce the signal into the cell and activate signalling cascades. Diverse signalling cascades are activated and modulate the activity of the inflammatory cell, for example by initiating gene expression. Activated inflammatory cells produce inflammatory mediators that modulate the inflammatory response and regulate the progress of inflammation. The inflammatory response is usually beneficial and required for survival, but when inappropriately focused or regulated, or excessive or prolonged, the inflammatory reaction may be harmful to the host. This is the case in septic shock and in chronic inflammatory diseases like arthritis.

Nitric oxide (NO) is a small gaseous signalling molecule that is synthesized from amino acid L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). In mammalian cells there are three isoforms of the enzyme. The endothelial (eNOS) and neuronal (nNOS) isoforms are low output Ca^{2+} -dependent enzymes, which produce NO in a pulsative manner. Although eNOS and nNOS expression is believed mainly to be constitutive, there are several examples showing a modulation of eNOS and nNOS expression (Förstermann et al. 1998). In contrast, iNOS is a high output, Ca^{2+} -independent enzyme, which is expressed in most cells only after induction by proinflammatory cytokines and other agents including bacterial products. In inflammation, NO modulates immune responses and inflammatory process (Moilanen et al. 1999, Korhonen et al. 2005). Even though NO has many positive effects and is an important defence molecule against bacteria and viruses, NO has detrimental effects in chronic inflammation. Overproduction of NO is associated with the pathophysiology of inflammatory diseases such as

rheumatoid arthritis, osteoarthritis, asthma and sepsis (Kirkebøen and Strand 1999, Zamora et al. 2000, Ricciardolo et al. 2004, Cuzzocrea 2006, Vuolteenaho et al. 2007). In light of the results in various forms of experimentally-induced inflammation, compounds that inhibit iNOS expression or iNOS activity are considered potential drug candidates for the treatment of inflammatory diseases (Tinker and Wallace 2006).

Bacterial products and some cytokines induce iNOS expression in inflammatory and tissue cells including macrophages. Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is a known inducer of iNOS expression. Interferon γ (IFN γ) is one of the central cytokines involved in the induction of iNOS expression and NO production in macrophages. LPS and IFN γ activate transcription factors critical in iNOS expression.

The aim of the present study was to investigate the pharmacological control of iNOS expression and NO production in activated macrophages by inhibitors of Janus kinases 2 and 3, and to evaluate the anti-inflammatory effects of orazipone (a candidate drug) and simendans by investigating their effects on iNOS expression and NO production.

Review of the literature

Nitric oxide

The factor regulating the relaxation of the endothelial smooth muscle cells was first described as the endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). Later this factor was recognized to be nitric oxide (Ignarro et al. 1987, Palmer et al. 1987).

Nitric oxide (NO) is a small gaseous signalling molecule. NO is involved in various physiological and pathophysiological conditions and has both detrimental and beneficial effects in the human body. NO regulates vasorelaxation and platelet aggregation. It acts as a neurotransmitter in NANC (non adrenergic, non cholinergic) nerves and in the central nervous system. NO regulates neutrophil activation and cell growth and induces apoptosis. NO has an important role in host defence mechanisms. NO mediates the toxicity of natural killer cells and regulates T cell activation. Nitrosylation of proteins by NO-driven radicals regulates protein activity and function. (Korbut and Guzik 2005)

In inflammation, NO modulates various vascular and cellular responses (Moilanen et al. 1999, Korhonen et al. 2005), and its overproduction is associated with the pathophysiology of inflammatory diseases such as asthma (Sanders 1999, Ricciardolo et al. 2004) and arthritis (Cuzzocrea 2006, Vuolteenaho et al. 2007).

Inflammation and nitric oxide

Inflammation is a host defence mechanism that protects the body against pathogens. The inflammatory response is usually beneficial and required for survival. However, when the inflammatory response is inappropriately focused

or regulated, or excessive or prolonged, it may be harmful to the host. This is the case in septic shock and in chronic inflammatory diseases like arthritis.

The inflammatory response can be divided into two phases, i.e. acute and chronic inflammation. The acute phase begins rapidly after the threat (e.g. tissue damage or a pathogen) is recognized and may last from minutes to a few days. In the acute phase of inflammation, blood vessels dilate leading to increased blood flow to the site of inflammation and the permeability of capillaries increases allowing fluid and plasma proteins to enter the injured tissue. Vasodilation and increased permeability of capillaries finally result in the typical symptoms of inflammation, namely swelling, redness, heat and pain. In the acute phase, leukocytes, predominantly neutrophils, also stick to the vascular endothelium and migrate through the vascular wall into the inflamed tissue. Leukocytes migrate towards the higher concentration of chemoattractants (including bacterial products, components of the complement system, leukotrienes and cytokines) that are produced in the inflamed tissue. Leukocytes ingest offending agents, kill bacteria and other microbes, and destroy necrotic tissue and toxins (Kumar et al. 2005).

When the acute phase turns into chronic inflammation, monocytes have entered the site of the inflammation and have differentiated into macrophages. Macrophages destroy foreign agents by phagocytosis and produce pro-inflammatory cytokines and other inflammatory mediators. Lymphocytes represent the adaptive immune response in the inflamed tissue. They are activated by antigens and produce specific antibodies to destroy foreign agents (Kumar et al. 2005).

Inflammation triggers the induction of inflammation-related enzymes and the production of proinflammatory cytokines in inflammatory cells including macrophages. Inflammation induces the expression of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2), which produce inflammatory mediators NO and prostaglandin E₂ respectively. The production of proinflammatory cytokines e.g. tumor necrosis factor (TNF) α and interleukins (IL) IL-1, IL-6 and IL-8 is related to inflammation (Kumar et al. 2005).

NO is an important molecule in host defence against infectious organisms. It regulates the functional activity, growth and death of many immune and

inflammatory cells including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils and natural killer cells (Coleman 2001). At the sites of inflammation NO is rapidly oxidized to reactive nitrogen species (RNOS), which mediate many of the effects of NO in immunological cells. RNOS can modify the activity of signalling proteins by nitrosylation of thiols or amines (Coleman 2001). S-nitrosation is also a negative feedback mechanism by which NO downregulates the inflammatory processes that lead to the production of NO (Laroux et al. 2001, Korhonen et al. 2005). NO mediates toxic effects when NO and superoxide (O_2^-) anion combine to form peroxynitrite ($ONOO^-$) under conditions of both nitrosative and oxidative stress (Coleman 2001, Korhonen et al. 2005).

Nitric oxide synthases (NOS)

NO is synthesized from amino acid L-arginine in a reaction catalyzed by nitric oxide synthase (NOSs, EC 1.14.13.39) (Fig. 1). In mammalian cells there are three isoforms of the enzyme. Neuronal nNOS (also known as NOS I) and endothelial eNOS (NOS III) are expressed mainly constitutively but can be modulated by different agents (Förstermann et al. 1998). The third isoform, iNOS (NOS II), is induced in response to proinflammatory cytokines and bacterial products in inflammatory and tissue cells (MacMicking et al. 1997, Geller and Billiar 1998, Alderton et al. 2001, Kleinert et al. 2004).

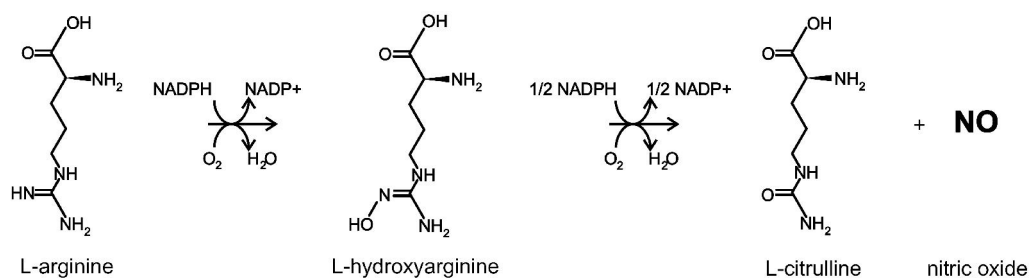


Figure 1. Synthesis of nitric oxide from L-arginine catalyzed by NOS.

cell wall component of gram negative bacteria, induce the transcription of iNOS. Proinflammatory cytokines including IFNs α , β and γ , IL-1 β and TNF- α are known inducers of iNOS expression, but iNOS promoters seem to respond to cytokines in a cell and tissue specific manner (Wort et al. 2001).

Inhibitors of iNOS

Overproduction of NO by iNOS is associated with inflammatory diseases and conditions like arthritis, asthma and septic shock (Zamora et al. 2000). However, NO production by the constitutively expressed isoforms of NOS is crucial to regulate normal functions in the body (e.g. blood pressure and neurotransmission) (Korbut and Guzik 2005). The development of an inhibitor that selectively inhibits NO production by the inducible isoform of NOS has therefore become a feasible target. However, the development of specific iNOS inhibitors has proved a challenging task. iNOS inhibitors developed so far include L-arginine analogs, guanidines, isothioureas and amidines together with compounds that inhibit iNOS dimerization or compete with essential cofactors of iNOS (Tinker and Wallace 2006).

Compounds resembling L-arginine competitively inhibit NO production. Their selectivities over the three isoforms of NOS vary (Table 1). L-NIL, which has 20–50 fold selectivity towards iNOS when compared to nNOS or eNOS, has been described to both ameliorate (Connor et al. 1995, Boileau et al. 2002, Hallinan et al. 2002) and exacerbate (McCartney-Francis et al. 2001, Veihelmann et al. 2002) inflammation in animal models of arthritis (Table 2). 1400W is a fairly selective iNOS inhibitor (Garvey et al. 1997, Paige and Jaffrey 2007). It has been described to reduce pain (da S.Rocha et al. 2002, Castro et al. 2006) in addition to being ineffective (Sakaguchi et al. 2004) in animal models of arthritis (Table 2). Recently described potent and specific iNOS inhibitors GW273629 and GW274150 (Alderton et al. 2005) functioned successfully in animal models of arthritis (Cuzzocrea et al. 2002, Bainbridge et al. 2006) (Table 2) and in other animal models of diseases or disorders including renal ischemia/reperfusion injury, septic shock, lung injury and pain (McDonald et al.

2002, Chatterjee et al. 2003, Genovese et al. 2005, De Alba et al. 2006, Albuszies et al. 2007).

Inhibitors of iNOS induction (i.e. gene expression) are potent inhibitors of NO production. However, inhibition of signalling pathways leading to iNOS expression usually interferes with other regulatory pathways in gene expression (Tinker and Wallace 2006).

Table 1. *L-arginine (substrate for NOS) and its analogs used as iNOS inhibitors. The isoform selectivities of the inhibitors are presented. (Modified from Alderton et al. 2001, Vuolteenaho et al. 2007)*

compound	structure	selectivity (fold)	
		iNOS vs. nNOS	iNOS vs. eNOS
L-arginine			
L-NMMA		0.7	0.5
L-NIL		23	49
L-NIO		20	30-50
1400W		32	>4000
aminoguanidine		5.5	11
GW273629		78	>125
GW274150		104	333

The formation of active NOS enzyme requires stable dimerization of two identical subunits. The interfaces between the two subunits are less conserved

than the active sites of different NOS isoforms. Therefore pharmacological compounds that specifically inhibit iNOS dimerization are under development (McMillan et al. 2000, Paige and Jaffrey 2007). PPA250, an inhibitor of iNOS dimerization, has been tested on an animal model of arthritis with promising results (Ohtsuka et al. 2002).

Table 2. Examples of the effects of iNOS inhibitors in animal models of arthritis.

iNOS inhibitor	arthritis model	result	reference
Prodrug of L-NIL	acute and chronic models of inflammation in rodents	inhibited inflammation and did not elevate systemic blood pressure	(Hallinan et al. 2002)
L-NIL	osteoarthritis model in dogs	anti-inflammatory effects	(Boileau et al. 2002)
L-NIL	streptococcal cell wall-induced arthritis in rats	exacerbation of disease	(McCartney-Francis et al. 2001)
L-NIL	antigen induced arthritis in mice	exacerbation of the acute inflammatory response	(Veihelmann et al. 2002)
aminoguanidine or L-NIL	adjuvant-induced arthritis in rats	suppressed joint inflammation	(Connor et al. 1995)
aminoguanidine	carrageenan granuloma air pouch and Freund's adjuvant-induced arthritis in rats	anti-inflammatory effects	(Gad and Khattab 2000)
aminoguanidine or 1400W	zymosan-induced arthritis in rats	reduced pain **	(da S.Rocha et al. 2002)
L-NAME / L-NMMA	adjuvant-induced arthritis in rats	anti-inflammatory effects	(Oyanagui 1994)
L-NAME or 1400W	experimental model of osteoarthritis in rats	reduced joint pain	(Castro et al. 2006)
1400W	type II collagen-induced arthritis in mice	no effect on arthritic inflammation or bone destruction	(Sakaguchi et al. 2004)
PPA250	collagen-induced arthritis in mice and adjuvant arthritis in rats	anti-inflammatory effect (suppressed the development of a destructive polyarthritis)	(Ohtsuka et al. 2002)
GW274150	collagen-induced arthritis in mice	attenuated the degree of chronic inflammation and tissue damage	(Cuzzocrea et al. 2002)
GW274150	collagen-induced arthritis in mice	reduced disease severity	(Bainbridge et al. 2006)
ttCH*	adjuvant-induced arthritis in rats	anti-inflammatory effects	(Rojas et al. 2003)
S-(2-aminoethyl)-isothiourrea	adjuvant-induced arthritis in rats	effectively reduced the paw swelling	(Yonekura et al. 2003)

* 2,4,6-trimethoxy-2V-trifluoromethylchalcone **only when given as a pretreatment

Regulation of iNOS expression

Once iNOS is expressed, it produces large amounts of NO (in a micromolar range) for prolonged periods. NO production through iNOS pathway is regulated mainly at the level of iNOS expression (Kleinert et al. 2004, Korhonen et al. 2005). iNOS expression is regulated by transcriptional, post-transcriptional or post-translational mechanisms.

Transcriptional regulation

iNOS promoter

Murine iNOS promoter region was cloned in 1993 by two groups (Xie et al. 1993, Lowenstein et al. 1993). Human iNOS promoter was described one year later (Chartrain et al. 1994). Murine and human iNOS promoters share some homologies, but their regulation seems to differ to some extent. A 1 kb fragment of the 5'-flanking sequence of the murine iNOS gene displayed nearly full promoter activity in transfection experiments (Xie et al. 1993, Lowenstein et al. 1993). By contrast, a 1 kb fragment of the 5'-flanking sequence of the human iNOS gene displayed only basal but not cytokine inducible promoter activity in transfection experiments using human cells. A 2-fold inducibility was seen using fragments containing 3.6 kb 5'-flanking sequence. In transfection experiments using the "full" human iNOS promoter (containing 16 kb of the 5'-flanking sequence) a maximal 20-fold cytokine-related inducibility was seen (de Vera et al. 1996) (Fig. 3). This relatively low inducibility of the 16 kb promoter fragment parallels the low inducibility seen for the endogenous human iNOS promoter activity in nuclear run-on experiments (de Vera et al. 1996). Both murine and human iNOS promoters contain TATA-box about 30 bp from the transcriptional start site and a NF- κ B binding site nearby. In contrast to the murine proximal NF- κ B site (Xie and Nathan 1994) the homologous site in the human iNOS promoter seems not to be functional in human cells (de Vera et al. 1996). In addition both the murine and the human iNOS promoter contain several upstream NF- κ B binding sites. In the human promoter combined NF- κ B

/STAT1 α binding sites at -5.2 to -5.8 kb seem to be essential for cytokine mediated induction of the promoter activity (Taylor et al. 1998, Ganster et al. 2001). Binding sites for IFN γ inducible factors (GAS, gamma activated site and ISRE, interferon-stimulated response element) which are recognized by the signal transducer and activator (STAT) 1 α or the interferon regulatory factor (IRF) 1 are located in the upstream regions of both human and murine iNOS promoter (Kleinert et al. 2004). In addition to NF- κ B and STAT1 α / IRF1 responsive elements binding sites for several other transcription factors like cAMP responsive element binding protein (CREB), CCAAT-enhancer box binding protein (C/EBP), activating protein-1 (AP-1), peroxisome proliferator activated receptor (PPAR), hypoxia inducible factor (HIF) 1 and STAT3 have been described in human or murine iNOS promoter (Kleinert et al. 2004).

NF- κ B binding region is required for iNOS induction in murine cells (Xie et al. 1994, Diaz-Guerra et al. 1996, Kleinert et al. 1996b). NF- κ B and STAT1 are important transcription factors for both murine and human iNOS (Korhonen et al. 2005). In addition, there are several reports showing the additional positive involvement of transcription factors like C/EBP (Sakitani et al. 1998, Guo et al. 2003), T-cell factor 4 (Du et al. 2006), octamer factor (Goldring et al. 1996, Sawada et al. 1997), hypoxia induced factor (HIF) (Melillo et al. 1997, Jung et al. 2000), krüppel like factor (KLF) 4 (Warke et al. 2003, Feinberg et al. 2005) or nuclear factor of activated T cells (NFAT) c1 (Obasanjo-Blackshire et al. 2006) in the regulation of murine or human iNOS promoter. In addition, the reduction of murine or human iNOS promoter activity by transcription factors like AP-1 (Kizaki et al. 2001, Pance et al. 2002), upstream stimulatory factor (USF) (Gupta and Kone 2002), NF- κ B-repressing factor (NRF) (Feng et al. 2002), IRF2 (Paludan et al. 1999), PPAR γ (Fahmi et al. 2001), STAT3 (Yu et al. 2002, Yu and Kone 2004) and STAT6 (Coccia et al. 2000) has been described.

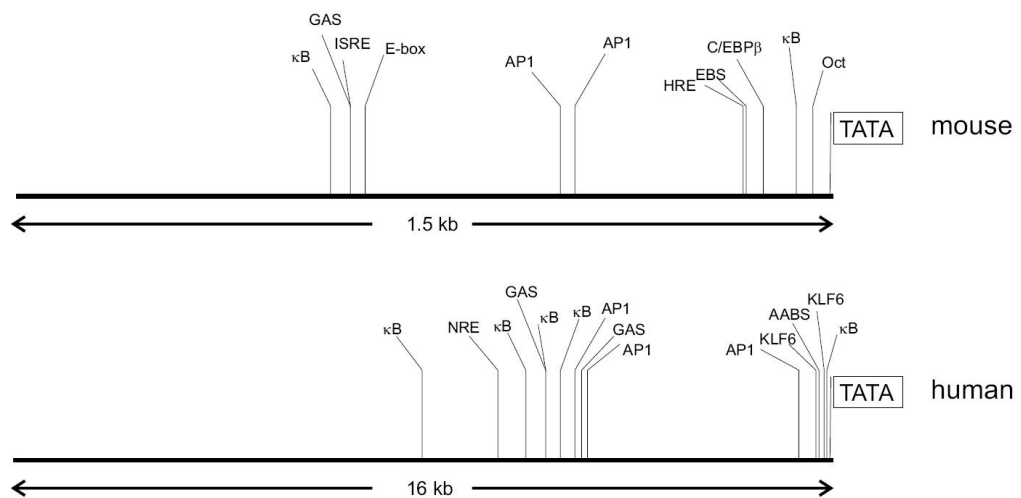


Figure 3. Schematic presentation of murine and human *iNOS* promoters and the binding sites of transcription factors. κ B = nuclear factor κ B GAS=gamma activated site; ISRE=interferon-stimulated response element; AP1=activator protein 1; HRE=hypoxia responsive enhancer; EBS=Est binding sequence; Oct=octamer factor; C/EBP β =CAAT/enhancer binding protein β ; I κ B= IRF-1; TATA=TATA box; AABS=A-activator binding site; KLF6 = Krüppel-like factor 6. (Modified from Kleinert et al. 2003, Lahti 2004)

JAK-STAT pathway

One of the intracellular signal transduction pathways that is activated by both LPS and IFN γ is Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway (Platanias 2005). The JAK family of tyrosine kinases includes JAK1, JAK2, JAK3 and Tyk2. A typical activation of JAK-STAT pathway begins on the cell surface when IFN γ binds to type II interferon receptor, which consists of two subunits, IFNGR1 and IFNGR2 (Platanias 2005) (Fig. 4). Receptor activation leads to the ligation of the two different receptor subunits, and this results in the formation of JAK heterodimers and their subsequent autophosphorylation. Upon activation, JAK2 is phosphorylated on tyrosine residues Tyr1007/Tyr1008 and JAK3 on Tyr980/Tyr981. JAKs phosphorylate STAT transcription factors. The STAT family consists of seven members (STAT 1, 2, 3, 4, 5a, 5b and 6). IFN γ -signalling preferentially leads to activation of STAT1 (Ivashkiv and Hu 2004), which is phosphorylated on Tyr701 by JAK (Leonard and O'Shea 1998). Phosphorylation of STAT1 induces

STAT1 dimerization, nuclear translocation and initiation of transcription of gamma activated site (GAS) -driven genes like iNOS (Kisseleva et al. 2002).

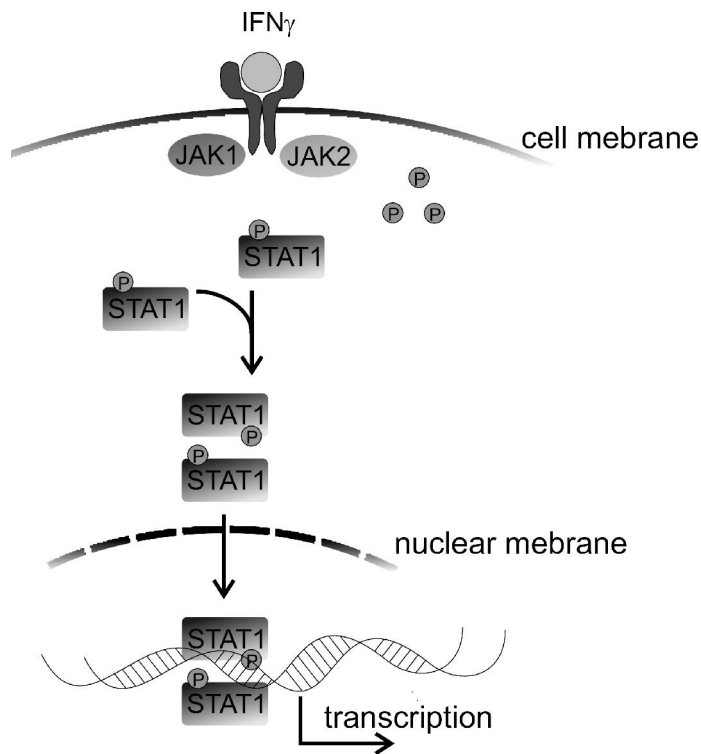


Figure 4. Activation of the JAK-STAT-pathway by IFN γ . A simplified presentation of the events after stimulation with IFN γ leading to STAT1 phosphorylation, nuclear translocation and gene transcription. (Modified from Plataniias 2005)

Two splicing variants of STAT1, α (91 kDa) and β (84 kDa), have been described (Pellegrini and Dusanter-Fourt 1997). STAT1 α has been reported to act as a key transcription factor in IFN γ -dependent mouse iNOS expression (Blanchette et al. 2003). After IFN γ -stimulation, STAT1 α is activated rapidly, in terms of minutes, in J774 murine macrophages (Gatto et al. 2004).

NF- κ B pathway

NF- κ B, another important transcription factor in the induction of iNOS, is merely involved in lipopolysaccharide (LPS)-induced iNOS expression and has a minor role following IFN γ -stimulation (Xie et al. 1994, Blanchette et al. 2003).

LPS is recognized by Toll-like receptor 4 (TLR4) on cell surface (Poltorak et al. 1998, Doyle and O'Neill 2006) (Fig. 5). TLR4 activation leads to the activation of I κ B kinase complex which phosphorylates I κ B and thus marks it for degradation (Doyle and O'Neill 2006). Degradation of I κ B releases NF- κ B dimer, which consists of subunits p65 and p50. Free NF- κ B translocates to the nucleus, binds to the specific κ B sites on DNA and initiates transcription (Baeuerle 1998, Doyle and O'Neill 2006).

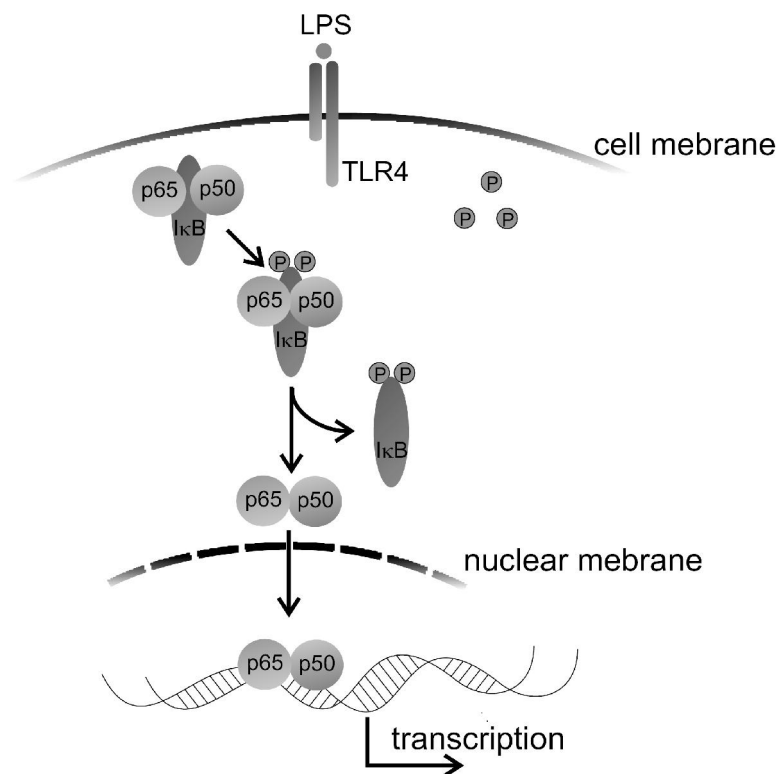


Figure 5. Activation of the NF- κ B-pathway by LPS. Simplified presentation of the events after stimulation with LPS leading to NF- κ B activation and gene transcription. (Modified from Baeuerle 1998)

Other pathways

Mitogen-activated protein kinase (MAPK) pathways are signal transduction cascades that lead to serial activation of serine and threonine kinases and finally to the activation of various transcription factors including activator protein 1 (AP-1). MAPK pathways are activated by various stimuli including cellular stress, growth factors and cytokines. Three most characterized MAPK pathways

lead to the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 kinase (Johnson and Lapadat 2002) which have a role in the regulation of iNOS expression in inflammation (Kristof et al. 2001, Lahti et al. 2003, Korhonen et al. 2005).

Interferon regulatory factor (IRF) 1 is a transcription factor that is inducible by IFNs and other signals. IRF-1 stimulates the expression of proinflammatory cytokines and IFN-inducible genes including iNOS (Taniguchi et al. 2001). NO production was severely impaired in cells derived from mice deficient in IRF1 (Kamijo et al. 1994), which implies that IRF1 has a role in iNOS expression.

Protein kinase A (PKA) and 3'-5'-cyclic adenosine monophosphate (cAMP) have been reported to regulate iNOS expression. The available data on the effect of the PKA/cAMP pathway on iNOS expression are quite diverse. Reports have documented both suppressive and enhanced effects of cAMP or cAMP-elevating compounds (e.g. inhibitors of phosphodiesterases) on iNOS expression depending on cell type, compound and stimulus used (Markovic et al. 2003).

Protein kinase C (PKC) isoenzymes have been proposed to be involved in the regulation of LPS- and cytokine-induced expression of inflammatory genes including iNOS (Chen et al. 1998a, Chen et al. 1998b, Carpenter et al. 2001, Banan et al. 2003). However, PKCs seem to regulate signalling in innate immunity in a cell specific and an isoenzyme specific manner (Tan and Parker 2003). Inhibition of classical PKC isoenzymes was shown to downregulate iNOS expression and NO production in murine J774 macrophages (Salonen et al. 2006).

Regulation of iNOS mRNA stability

In addition to transcriptional regulation, iNOS expression is regulated at the level of mRNA stability (Kleinert et al. 2004, Korhonen et al. 2005, Söderberg 2005). In murine J774 macrophages, pharmacological compounds dexamethasone and SP600125 (anthra(1,9-*cd*)pyrazol-6(2H)-one), an inhibitor of c-Jun N-terminal kinase (JNK) (Bennett et al. 2001), have been shown to reduce LPS-induced iNOS expression by destabilizing the mRNA (Korhonen et al. 2002, Lahti et al. 2003). IFN γ , which regulates iNOS expression at

transcriptional level (Kleinert et al. 2004, Korhonen et al. 2005) has also been shown to lengthen iNOS mRNA half-life in LPS-stimulated murine macrophages (Korhonen et al. 2002).

The 3'-untranslated region (3'-UTR) of iNOS mRNA is considered the major element on the mRNA that participates in the regulation of iNOS mRNA stability. Recently, several trans-acting proteins that regulate iNOS mRNA stability by interacting with the 3'-UTR of iNOS mRNA have been found. These include tristetraprolin (TTP), KH-type splicing regulatory protein (KSRP), a member of the Hu family of RNA binding proteins (HuR), ARE/poly(U)-binding/degradation factor 1 (AUF1), and heterogeneous nuclear ribonucleoprotein I (hnRNPI; also known as the polypyrimidine tract-binding protein PTB) and hnRNPL. (Kleinert et al. 2004)

TTP is a zinc-finger protein containing two Cys-Cys-Cys-His zinc-finger domains, which bind to AU-rich elements in the 3'-untranslated regions (3'-UTR) of mRNA and regulate their stability (Blackshear 2002). TTP has been shown to destabilize many mRNAs by binding the AU-rich elements on their 3'-UTR and promoting deadenylation (Lai et al. 1999, Lai et al. 2003). By contrast, TTP was shown to have a stabilizing affect on human iNOS mRNA even though no direct binding to human iNOS 3'-UTR was observed (Fechir et al. 2005). Recently JNK was shown to regulate TTP protein expression and the stabilization of human iNOS mRNA (Korhonen et al. 2007). KSRP has been shown to have a binding site on human iNOS 3'-UTR and to destabilize iNOS mRNA thereby decreasing iNOS expression (Linker et al. 2005). HuR is another protein that stabilizes mRNAs by binding to AU-rich elements (Brennan and Steitz 2001). HuR has been shown to bind and stabilize human iNOS mRNA via the 3'-UTR (Rodriguez-Pascual et al. 2000, Kleinert et al. 2002). AUF1 was shown to downregulate cytokine-induced human iNOS expression (Kleinert et al. 2002). hnRNPI (PTB) was shown to bind to the 3'-UTR of human iNOS mRNA and to stabilize it, which resulted in increased iNOS expression (Pautz et al. 2006). However, hnRNPI and hnRNPL were shown to destabilize murine iNOS mRNA and bind to the 3'-UTR of the mRNA (Söderberg et al. 2007).

Post-translational regulation

Substrate availability, tetrahydrobiopterin (BH₄) availability, phosphorylation of iNOS, dimerization and protein interactions, iNOS protein degradation and NO autoregulation are known post-translational mechanisms regulating iNOS expression, protein stability and/or activity. NO is produced from L-arginine by NOS, and the availability of arginine is a rate limiting step in NO synthesis in certain conditions. In NO producing cells, arginine uptake, recycling and degradation modulate the production of NO (Mori and Gotoh 2000). BH₄ is a cofactor that is essential for iNOS activity. BH₄ has also been reported to stabilize iNOS mRNA (Linscheid et al. 1998). Phosphorylation of iNOS protein at tyrosine residues may contribute to the enzymatic activity of iNOS (Pan et al. 1996).

Dimerization of iNOS protein into active complex is a prerequisite of iNOS enzyme activity and NO production. Dimerization of iNOS is inhibited by cellular proteins Kalirin and NOS-associated protein 1.10 kd (NAP110) (Zhang et al. 2003). Inhibition of iNOS dimerization by a pharmacological inhibitor led to reduced iNOS expression as a result of co-translational mechanisms (Kolodziejewski et al. 2004).

Once iNOS protein is expressed, its levels are regulated by protein degradation. Degradation is regulated by the degree of ubiquitination of iNOS protein. When ubiquitination reaches a critical level, iNOS protein is directed into degradation by proteasome (Felley-Bosco et al. 2000, Musial and Eissa 2001). Caveolin-1 (Felley-Bosco et al. 2000), transforming growth factor (TGF) β (Mitani et al. 2005, Vuolteenaho et al. 2005) and PPAR (peroxisome proliferator-activated receptor) α agonists (Paukkeri et al. 2007) have been proposed to accelerate iNOS degradation via the ubiquitin-proteasome pathway. Calpain, a cysteine protease, may also have a role in iNOS protein degradation (Walker et al. 1997).

NO itself mostly downregulates the induction of iNOS gene (Kleinert et al. 2003). Regulation by the negative feedback seems to be a protective mechanism against NO overproduction, cell destruction and inflammation.

JAK inhibitors

AG-490

Tyrphostin AG-490 (α -cyano-(3,4-dihydroxy)-N-benzylcinnamide; Fig. 6) has earlier been reported to act as a specific JAK2 inhibitor in acute lymphoblastic leukaemia cells (Meydan et al. 1996). AG-490 was shown to prevent JAK2 tyrosine autophosphorylation in a concentration-dependent manner at 1-50 μ M drug concentrations in *in vitro* kinase assay. AG-490 was shown not to inhibit SYK (spleen tyrosine kinase), LYN (protein tyrosine kinase related to LCK and YES), BTK (Bruton's tyrosine kinase) and IRK (insulin receptor kinase). AG-490 has been described to inhibit the induction of iNOS expression in cells like murine macrophages (Ruetten and Thiemermann 1997, Marrero et al. 1998), murine skin derived dendritic cells (Cruz et al. 1999) and human epithelial A549/8 or DLD-1 cells (Kleinert et al. 1998).

However, AG-490 has been shown also to inhibit JAK3 when used at higher concentrations. AG-490 (40–80 μ M) decreased the phosphorylation of both JAK3 and STAT3 in anaplastic large cell lymphoma cells (ALCL) (Amin et al. 2003). AG-490 (50–100 μ M) was also reported to inhibit IL-2-induced tyrosine phosphorylation of JAK3, and subsequently activation of STAT5a and STAT5b in T cells (D10) (Wang et al. 1999, Kirken et al. 1999).

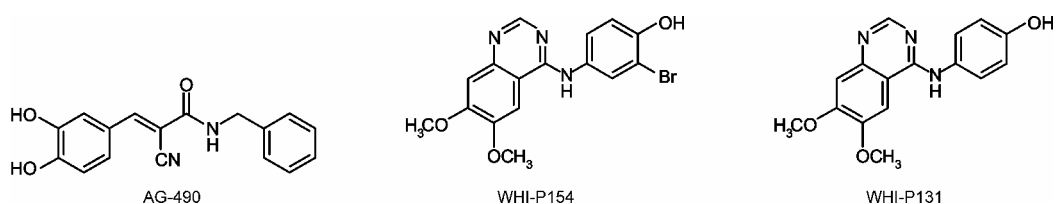


Figure 6. Chemical structures of JAK inhibitors AG-490, WHI-P154 and WHI-P131.

WHI-P154 and WHI-P131

WHI-P154, [4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline], and WHI-P131, [4-(4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline] (Fig. 6) were designed to specifically inhibit JAK3 and to act as apoptosis-inducing

antileukemic agents (Narla et al. 1998, Sudbeck et al. 1999). WHI-P154 did not inhibit JAK1 or JAK2 kinase activity at 25 or 75 $\mu\text{g/ml}$ (67 and 200 μM) drug concentrations in *in vitro* kinase assays but inhibited JAK3 at 0.5-100 $\mu\text{g/ml}$ (1.3-266 μM) (Sudbeck et al. 1999). WHI-P154 inhibited JAK3 kinase activity at 10 $\mu\text{g/ml}$ (26.6 μM) but not JAK1 or JAK2 activity even at 75 $\mu\text{g/ml}$ (200 μM) in kinase assay, suggesting that WHI-P154 is a specific JAK3 inhibitor (Goodman et al. 1998, Amin et al. 2003). However, in IL-4-stimulated U937 cells, WHI-P154 (25-250 μM) inhibited both JAK1 and JAK3 phosphorylation, and prevented STAT6 activation (Deszo et al. 2004).

Orazipone and its derivatives

Orazipone (OR-1384; 3-[4-(methylsulfonyl)benzylidene]pentane-2,4-dione) and its derivative OR-1958 (3-[3-chloro-4-(methylsulfonyl)benzylidene]pentane-2,4-dione) (Fig. 7) are novel thiol modulating compounds developed by the Finnish pharmaceutical company Orion Pharma. Orazipone and its derivative exert their effects most likely by forming reversible conjugates with the thiol groups of glutathione (GSH) and proteins (Wroblewski et al. 1998, Aho et al. 2001). The reaction with GSH is reversible, which makes orazipone and OR-1958 unique among thiol-modulating compounds. Orazipone has been shown to possess anti-inflammatory properties. Orazipone was shown to suppress LPS-induced release of proinflammatory cytokines IL-1 β , IL-8 and TNF- α in human monocytes (Nissinen et al. 1997) and to inhibit IL-1 β , TNF- α and IL-6 production in human monocytes (Wroblewski et al. 1998). Orazipone and OR-1958 were shown to inhibit TNF- α production in human mast cell line and to downregulate compound 48/80 induced histamine release from rat peritoneal mast cells (Vendelin et al. 2005). In addition, orazipone was shown to inhibit eosinophil accumulation in animal models of asthma (Ruotsalainen et al. 2000) and to induce apoptosis in human eosinophils (Kankaanranta et al. 2006). Orazipone has been tested in models of inflammatory bowel disease with promising results (Wroblewski et al. 1998) and reported to ameliorate intestinal radiation injury in a rat model (Boerma et al. 2006).

OR-2149, 3-[4-(methylsulfonyl)benzyl]pentane-2,4-dione, is a negative control compound for orazipone. It shares structural similarity with orazipone, but lacks the sulfhydryl binding property. The difference between orazipone and its negative control compound OR-2149 is in a single bond. In OR-2149 the bond is reduced, whereas in orazipone the bond is a double bond (Fig. 7).

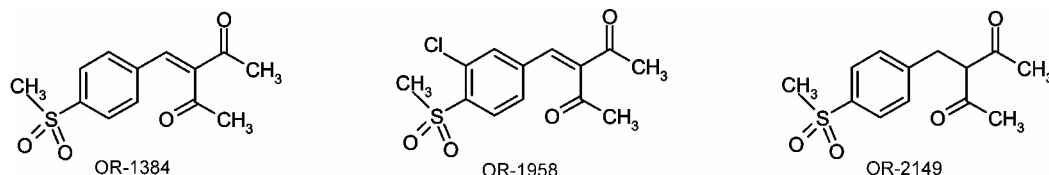


Figure 7. Chemical structures of orazipone (OR-1384), orazipone derivative (OR-1958) and the non-thiol modulating control compound (OR-2149).

Simendans

Levosimendan, 2-[[4-[(4R)-4-methyl-6-oxo-4,5-dihydro-1H-pyridazin-3-yl]-phenyl]hydrazinylidene]propanedinitrile (Fig. 8), is a Ca^{2+} sensitizer developed by the Finnish pharmaceutical company Orion Pharma. Levosimendan (Simdax®) is one of the calcium sensitizing agents developed for the treatment of decompensated heart failure (Mathew and Katz 1998). In addition to sensitizing troponin C to calcium it opens ATP sensitive potassium channels (K_{ATP}), which causes vasodilation (Yokoshiki et al. 1997). Levosimendan has been reported to inhibit phosphodiesterases (PDEs) and to be a more selective inhibitor of PDE III than PDE IV (IC_{50} for PDE 1.4 nM and IC_{50} for PDE IV 11 μM) (Szilagyi et al. 2005). Dextrosimendan (Fig. 8) is a stereoisomer of levosimendan. [(S)-(4-(6-methyl-2-oxo-3,6-dihydro-2H-1,3,4-thiadiazin-5-yl)-phenyl)hydrazono]propanedinitrile, also known as OR-1560 (Fig. 8), is a structural analog of simendans.



Figure 8. Chemical structures of levosimendan, dextrosimendan and simendan analog OR-1560.

Levosimendan has been shown to have about 10-fold affinity to myocardial troponin C when compared to dextrosimendan (Sorsa et al. 2004). Levosimendan was more potent calcium-sensitizer than dextrosimendan (potency difference of 76) and the potency difference was even greater when the inhibitory effects on PDE III were studied (levosimendan was 427 times more potent) (Kaheinen et al. 2006). The pure enantiomer, levosimendan, has been found not to isomerize *in vivo* (Wikberg et al. 1996). Levo- and dextrosimendans have been shown to act as K_{ATP} channel openers at similar concentrations (Haikala et al. 2005). Other K_{ATP} channel openers (diazoxide and iptakalim) have been shown to decrease iNOS expression in BV-2 cells (Liu et al. 2005) and in rats (Yang et al. 2006) exposed to rotenone.

Levosimendan has anti-inflammatory properties (Paraskevaidis et al. 2005). It has been shown to decrease plasma levels of TNF- α , IL-6, soluble FAS ligand and N-terminal-pro-B-type natriuretic peptide in patients with decompensated heart failure (Parissis et al. 2004, Kyrzopoulos et al. 2005). In addition, simendans reduced swelling when carrageenan-induced paw edema was used as a model of acute inflammation in rats (Haikala et al. 2005). Simendans were also shown to reduce lung eosinophilia in a mouse model of allergic inflammation and to induce apoptosis in human eosinophils (Kankaanranta et al. 2007). In addition, levosimendan has been reported to have beneficial effects in experimental models of septic shock (Oldner et al. 2001, Faivre et al. 2005) and recently it was reported to ameliorate myocardial depression in a patient with pneumococcal septic shock (Ramaswamykanive et al. 2007).

Aim of the study

Increased iNOS expression and NO production in macrophages and other cells is involved in the pathogenesis of various inflammatory diseases. The aim of the present study was to investigate pharmacological means and mechanisms to target excessive iNOS expression and NO production in macrophages exposed to proinflammatory stimuli.

The detailed aims of the present study were:

1. to evaluate the importance of JAK-STAT pathway in the regulation iNOS expression and NO production in activated macrophages by using JAK inhibitors and to characterize the mechanisms involved. (I–II)
2. to study the effects and mechanisms of action of a novel sulfhydryl modulating drug candidate, orazipone, on iNOS expression and NO production in cells activated by proinflammatory stimuli. (III)
3. to test the anti-inflammatory properties of levosimendan, dextrosimendan and their structural analog OR-1560 by studying their effects on iNOS expression and NO production in cells activated by proinflammatory stimuli and to describe the molecular mechanism involved in the effects of simendans. (IV)

Materials and methods

Materials

JAK inhibitors AG-490 [tyrphostin B42; α -Cyano-(3,4-dihydroxy)-N-benzylcinnamide], WHI-P154 (4-[(3'-Bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline) and WHI-P131 [4-(4'-Hydroxyphenyl)amino]-6,7-dimethoxyquinazoline] were from Merck Chemicals Ltd. (Nottingham, UK) and MG 132 [N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide] was from Tocris Bioscience (Ellisville, MO, USA). Anti-iNOS, anti-actin, anti-COX-2, anti-lamin A/C, anti-NF- κ B p65 and anti-STAT1 α p91 primary antibodies and secondary HRP-conjugated polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-STAT1 (Tyr701) antibody (Cell Signaling Technology Inc., Beverly, MA, USA) and recombinant mouse γ -interferon, tumor necrosis factor α , interleukin 1 β (R&D systems, Minneapolis, MN, USA) were obtained as noted. Orazipone (OR-1384; 3-[4-(methylsulfonyl)benzylidene]pentane-2,4-dione), OR-1958 (3-[3-chlorine-4-(methylsulfonyl)benzylidene]pentane-2,4-dione), the non-thiol modulating control compound (OR-2149; 3-[4-(methylsulfonyl)benzyl]-pentane-2,4-dione), levosimendan [2-[[4-[(4R)-4-methyl-6-oxo-4,5-dihydro-1H-pyridazin-3-yl]phenyl]hydrazinylidene]propanedinitrile], its stereoisomer dextrosimendan and simendan analog (OR-1560; [(S)-(4-(6-methyl-2-oxo-3,6-dihydro-2H-1,3,4-thiadiazin-5-yl)phenyl)hydrazono]propanedinitrile) were kindly provided by Orion Pharma (Espoo, Finland). Lipopolysaccharide from *Escherichia coli* 0111:B4 and all other reagents were from Sigma Chemical Co. (St Louis, USA). Orazipone, its derivatives and simendans were dissolved in dimethylsulfoxide just prior to experiments. The other drug compounds were dissolved in dimethylsulfoxide, aliquoted and stored at -20 °C. For cell experiments, the drugs were further diluted into culture medium 1:1000. The

final concentration of dimethylsulfoxide at incubation conditions was 1 %. Equal volume of solvent was included in all cell culture incubations.

Cell culture

J774 (TIB-67; ATCC, Manassas, Virginia, USA) murine macrophages were cultured at 37 °C in 5 % CO₂ atmosphere in Dulbecco's modified Eagle's medium with glutamax-I (Lonza, Verviers Sprl, Verviers, Belgium) containing 10 % heat-inactivated fetal bovine serum (Lonza), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK).

A549 (CCL-185; ATCC, Manassas, Virginia, USA) human alveolar epithelial cells were cultured at 37 °C in 5 % CO₂ atmosphere in Ham's F12K (Kaighn's modification) medium (Invitrogen, Paisley, UK) containing 10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen).

A549/8 cells had been stably transfected with plasmid pXP2-16kb containing full length human iNOS promoter (16 kb) and partial (1-38) 5'-UTR region in front of luciferase gene (de Vera et al. 1996) to generate A549/8-Luc cells expressing luciferase reporter gene under control of iNOS full length promoter. The A549/8-Luc cells were kindly provided by Prof. Hartmut Kleinert, Johannes Gutenberg University, Mainz, Germany. A549/8-Luc cells were cultured at 37 °C in 5 % CO₂ atmosphere in Dulbecco's modified Eagle's medium with 1 mM sodiumpyruvate (Lonza, Verviers Sprl, Verviers, Belgium) containing 5 % heat-inactivated fetal bovine serum (Lonza), 2.5 µg/ml Polymyxin B and 0.5 mg/ml G418 disulfate salt for selection.

L929 (CCL-1; ATCC, Manassas, VA, USA) murine fibroblasts were cultured at 37 °C in 5 % CO₂ atmosphere in Eagle's Minimum Essential Medium with L-glutamine containing 10 % heat-inactivated fetal bovine serum and supplemented with sodium bicarbonate (0.15 %), non-essential amino acids (1 mM each), sodium pyruvate (1 mM) (all from Lonza) and 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen).

L929-pGL4(miNOS-prom)neo and J774-pGL4(miNOS-prom)neo cell lines had been stably transfected with plasmid pGL4(miNOS-prom)neo. The plasmid had been constructed by inserting the *KpnI*–*HindIII* fragment (containing full length murine iNOS promoter and part of exon 1 of iNOS gene) of plasmid pGL-MNOS II-5'-Luc (Kleinert et al. 1996) into the *KpnI/HindIII* site of firefly luciferase reporter plasmid pGL4.17(luc2/neo) (Promega, Madison, WI, USA) generating pGL4(miNOS-prom)neo. L929 cells similarly transfected with the backbone vector pGL4.17(luc2/neo) lacking iNOS promoter insert were used as a control.

L929-pNFκB(luc)neo and L929-pGAS(luc)neo reporter cell lines had been stably transfected with luciferase reporter constructs for NF-κB [pNFκB(luc)neo] and STAT1 [pGAS(luc)neo] respectively. The plasmids were provided by Professor Hartmut Kleinert (Johannes Gutenberg University, Mainz, Germany). pNFκB(luc)neo contained five NF-κB binding sites and pGAS(luc)neo contained four GAS (γ-activated site) sites to drive luciferase expression.

All transfected cell lines were cultured under similar conditions as untransfected cells (see above). Culture media for transfected cells was supplemented with 400 μg/ml (for fibroblasts) or 200 μg/ml (for macrophages) of G 418 disulfate salt for selection.

Cells were seeded on 24-well plates for nitrite measurement and RT-PCR, on 6-well plates for Western blot and on 10-cm dishes for nuclear extract preparation, and were grown for 72 h to confluence before the commencement of the experiments unless otherwise stated.

Toxicity of the tested compounds was ruled out by measuring cell viability using Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Nitrite assays

After 24 h incubation the culture medium was collected for the nitrite measurement, which was used as a measure of NO production. Culture medium (100 μl) was incubated with 100 μl of Griess reagent (0.1 % naphthaethylenediamine dihydrochloride, 1 % sulfanilamine, 2.4 % H₃PO₄) and

the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard. (Green et al. 1982)

Enzyme linked immunosorbent assay

TNF- α and IL-6 were measured in the culture medium by enzyme linked immunosorbent assay (ELISA) using reagents from R & D Systems Europe, Abingdon, UK.

Preparation of cell lysates

At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) containing 2 mM sodiumorthovanadate. For pSTAT1 Western blot the cells were solubilised in cold lysis buffer (1 % NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM sodiumorthovanadate, 80 μ M leupeptin, 1 μ g/ml aprotinin, 1 mM NaF, 1 μ g/ml pepstatin, 2 mM sodiumpyrophosphate, 0.25 % sodiumdeoxycholate and 10 μ M N-octyl- β -D-glucopyranoside). After incubation for 15 min on ice, lysates were centrifuged (13 500 g, 5 min). The protein content of the supernatants was measured by the Coomassie blue method (Bradford 1976).

For iNOS Western blot the cells were resuspended in lysis buffer containing 1 % Triton X, 50 mM NaCl, 10 mM Tris-base pH 7.4, 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 1 mM sodiumorthovanadate, 40 μ M leupeptin, 50 μ g/ml aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate, 10 μ M N-octyl- β -D-glucopyranoside. Otherwise the lysis was performed as described above.

Preparation of nuclear extracts

At indicated time points the cells were rapidly washed with ice-cold PBS and solubilised in hypotonic buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiotreitol, 0.2 mM phenylmethylsulfonylfluoride,

10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1 mM sodiumorthovanadate, 1 mM NaF). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei separated by centrifugation at 4 °C, 21 000 g for 10 s. The pellet was resuspended in buffer C (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiotreitol, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1 mM sodiumorthovanadate, 1 mM NaF) and incubated on ice for 20 min. Nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4 °C, 21 000 g for 2 min. The protein content of the supernatant was measured by the Coomassie blue method (Bradford 1976). For electrophoretic mobility shift assay, the samples were stored at -70 °C. For Western blot analysis, the samples were boiled in SDS sample buffer and stored at -20 °C.

Western blotting

Protein (20 µg of lysates or nuclear extracts) was loaded on 8 % SDS-polyacrylamide electrophoresis gel and was electrophoresed for 2 h at 120 V in buffer containing 25 mM Tris base, 250 mM glycine and 0.1 % SDS. After electrophoresis the proteins were electrically transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) in buffer containing 25 mM Tris, 192 mM glycine, 20 % methanol and 0.005 % SDS. After transfer, the membrane was blocked in TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.1 % Tween-20) containing 5 % skimmed milk for 1 h at room temperature. The membrane was incubated with primary antibody in the blocking solution at 4 °C overnight (for anti-pSTAT1 milk was replaced with bovine serum albumin). Thereafter the membrane was washed three times with TBST for 5 min, incubated with secondary antibody in the blocking solution for 50 min at room temperature, and washed three times with TBST for 5 min. Bound antibody was detected using Super Signal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro,

CA, USA). The quantitation of the chemiluminescent signal was carried out with FluorChem™ software version 3.1.

RNA extractions and quantitative RT-PCR

Cell homogenization, RNA extraction and quantitative reverse transcription/real time (RT) -PCR were performed as described in (Lahti et al. 2003). In case of PCR primers that were complementary to a region in a single exon, the extracted RNA was treated with DNase I (Fermentas UAB, Vilnius, Lithuania) to get rid of contaminating DNA possibly present in the RNA sample. When the used primers recognized a region in the exon-exon boundary, DNase I treatment was not performed. Primers and probes [6-FAM (6-carboxy-fluorescein) as 5'-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3'-quencher] were designed using Express Software (Applied Biosystems) and their sequences are described in Table 3. The primers were used at 300 nM and the probes at 150 nM concentrations (luciferase probe 200 nM). All primers and probes were purchased from Metabion. Thermal cycling conditions were: incubation at 50 °C for 2 min, 95 °C for 10 min, thereafter 40 cycles of denaturation at 92 °C for 15 s and annealing/extension at 60 °C for 1 min. The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin #2. Each sample was determined in duplicate.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was used to analyse protein binding to specific oligonucleotides. Single-stranded DNA oligonucleotide (5'-CCTTTTCCCCTAACACT-3') and its complementary DNA oligonucleotide containing the GAS region (STAT1 binding site) found in iNOS promoter (Gao et al. 1997) (Oligomer, Helsinki, Finland) were annealed in annealing buffer (Ambion, Austin, TX, USA) by heating at 95 °C for 5 minutes and then slowly cooling down to generate double-stranded NOS-GAS oligonucleotide. NOS-

GAS oligonucleotides and transcription factor consensus oligonucleotides for NF- κ B (Promega, Madison, WI, USA) were 5'-end-labelled with γ -P³²-ATP (GE Healthcare Europe GmbH, Munich, Germany) using T4 polynucleotide kinase (Promega, Madison, WI, USA). For binding reactions, 5 μ g of nuclear extracts were incubated in a 20 μ l reaction volume containing 0.1 mg/ml (for NF- κ B oligonucleotides) or 0.01 mg/ml (for NOS-GAS oligonucleotides) (poly)dI-dC, 1mM dithiothreitol, 10mM Tris-HCl, pH 7.5, 1mM EDTA, 40mM KCl and 10 % glycerol for 20 min at room temperature. P³²-labelled 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.

Actinomycin D assay

Actinomycin D assay was used to evaluate mRNA decay. Actinomycin D, an inhibitor of transcription, was added into the cell culture medium to stop transcription. After the addition of actinomycin D, the decay of mRNA of interest was followed by measuring the remaining RNA levels by quantitative RT-PCR and relating it to the mRNA levels measured at the time of addition of the transcriptional blocker actinomycin D.

Statistics

Results are expressed as mean \pm standard error of mean (SEM). When indicated, statistical analysis was carried out by analysis of variance supported by Dunnett adjusted significance levels. Differences were considered significant at $P < 0.05$.

Table 3. Primer and probe sequences

Gene	Oligonucleotide	Sequence 5' → 3'
luciferase*	forward primer	ACGGCTTCGGGCATGTTCA
	reverse primer	CTCCTCCTCGAAGCGGTACA
	probe	TTGATCTGCGGGCTTTCGGGTCCGT
luciferase**	forward primer	AAAAAGTTGCGCGGAGGAG
	reverse primer	TTTTTCTTGCGTCGAGTTTCC
	probe	TGTGTTGTGGACGAAAGTACCGAAAGGTCTTAC
human iNOS	forward primer	GCAGGTCGAGGACTATTTCTTCA
	reverse primer	TCCTTCTTCGCCCTCGTAAGGA
	probe	TCAAGAGCCAGAAAGCGCTATCACGAAGATA
human GAPDH	forward primer	AAGGTCGGAGTCAACGGATT
	reverse primer	GCAACAATATCCACTTTACCAGAGTTAA
	probe	CGCCTGGTCACCAGGGCTGC
murine iNOS	forward primer	CCTGGTACGGGCATTGCT
	reverse primer	GTCATGCGGGCCTCCTT
	probe	CAGCAGCGGGCTCCATGACTCCC
murine GAPDH	forward primer	GCATGGCCTTCCGTGTTTC
	reverse primer	GATGTCATCATACTTGGCAGGTTT
	probe	TCGTGGATCTGACGTGCCGCC

*L929-pGL4(miNOS-prom)neo and J774-pGL4(miNOS-prom)neo cells

**A549/8 and L929-pNFκB(luc)neo and L929-pGAS(luc)neo cells

Summary of the results

JAK inhibitors decrease iNOS expression and inhibit STAT1 activation (I, II)

One of the main cytokines involved in the induction of iNOS expression and NO production in macrophages is interferon γ (IFN γ). Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is another inducer of iNOS (Alderton et al. 2001, Bogdan 2001, Kleinert et al. 2004). In the present study, J774 macrophages were stimulated with either IFN γ or LPS. Both stimuli induced the expression of iNOS, which was observed as increased cellular levels of iNOS mRNA and protein. JAK3 inhibitors WHI-P154 and WHI-P131, and JAK2 inhibitor AG-490 decreased iNOS protein expression in LPS-stimulated macrophages (Fig. 9) and similar inhibition was observed in macrophages activated by IFN γ . The JAK inhibitors were found to inhibit nitric oxide production into the culture medium in both IFN γ - and LPS-stimulated macrophages in a concentration-dependent manner.

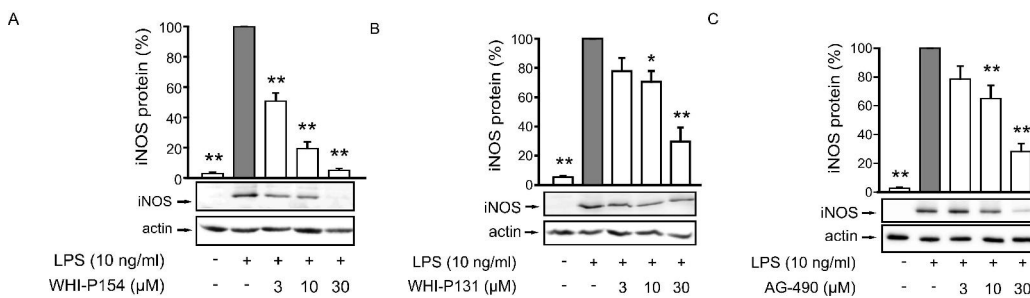


Figure 9. JAK inhibitors WHI-P154 (A), WHI-P131 (B) and AG-490 (C) inhibited LPS-induced iNOS protein expression in J774 macrophages. The results are expressed as mean + SEM, $n=7$ in A, $n=4$ in B and $n=12$ in C. ** $P<0.01$ when compared to cells treated with LPS alone. (Reprinted with permission from Sareila et al. 2008 *Int Immunopharmacol* 8:100-108 © Elsevier Ltd.)

Inhibition of JAK-STAT pathway

JAK-STAT pathway is activated in response to cytokines and LPS (Platanias 2005). When the cells were exposed to IFN γ , STAT1 was phosphorylated and translocated into the nucleus within 5–15 minutes in J774 macrophages. When LPS was added into the cell culture, STAT1 activation was observed after 2 h incubation and remained elevated for up to 6 h. JAK2 inhibitor AG-490 and JAK3 inhibitors WHI-P154 and WHI-P131 were found to inhibit STAT1 activation. AG-490 and WHI-P154 decreased STAT1 nuclear translocation in IFN γ -stimulated macrophages (Fig. 10). AG-490 and the JAK3 inhibitors WHI-P154 and WHI-P131 inhibited STAT1 phosphorylation and nuclear translocation in LPS-stimulated macrophages (Fig. 11). Accordingly, WHI-P154 was shown to inhibit nuclear levels of phosphorylated STAT1 in LPS-stimulated cells.

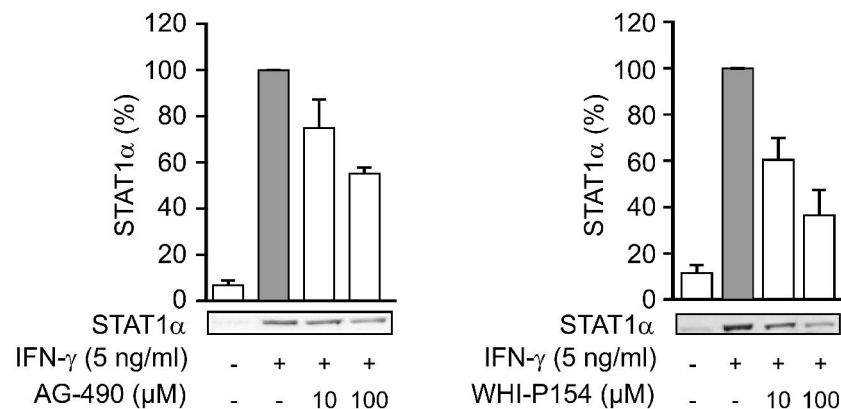


Figure 10. JAK inhibitors AG-490 and WHI-P154 decreased nuclear translocation of STAT1 α in IFN γ -stimulated J774 macrophages. The results are expressed as mean + SEM ($n = 2-3$ for AG-490 and $n = 4$ for WHI-P154). (Reprinted with permission from: Sareila et al. 2006, *Mediators Inflamm* 2006: Article ID 16161, 1–7 © Hindawi Publishing Corporation)

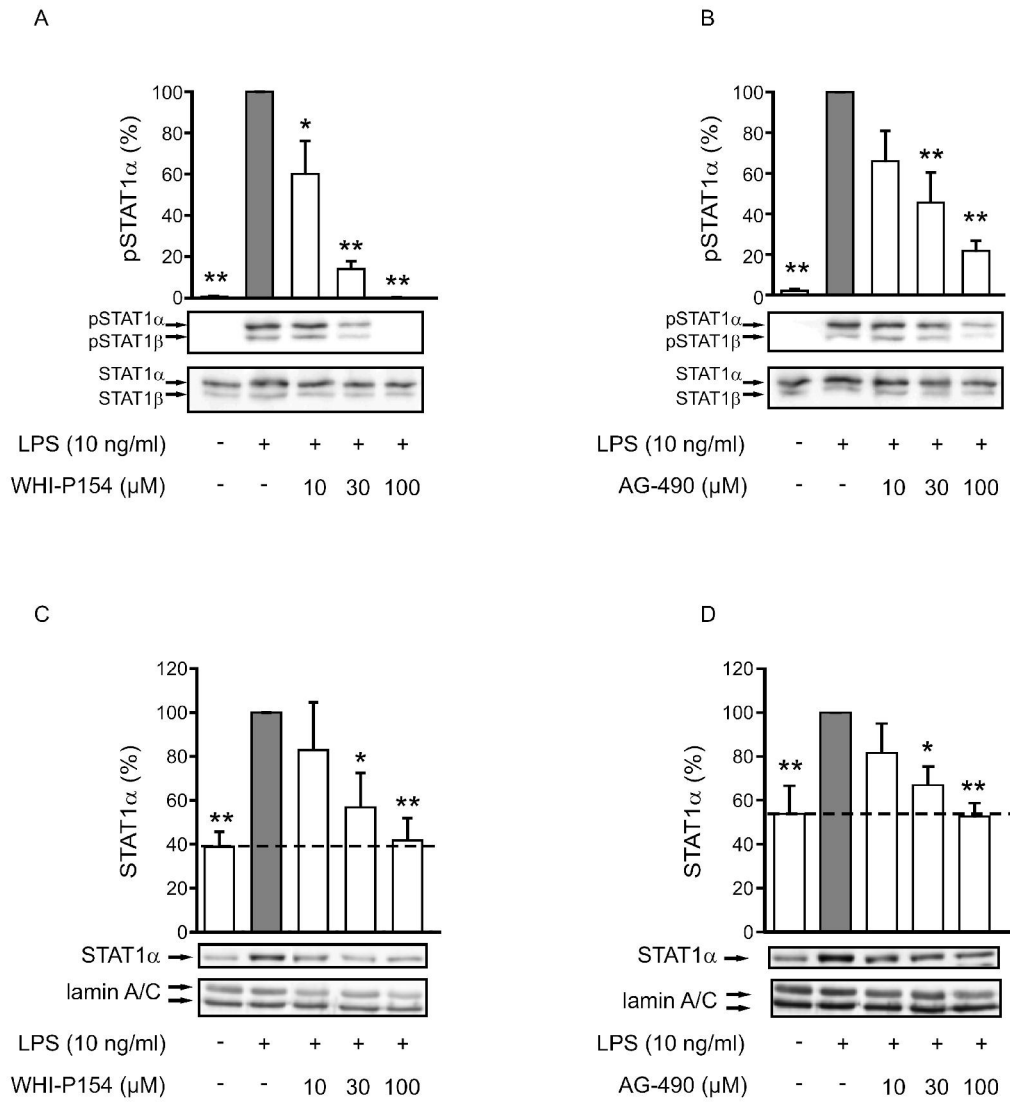


Figure 11. JAK inhibitors WHI-P154 and AG-490 decreased STAT1 activation in LPS-stimulated J774 macrophages. WHI-P154 and AG-490 inhibited STAT1α phosphorylation (A-B) and nuclear translocation (C-D). The values are mean + SEM, n=3 in A and n=6 in B-D. *P<0.05 and **P<0.01 when compared to cells treated with LPS only. (Reprinted with permission from Sareila et al. 2008 *Int Immunopharmacol* 8:100-108 © Elsevier Ltd.)

Inhibition of iNOS mRNA expression and murine iNOS promoter activity

The effects of JAK inhibitors on iNOS mRNA expression were studied. Both AG-490 and WHI-P154 decreased iNOS mRNA expression in J774 macrophages stimulated by either IFN γ or LPS (Fig. 12 A-B). The JAK inhibitors did not affect iNOS mRNA decay, when measured by actinomycin D assay (Fig. 12 C).

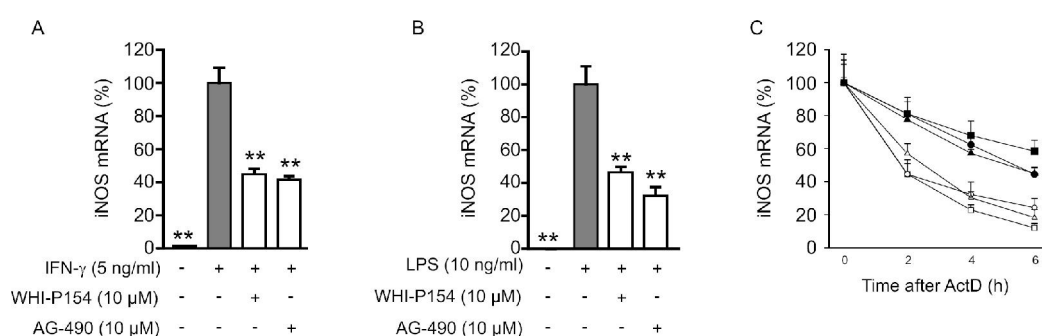


Figure 12. JAK inhibitors WHI-P154 and AG-490 inhibited iNOS mRNA expression but did not affect iNOS mRNA decay in J774 macrophages stimulated with IFN γ or LPS. $**P < 0.01$ when compared to cells treated with IFN γ (A) or LPS (B) alone. (C) Degradation of iNOS mRNA was measured by actinomycin D (ActD) assay. (\square = IFN γ 5 ng/ml, Δ = IFN γ + WHI-P154 10 μ M, \circ = IFN γ + AG-490 10 μ M, \blacksquare = LPS 10 ng/ml, \blacktriangle = LPS + WHI-P154 10 μ M, \bullet = LPS + AG-490 10 μ M). The results are expressed as mean + SEM, $n=3$. (Reprinted with permission from: Sareila et al. 2006, *Mediators Inflamm* 2006: Article ID 16161, 1–7 © Hindawi Publishing Corporation and Sareila et al. 2008 *Int Immunopharmacol* 8:100-108 © Elsevier Ltd.)

Since JAK inhibitors decreased iNOS mRNA expression but did not affect iNOS mRNA stability, the effect of JAK3 inhibitor WHI-P154 on murine iNOS promoter activity was studied. WHI-P154 was found to inhibit murine iNOS promoter activity in cytokine-stimulated L929 pGL4(miNOS-prom)neo cells, which express luciferase gene under the control of full length murine iNOS promoter (Fig. 13).

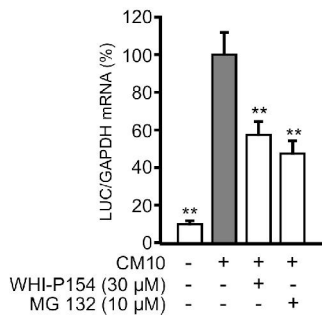


Figure 13. *JAK3* inhibitor WHI-154 decreased murine *iNOS* promoter activity in L929 pGL4(miNOS-prom)neo cells. The cells were stimulated with proinflammatory cytokines (CM10; IFN γ , TNF α and IL-1 β ; 10 ng/ml each) in the presence or in the absence of WHI-P154 or an NF- κ B inhibitor MG 132 (which was used as a control compound). Luciferase mRNA level (a marker of *iNOS* promoter activity) in LPS-treated cells was set as 100 % and the other values were related to that. The values are expressed as mean + SEM, n=4. ** P<0.01 when compared to cells treated with CM alone.

To test if *JAK3* inhibitor WHI-P154 affects the production of other inflammatory factors in J774 macrophages exposed to LPS, TNF- α production and COX-2 expression were studied. WHI-P154 (3–30 μ M) did not affect LPS-induced COX-2 expression, whereas TNF- α production was slightly decreased when 30 μ M WHI-P154 was added into the culture medium (Fig. 14).

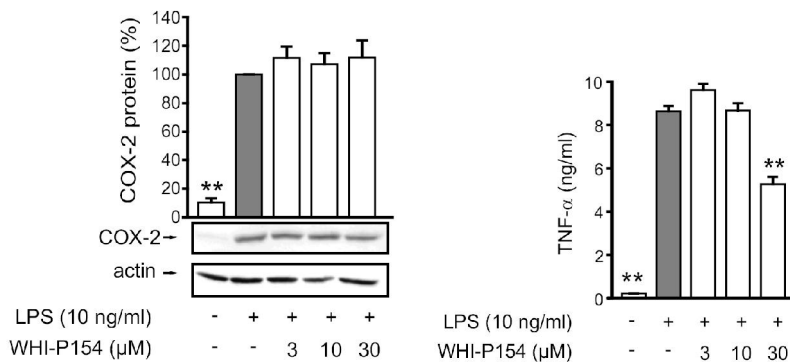


Figure 14. *JAK3* inhibitor WHI-P154 did not affect COX-2 protein expression but inhibited TNF- α production in J774 macrophages exposed to LPS. The results are expressed as mean + SEM, n=4-8. **P<0.01 when compared to cells treated with LPS only. (Reprinted with permission from Sareila et al. 2008 *Int Immunopharmacol* 8:100-108 © Elsevier Ltd.)

Orazipone inhibits the activation of transcription factors NF- κ B and STAT1, and the transcription of iNOS (III)

In Study III, the effects of orazipone (OR-1384), a novel thiol-modulating compound, on LPS-induced NO production in J774 macrophages was investigated. LPS-induced NO production was inhibited by OR-1384 in a concentration-dependent manner (Fig 15 A). Orazipone derivative OR-1958 had a similar effect but the non-thiol modulating control compound OR-2149 was ineffective. When orazipone was added into the culture 6 h after LPS, NO production was not altered. These data suggest that orazipone does not inhibit iNOS enzyme activity.

To study whether the reduction in NO production by orazipone is due to decreased iNOS protein expression, iNOS protein levels in LPS-stimulated J774 cells were measured by Western blot. Orazipone inhibited LPS-induced iNOS expression in a concentration-dependent manner (Fig. 15 B). The non-thiol modulating control compound OR-2149 did not affect LPS-induced iNOS expression.

Similar inhibition was observed when iNOS mRNA expression was studied. iNOS mRNA was measured by quantitative RT-PCR after 3 h incubation with a combination of LPS and the drugs. LPS-induced iNOS mRNA expression was dose-dependently inhibited by orazipone (OR-1384) and a significant reduction was also obtained with its derivative OR-1958 (Fig. 15 C). iNOS mRNA expression was not affected by the non-thiol modulating control compound OR-2149.

In addition to transcriptional regulation, iNOS expression in inflammatory cells is regulated at the level of iNOS mRNA degradation (Kleinert et al. 2004, Korhonen et al. 2005). The decay of iNOS mRNA was measured by actinomycin D assay. Orazipone did not affect iNOS mRNA decay (Fig. 15 D).

Orazipone inhibited the activation of NF- κ B (Fig. 16 A), which is an important transcription factor in iNOS expression (Xie et al. 1994). The non-thiol modulating control compound (OR-2149) did not alter LPS-induced NF- κ B activation. In addition to NF- κ B, transcription factor STAT1 mediates LPS-induced iNOS expression (Gao et al. 1998). Orazipone and its derivative

OR-1958 inhibited LPS-induced nuclear translocation of STAT1 α , whereas the non-thiol modulating control compound OR-2149 had no effect (Fig. 16 B).

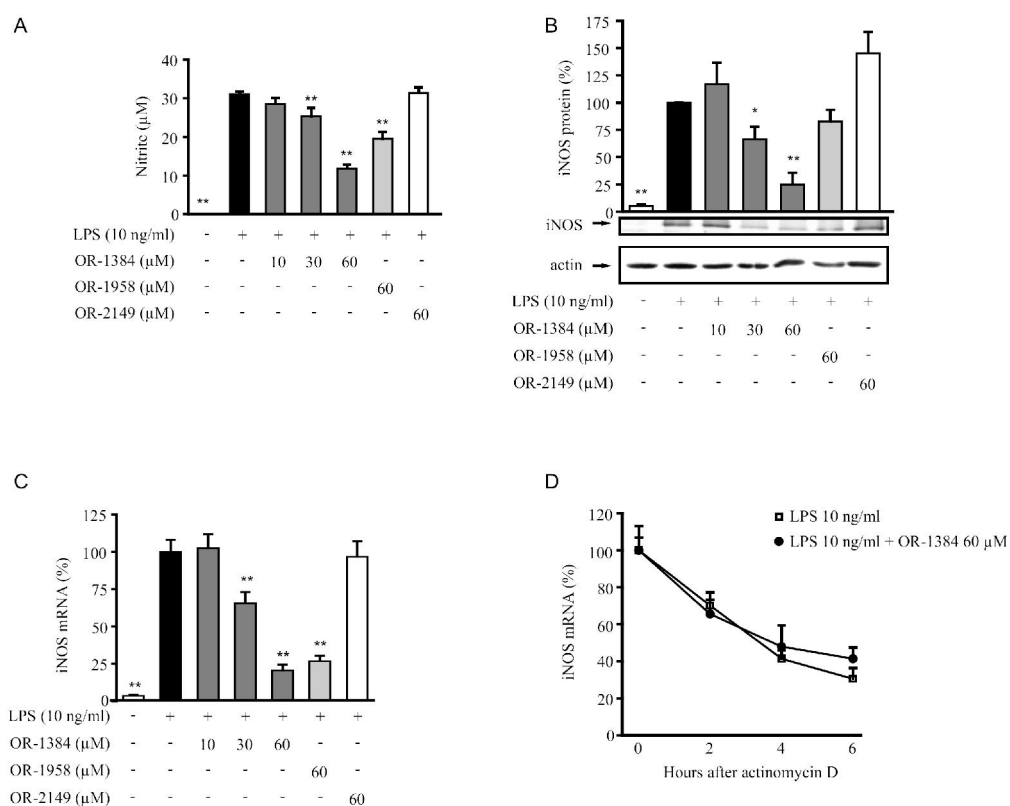


Figure 15. Orazipone (OR-1384) inhibited NO production (A), iNOS protein (B) and mRNA (C) expression, but did not affect iNOS mRNA decay (D) in LPS-stimulated J774 macrophages. Orazipone derivative OR-1958 had a similar effect as orazipone but the non-thiol modulating control compound OR-2149 was ineffective. The results are expressed as mean + SEM, $n=12-31$ in A and $n=3-7$ in B-D. (Reprinted with permission from Sareila et al. 2008, *J Pharmacol Exp Ther*, 324:858–866. © The American Society for Pharmacology and Experimental Therapeutics)

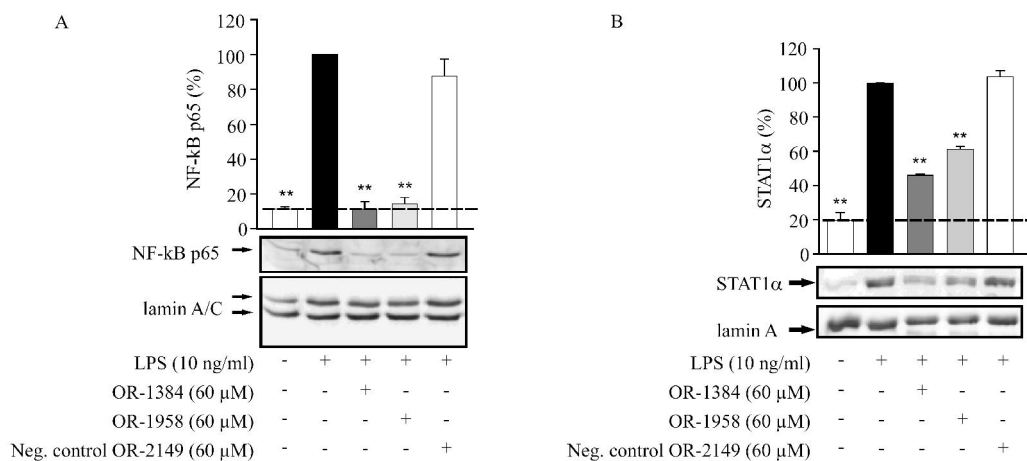


Figure 16. Orazipone (OR-1384) and its derivative OR-1958 inhibited nuclear translocation of NF- κ B (A) and STAT1 α (B) in LPS-stimulated J774 macrophages but the non-thiol modulating control compound OR-2149 was ineffective. Nuclear levels of NF- κ B p65 or STAT1 α in LPS-treated cells were set as 100 % and the other values were related to those values. The results are expressed as mean + SEM, n=3. ** P<0.01 when compared to cells treated with LPS alone. (Reprinted with permission from Sareila et al. 2008, *J Pharmacol Exp Ther*, 324:858–866. © The American Society for Pharmacology and Experimental Therapeutics)

To investigate the mechanism by which orazipone lowered iNOS expression and NO production, the effects of orazipone on iNOS and luciferase mRNA expression in human alveolar epithelial A549/8-pNOS2(16)Luc cells were studied. A549/8-pNOS2(16)Luc cells are genetically modified to express luciferase reporter gene under the control of full-length human iNOS promoter (16 kb) (Hausding et al. 2000). The cells were stimulated with a combination of proinflammatory cytokines TNF α , IFN γ and IL-1 β (10 ng/ml each). Cytokine mixture induced iNOS mRNA expression, which was inhibited by orazipone (OR-1384; 60 μ M) and PDTC (100 μ M; an NF- κ B inhibitor) (Fig. 17). In A549/8-pNOS2(16)Luc cells, luciferase gene expression is controlled by full length human iNOS promoter. In this experiment, luciferase mRNA was used as a reporter of iNOS promoter activity. Unstimulated cells expressed a basal level of luciferase mRNA as a marker of some iNOS promoter activity in resting cells, which was expected in light of earlier data (Kleinert et al. 2004). In order to examine cytokine-induced portion of luciferase mRNA expression, the basal expression was subtracted from the luciferase expression in cells stimulated with

cytokine mixture. The cytokine mixture induced luciferase mRNA expression, which was decreased by both orazipone and PDTC in a very similar manner (Fig. 17).

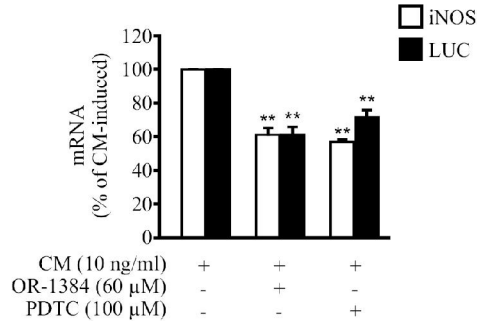


Figure 17. Orazipone (OR-1384) and an NF- κ B inhibitor PDTC inhibited iNOS mRNA expression and iNOS promoter activity in human alveolar epithelial A549/8-pNOS2(16)Luc cells. mRNA expression in cells stimulated by proinflammatory cytokines (CM) were set as 100 % and the other values were related to those values. The results are expressed as mean + SEM (n=3). ** P<0.01 when compared to cells treated with cytokine mixture alone. (Reprinted with permission from Sareila et al. 2008, *J Pharmacol Exp Ther*, 324:858–866. © The American Society for Pharmacology and Experimental Therapeutics)

Because orazipone reacts with thiols in e.g. glutathione, at least part of its effect on NO production could be mediated through inactivation of glutathione. The effects of orazipone on iNOS mRNA expression were eliminated when an excess of exogenously added thiols (N-acetyl-L-cysteine or glutathione) was present in the culture (Fig. 18). This observation supports the role of thiol-binding property of orazipone in the mechanism leading to lowered iNOS expression. Orazipone may react with glutathione and/or thiol groups in intracellular signal transduction proteins. This idea is further supported by the results showing that the non-thiol modulating derivative of orazipone (OR-2149) did not affect NF- κ B or STAT1 activation, iNOS expression or NO production.

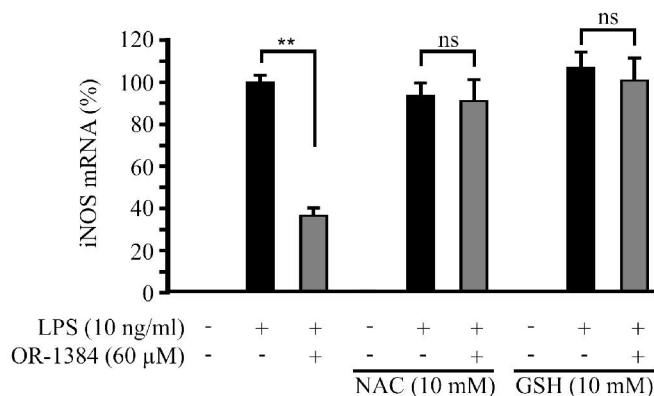


Figure 18. The inhibitory effect of orazipone (OR-1384) on LPS-induced iNOS mRNA expression was eliminated in the presence of exogenous thiols (N-acetyl-L-cysteine, NAC; glutathione, GSH) in J774 macrophages. LPS-induced iNOS mRNA expression was set as 100 % and the other values were related to that. The results are expressed as mean + SEM, n=3. ** P<0.01, ns = non significant. (Reprinted with permission from Sareila et al. 2008, *J Pharmacol Exp Ther*, 324:858–866. © The American Society for Pharmacology and Experimental Therapeutics)

Simedans decrease NO production by inhibiting iNOS promoter activity (IV)

Levosimendan is a calcium-sensitizer used for the treatment of decompensated heart failure (Mathew and Katz 1998). In addition, it has recently been reported to have beneficial effects in experimental models of septic shock (Oldner et al. 2001, Faivre et al. 2005). The effects of levosimendan, its stereoisomer dextro-simendan and their structurally related analog (OR-1560) on iNOS expression and NO production in murine macrophages were studied. Simedans (1–30 μM) inhibited LPS-induced NO production (Fig. 19) and iNOS protein expression in a dose-dependent manner (Fig. 20; only one concentration is shown).

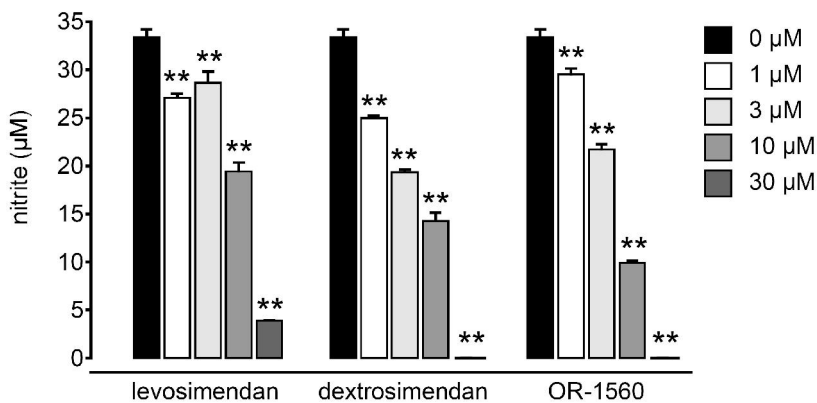


Figure 19. Simendans inhibited LPS-induced NO production in a dose-dependent manner in J774 macrophages. The cells were stimulated with LPS in the presence of the indicated concentrations of levosimendan, dextrosimendan or simendan analog OR-1560. NO production was not detected in un-stimulated cells. The results are expressed as mean + SEM, n=6. ** P<0.01 when compared to cells treated with LPS alone.

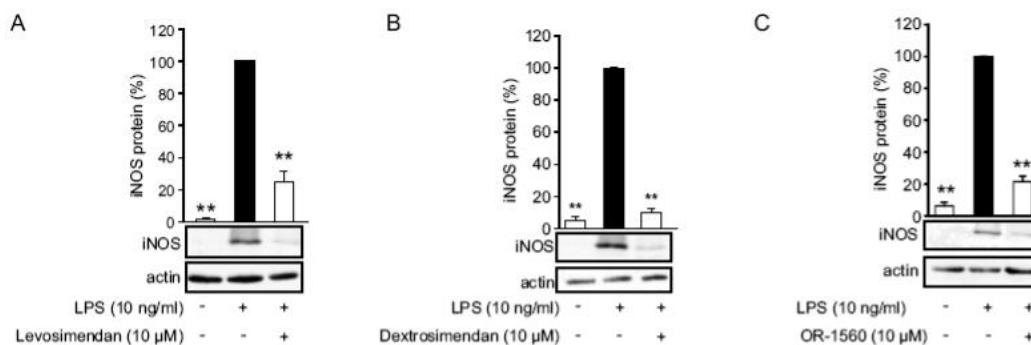


Figure 20. Simendans inhibited LPS-induced iNOS expression in J774 macrophages. LPS-induced iNOS protein expression was set as 100 % and the other values were related to those values. The values are expressed as means + SEM, n=3–6. ** P<0.01 when compared to cells treated with LPS alone.

Simendans were found to inhibit iNOS mRNA expression in both J774 macrophages (Fig. 21 A) and L929 fibroblasts. Simendans did not affect iNOS mRNA decay when measured by actinomycin D assay in LPS-stimulated J774 macrophages (Fig. 21 B) and in L929 fibroblasts stimulated with a combination of pro-inflammatory cytokines (TNF- α , IL-1 β and IFN γ).

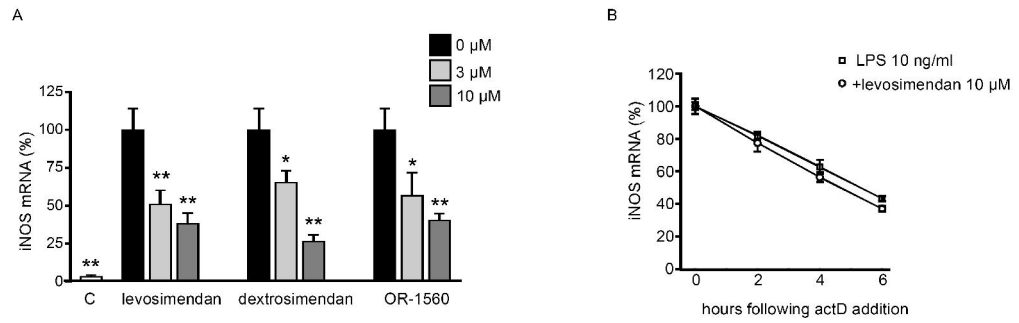


Figure 21. Simendans inhibited *iNOS* mRNA expression (A) but did not affect *iNOS* mRNA decay (B) in J774 macrophages. LPS-induced *iNOS* mRNA expression (A) or *iNOS* mRNA level at the time of addition of actinomycin D (ActD) (B) were set as 100 % and the other values were related to those values. The results are expressed as mean + SEM, $n=3-6$. C=unstimulated cells. ** $P<0.01$ and * $P<0.05$ when compared to cells treated with LPS alone.

Because simendans decreased *iNOS* mRNA expression, their effects on *iNOS* promoter activity were studied using stably transfected J774 macrophages and L929 fibroblasts, which expressed luciferase reporter gene under the control of full length murine *iNOS* promoter. Dextrosimendan and levosimendan (10 μM) decreased *iNOS* promoter activity (Fig. 22) and the effect was comparable to that of an NF- κB inhibitor MG 132, which was used as a control compound (Fig. 22 B).

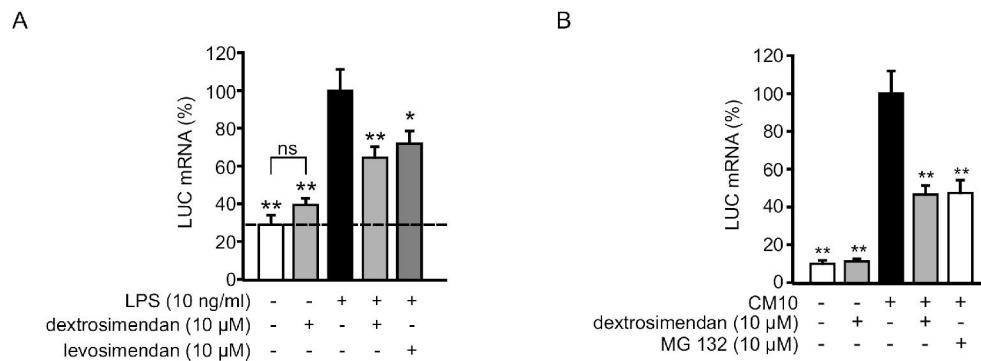


Figure 22. Simendans inhibited *iNOS* promoter activity in J774 (A) and L929 (B) cells stably transfected with the construct *pGLA(miNOS-prom)neo*. Luciferase (LUC) mRNA levels (a marker of *iNOS* promoter activity) in stimulated cells were set as 100 % and the other values were related to those values. The results are expressed as mean + SEM, $n=4$. * $P<0.05$ and ** $P<0.01$ when compared to LPS/CM10-induced levels. (CM10=cytokine mixture; ns=non significant)

Because simendans decreased iNOS mRNA and protein expression, their effects on NF- κ B and STAT1, the important transcription factors for iNOS expression, were studied. Nuclear translocation was measured by Western blot and DNA binding activity was measured by EMSA. Simendan concentrations that decreased iNOS expression were found to be ineffective when LPS-induced nuclear translocation of NF- κ B or STAT1 α was studied. Likewise, the DNA binding activity of transcription factors NF- κ B and STAT1 was not affected in the presence of simendans (Fig. 23).

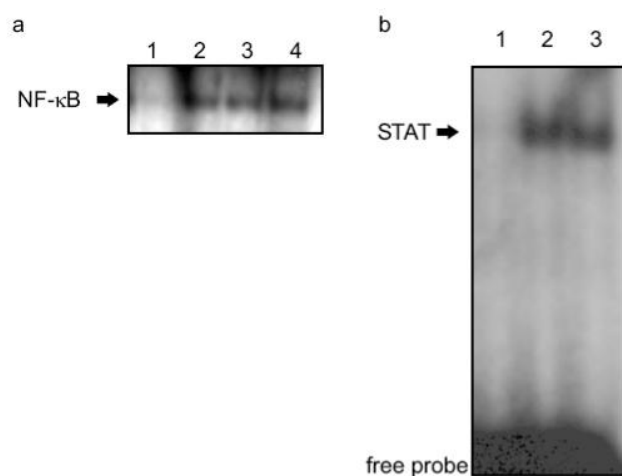


Figure 23. Simendans did not affect NF- κ B and STAT1 activation in J774 macrophages as measured by EMSA. (a) 1. control, 2. LPS, 3. LPS + dextrosimendan, 4. LPS + levosimendan. (b) 1. control, 2. LPS + IFN γ , 3. LPS + IFN γ + dextrosimendan. One representative blot of three experiments with similar results is shown.

The effects of simendans on the transcriptional activities of NF- κ B and STAT1 were studied using cell lines stably transfected with luciferase reporter constructs. In L929-pNF κ B(luc)neo cell line expressing luciferase reporter gene under the control of NF- κ B-responsive promoter, levo- and dextrosimendan were found to decrease NF- κ B-dependent transcription induced by proinflammatory cytokines (Fig. 24). Simendans did not modulate GAS-mediated luciferase mRNA expression in activated L929-pGAS(luc)neo cells (Fig. 24).

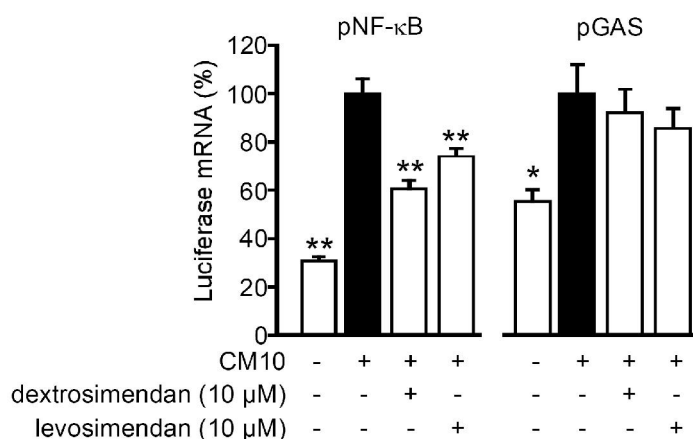


Figure 24. Simendans inhibited NF-κB-mediated but not GAS-mediated transcription. Cytokine-induced luciferase mRNA expression (a marker of promoter activity) in L929-pNFκB(luc)neo and L929-pGAS(luc)neo cells were separately set as 100 % and the other values in the same cell line were related to those values. The results are expressed as mean + SEM, n=3. * P<0.05 and ** P<0.01 when compared with cells treated with cytokine mixture alone.

Comparison of the inhibitors used in the present study

The effects of a series of compounds on iNOS expression and NO production in murine J774 macrophages were studied. Murine macrophages were stimulated with either IFNγ or LPS. All compounds, except the negative control compound of orazipone (OR-2149), were found to inhibit NO production and iNOS expression (Table 4). Different mechanisms were found to be involved in the down-regulation of iNOS expression and NO production when different inhibitors were used. NF-κB activation was inhibited by orazipone and its analog, but JAK inhibitors and simendans did not affect NF-κB activation. STAT1 activation was decreased by orazipone and its analog and JAK inhibitors WHI-P154 and AG-490. Even though simendans did not affect activation (i.e. nuclear translocation) or DNA binding of NF-κB or STAT as measured by Western blot or EMSA in nuclear extracts, they inhibited NF-κB-dependent transcription along with their inhibitory effect on iNOS promoter activity. Although regulation of iNOS mRNA stability is an important mechanism regulating iNOS expression, none of the used compounds affected the decay of

iNOS mRNA. Differential effects on COX-2 expression and TNF- α production were observed.

Table 4. *Effects of the compounds used on iNOS mRNA and protein expression, on NO production and on the activation of the transcription factors regulating iNOS expression in J774 macrophages. The effects on COX-2 expression and on TNF- α production are also shown. (\downarrow = inhibitory effect, \uparrow = increasing effect and \leftrightarrow = no effect; nd = not determined)*

inhibitor	Effect on							
	activation of		iNOS mRNA expression	iNOS mRNA decay	iNOS protein expression	NO production	COX-2 expression	TNF- α production
	STAT1	NF- κ B						
AG-490	\downarrow	\leftrightarrow	\downarrow	\leftrightarrow	\downarrow	\downarrow	\uparrow	\uparrow
WHI-P154	\downarrow	\leftrightarrow	\downarrow	\leftrightarrow	\downarrow	\downarrow	\leftrightarrow	\downarrow
WHI-P131	nd	nd	nd	nd	\downarrow	\downarrow	\downarrow	\downarrow
Orazipone	\downarrow	\downarrow	\downarrow	\leftrightarrow	\downarrow	\downarrow	nd	nd
OR-1958	\downarrow	\downarrow	\downarrow	\leftrightarrow	\downarrow	\downarrow	nd	nd
OR-2149	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	nd	nd
PDTC	nd	\downarrow	\downarrow	nd	\downarrow	\downarrow	nd	nd
L-simendan	\leftrightarrow	\downarrow^*	\downarrow	\leftrightarrow	\downarrow	\downarrow	\uparrow	\leftrightarrow
D-simendan	\leftrightarrow	\downarrow^*	\downarrow	\leftrightarrow	\downarrow	\downarrow	\uparrow	\leftrightarrow
OR-1560	\leftrightarrow	nd	\downarrow	\leftrightarrow	\downarrow	\downarrow	\uparrow	\leftrightarrow

* NF- κ B-responsive promoter activity was inhibited even though the activation of NF- κ B was not affected when measured by nuclear translocation or EMSA.

Discussion

Methodology

In cell culture conditions, most of the NO produced is oxidized to nitrite instead of the oxidation product nitrate in e.g. blood (Ignarro et al. 1993, Moshage et al. 1995). Therefore nitrite was measured as a marker of NO production in cell culture media. The contribution of the nitrite and/or phenol red and other possible interfering substrates in the cell culture media was eliminated by diluting the standards of the nitrite measurement into the complete cell culture medium used in the experiment. In addition, iNOS inhibitor 1400W (Garvey et al. 1997) was used to ensure that nitrite production observed was prevented when iNOS inhibitor was added to the cells.

The cytotoxicity of the drugs was ruled out by modified XTT test (Cell Proliferation Kit II) which measures the metabolic activity of viable cells. This assay was used to exclude the possibility that the observed effect could be due to reduced cell viability. In some experimental setups, the integrity of nuclear lamin (the fragmentation of which is widely used as a marker of apoptosis) was used to confirm that cells were not apoptotic after drug treatments.

Standard molecular biology methods were used to measure protein expression (Western blot) and RNA levels (quantitative real time RT-PCR). Western blot of nuclear extracts was used to measure the presence of a certain transcription factor in the nuclear compartment at a certain time point. The protein level of the transcription factor in the nucleus in the presence of an inhibitor was compared to the protein level in the absence of the inhibitor. The transcription factors measured were NF- κ B and STAT1. Upon activation, NF- κ B is released from its inhibitory subunit (Doyle and O'Neill 2006) and STAT1 is phosphorylated (Kisseleva et al. 2002). The transcription factors translocate to the nucleus only when these activation events have occurred. This method was used to measure the inhibition of events related to the activation of the

transcription factor prior to their entry into the nucleus. The method ignores the possibility that the DNA binding activity of the transcription factor may have been affected. Therefore, activation and DNA binding of transcription factors were also measured by electrophoretic mobility shift analysis (EMSA) in certain experiments. The nuclear proteins were extracted and subjected to EMSA analysis, which means that (in the case of NF- κ B and STAT1) only the activated portion of those transcription factors was present in the assay. EMSA measures the binding activity of an activated transcription factor to a specific DNA fragment. However, mechanisms that do not affect DNA binding activity but affect the transcriptional activity (e.g. protein modification by phosphorylation) could not be excluded in EMSA. Therefore, the effects of levo- and dextrosimendan on NF- κ B and STAT1 were further studied in cell lines containing luciferase reporter constructs for NF- κ B and STAT1 responsive promoters.

Actinomycin D is widely used as an inhibitor of transcription, and actinomycin D assay is a standard method used to study mRNA decay. In the present study, actinomycin D assay was used to evaluate the decay of mRNA when transcription was blocked and the remaining mRNA levels were measured by quantitative RT-PCR. Our group has previously reported that actinomycin D may have a stabilizing effect on iNOS mRNA in J774.2 macrophages (Lahti et al. 2006). However, this effect was not observed in J774 macrophages in the present study supporting that actinomycin D assay could be reliably used to evaluate mRNA decay.

In the present study, luciferase mRNA expression was used to assess the activity of iNOS promoter and NF- κ B or STAT1 responsive promoters, which had been cloned at the front of the luciferase gene and the constructs had been transfected into L929 fibroblasts or into J774 macrophages. Measurement of luciferase mRNA levels instead of luciferase enzyme activity rule out the possibility that the drugs could affect the activity of the enzyme, folding of the newly synthesized protein, post-translational mechanisms or translation efficiency. Some chemical compounds have been shown to affect luciferase activity but not luciferase mRNA expression (Deroo and Archer 2002). In the present study, the effects of the drugs used on luciferase mRNA stability could

not be ruled out. However, luciferase is a commonly used reporter gene and the plasmids into which the promoters were cloned were designed for purposes of studying expression.

The importance of JAK-STAT pathway in the induction of iNOS expression following stimulation with either IFN γ or LPS

In the present study, JAK3 inhibitor WHI-P154 inhibited iNOS expression and NO production in a dose-dependent manner in both IFN γ - and LPS-stimulated J774 macrophages. JAK3 inhibition attenuated iNOS mRNA expression but did not affect iNOS mRNA decay, suggesting that JAK3 is involved in the transcriptional regulation of iNOS expression, which was a novel finding. The results confirmed recent observations that JAK2 inhibitor AG-490 inhibits both LPS- and IFN γ -induced iNOS expression in murine macrophages (Blanchette et al. 2003, Uto et al. 2005, Blanchette et al. 2007). Similar inhibition of LPS-induced iNOS expression or NO production by AG-490 has been reported in a mouse fetal skin dendritic cell line (Cruz et al. 1999, Cruz et al. 2001). IFN γ -induced iNOS expression or NO production have also been reported to be inhibited by AG-490 in primary astroglial cells (Dell'Albani et al. 2001) and in TH2.52 murine CD5⁺ B1-like cells (Koide et al. 2003). In the present study, JAK3 inhibitor WHI-P154 was found also to inhibit NO production and iNOS expression in cytokine-treated human A549 epithelial cells, indicating that it affects the upregulation of both human and murine iNOS.

In the present study, stimulation of J774 macrophages by either IFN γ or LPS led to the phosphorylation and nuclear translocation of STAT1, which was markedly attenuated by JAK3 inhibitor WHI-P154 and JAK2 inhibitor AG-490. On a molar basis WHI-P154 was a somewhat more potent inhibitor than AG-490. Similarly to the present results, AG-490 has previously been shown to prevent JAK2 phosphorylation and to decrease STAT1 phosphorylation in J774 cells (Blanchette et al. 2003) and to decrease activation of STAT1 pathway in B-cell chronic lymphocytic leukemia cells (Martinez-Lostao et al. 2005). Inhibition of STAT1 activation may well explain the suppressive effect of the

JAK inhibitors on iNOS levels, since LPS-induced iNOS expression was severely impaired in macrophages prepared from STAT1^{-/-} mice (Ohmori and Hamilton 2001) and LPS/IFN γ -induced iNOS expression and NO production was reduced in fibroblasts obtained from STAT1^{-/-} mice (Samardzic et al. 2001). STAT1 binding site (IFN γ -activated site, GAS) is necessary for full expression of iNOS in response to IFN γ and LPS (Meraz et al. 1996, Gao et al. 1997). Recently, STAT1 knock-down by siRNA or pharmacologic inhibition of JAKs was reported to lead to impaired induction of iNOS in rat intestinal epithelial cells stimulated by IFN γ and LPS (Stempelj et al. 2007). Consistent with these reports and the other results in the present study, WHI-P154 was found to inhibit murine iNOS promoter activity when studied in L929 pGL4(miNOS-prom)neo cell line.

LPS-induced iNOS expression and NO production have been reported to be inhibited by an isoquinoline compound THI 53 (2-naphtylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) in murine RAW 264.7 macrophages. Similarly to the present results, iNOS expression was shown to be regulated by STAT1 activity. In addition, THI 53 inhibited NF- κ B activation in macrophages and decreased plasma nitrite levels in mice exposed to LPS (Kim et al. 2007). Pharmacological inhibitors of JAK2 inhibited cytokine-induced STAT1 DNA binding, human iNOS promoter activity and iNOS expression in A549 human lung epithelial cells (Kleinert et al. 1998, Kleinert et al. 1999), and expression of a dominant negative mutant of STAT1 inhibited cytokine-induced human iNOS promoter activity (Ganster et al. 2001).

WHI-P154 was designed to specifically inhibit JAK3, and it has been shown to inhibit IL-2-triggered JAK3-dependent STAT activation in 32Dc11-IL-2R β -cells (Sudbeck et al. 1999). WHI-P131 (a WHI-P154-related JAK3 inhibitor), has been shown to inhibit STAT1 activation in B-cell chronic lymphocytic leukemia cells, in platelets and in mesenchymal stem cells (Tibbles et al. 2001, Martinez-Lostao et al. 2005, Song et al. 2005). WHI-P154 and WHI-P131 have been shown to be fairly specific for JAK3 and not to significantly inhibit JAK1, JAK2 or SYK (spleen tyrosine kinase), LYN (protein tyrosine kinase related to LCK and YES), BTK (Bruton's tyrosine kinase) and IRK (insulin receptor kinase) within the concentration range used in the present study (Goodman et al.

1998, Sudbeck et al. 1999). AG-490 have been reported to inhibit JAK3 in addition to inhibiting JAK2 (Wang et al. 1999, Kirken et al. 1999, Amin et al. 2003).

In addition to transcriptional regulation, iNOS expression is critically regulated at post-transcriptional level (Kleinert et al. 2004, Korhonen et al. 2005). In murine J774 macrophages, dexamethasone and SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), have been shown to reduce LPS-induced iNOS expression by destabilizing the mRNA (Korhonen et al. 2002, Lahti et al. 2003). By contrast, IFN γ has been shown to delay iNOS mRNA degradation when compared to iNOS mRNA induced by LPS alone (Korhonen et al. 2002). In the present study, the effects of WHI-P154 and AG-490 on iNOS mRNA decay were tested with actinomycin D assay. The decay of iNOS mRNA was not altered in the presence of JAK inhibitors in either IFN γ - or LPS-stimulated macrophages. However, both WHI-P154 and AG-490 reduced iNOS mRNA levels significantly. When the inhibitors were added to the culture 6 h after the stimulus, NO production was comparable to that induced by IFN γ or LPS alone. These data together suggest that the JAK inhibitors tested decreased iNOS transcription but did not regulate the mRNA degradation or affect iNOS enzyme activity.

The importance of NF- κ B in the induction of iNOS expression following inflammatory stimuli

LPS is a strong activator of the innate immune response and activates NF- κ B through Toll-like receptor pathway (Doyle and O'Neill 2006). NF- κ B is an important transcription factor in iNOS expression (Xie et al. 1994). In the present study, LPS was found to activate NF- κ B and induce iNOS expression in macrophages. JAK3 inhibitor WHI-P154 and JAK2 inhibitor AG-490 reduced iNOS expression without decreasing NF- κ B activation. Simendans (10 μ M) significantly inhibited iNOS expression and NO production in LPS-stimulated J774 macrophages. However, simendans did not inhibit NF- κ B activation when the nuclear translocation was measured by Western blot or when the DNA binding activity was measured by EMSA. By contrast, simendans were found to

inhibit NF- κ B responsive promoter activity and NF- κ B-dependent transcription, which may well explain the inhibitory effects of simendans on iNOS expression and NO production. Orazipone and its analog OR-1958 were potent inhibitors of NF- κ B and iNOS expression. Similarly, an NF- κ B inhibitor PDTC was shown to inhibit nuclear translocation of NF- κ B along with its inhibitory effect on iNOS promoter activity and iNOS mRNA expression. PDTC has earlier been demonstrated to inhibit LPS-induced NO-production in J774 macrophages (Ruetten et al. 1999, Vuolteenaho et al. 2001). These results together suggest that inhibition of NF- κ B leads to reduced iNOS transcription, but a significant reduction in iNOS expression can be achieved without affecting the signalling cascade leading to NF- κ B activation in J774 macrophages.

Orazipone inhibits activation of NF- κ B and STAT1, and iNOS expression

In Study III, orazipone was found to decrease iNOS expression and NO production in a concentration dependent manner in activated macrophages, which was a novel finding. LPS-induced NF- κ B activation and iNOS mRNA accumulation were inhibited by orazipone when measured after 30 min and 3 h incubation respectively. Orazipone inhibited human iNOS promoter activity in A549/8-pNOS2(16)Luc human epithelial cells. In these cells, orazipone and PDTC (an NF- κ B inhibitor) inhibited the expression of a reporter gene which was under the control of full length human iNOS promoter similarly as the expression of iNOS and the production of NO. NF- κ B is known to be an important transcription factor in human and murine iNOS expression (Kleinert et al. 2004, Korhonen et al. 2005) and inhibition of NF- κ B by orazipone or PDTC resulted in decreased iNOS mRNA expression along with inhibition of iNOS promoter activity. These data together suggest that inhibition of NF- κ B activation by orazipone is likely to contribute to its ability to inhibit iNOS expression and NO production.

Orazipone is a novel sulfhydryl modulating compound that forms reversible conjugates with thiol groups in proteins and glutathione and has anti-

inflammatory properties (Wroblewski et al. 1998). LPS-induced NO production has been shown to be decreased in murine RAW264.7 macrophages in which glutathione was depleted by inhibiting its synthesis (Srisook and Cha 2005). Because orazipone reacts with thiols in e.g. glutathione, at least part of its effect on NO production could be mediated through inactivation of glutathione. In the present study, the effects of orazipone were eliminated when an excess of glutathione or N-acetyl-L-cysteine was present in the culture, supporting the assumption that the effects of orazipone are mediated through its reactions with glutathione and/or thiol groups in intracellular signal transduction proteins. This idea is further supported by the present results showing that the non-thiol modulating derivative of orazipone (OR-2149) did not affect NF- κ B or STAT1 activation, iNOS expression or NO production. Recently, protein glutathionylation has been observed as an important post-translational modification which serves to transduce redox signals (Ghezzi 2005, Gallogly and Mieyal 2007). Therefore the existing literature was studied to ascertain if glutathionylation of transcription factors NF- κ B or STAT1 has been reported to have functional consequences which could explain the present results. No reports on glutathionylation of STAT1 were found. Glutathionylation of p50 subunit of NF- κ B has been reported to inhibit its DNA binding (Pineda-Molina et al. 2001). However, in the present study, orazipone was found to inhibit nuclear translocation of NF- κ B. Another explanation is that orazipone targets some of the TLR4-receptor activated signalling mechanisms upstream of NF- κ B and STAT1 either by a mechanism related or unrelated to glutathionylation.

In the present study, orazipone extensively reduced NF- κ B activation in J774 macrophages when measured after 30 min and iNOS mRNA expression when measured after 3 h incubation, whereas it was less potent as an inhibitor of iNOS protein expression and NO production which were determined after 24 h incubation. When the cells were incubated with LPS and orazipone for only 4 h to induce iNOS expression and then cultured in fresh medium (without LPS and orazipone) for another 20 h to collect the produced nitrite, orazipone was more potent as an inhibitor of NO production than when the cells were incubated with LPS and orazipone for 24 h. These observations may be explained by the chemical properties of orazipone. The reaction between glutathione and the thiol

modulating compounds orazipone and OR-1958 have been shown to be reversible (Nissinen et al. 1997). In addition, orazipone has been described to be labile in aqueous solutions (Vendelin et al. 2005). Therefore orazipone and OR-1958 may be partly degraded or their effects deteriorated during a prolonged time in the culture, and while LPS was present its effects may have overcome those of orazipone. It is possible that the experimental conditions (24 h incubations) may underestimate the potency of orazipone. This is in line with the studies in colitis models, where orazipone seemed to be more potent when administered locally (intracolonic administration) than when given systemically (Wroblewski et al. 1998).

To summarize, orazipone was found to inhibit iNOS expression and NO production and to inhibit transcription factors NF- κ B and STAT1 in cells activated by inflammatory stimuli. These observations may at least partly explain the anti-inflammatory effects of orazipone reported in experimental models of inflammatory diseases.

Simendans inhibit iNOS expression

In the present study, levosimendan and its enantiomer dextrosimendan were found to decrease NO production in macrophages and fibroblasts exposed to inflammatory stimuli, which was a novel finding. The effects of simendans were not cell type specific, since iNOS expression was inhibited in both macrophages and fibroblasts. The results suggest that simendans did not inhibit the activity of iNOS enzyme although they decreased iNOS protein and mRNA expression. Simendans did not increase iNOS mRNA decay when determined by actinomycin D assay. Nor did simendans inhibit activation or DNA binding of NF- κ B or STAT1, which are important transcription factors for iNOS, but they did inhibit the activity of NF- κ B responsive promoter in luciferase reporter assay. In addition, levo- and dextrosimendan were found to decrease iNOS promoter activity when determined by luciferase reporter assays in stably transfected macrophages and fibroblasts. There are several reports describing the regulation of the murine iNOS promoter by other transcription factors such as

IRF1 (positively) and IRF2 (negatively) (Kamijo et al. 1994, Coccia et al. 2000, Kielar et al. 2000), octamer factors (positively) (Goldring et al. 1996, Sawada et al. 1997), HIF-1 and NF-IL6 (both positively) (Dlaska and Weiss 1999), STAT3 (negatively) (Yu et al. 2002) and USF1/2 (negatively) (Gupta and Kone 2002). Even though the inhibitory effects of simendans on NF- κ B responsive promoter may explain their inhibitory effects on iNOS expression, it is possible that simendans may also regulate iNOS promoter activity by modifying the activity of the additional transcription factors mentioned above. According to the preliminary results obtained during the present study, simendans did not alter LPS-induced IRF1 activation in J774 macrophages.

In the present study, iNOS expression and NO production were significantly inhibited by simendans at 10 μ M concentrations, whereas these concentrations of simendans had smaller effects on IL-6 production and practically no effect on TNF- α synthesis. These results suggest that the anti-inflammatory effects of simendans are focused and regulatory and not non-specific in nature. Similar results have been obtained in patients with decompensated chronic heart failure, when levosimendan treatment was shown to decrease serum IL-6 levels but not TNF- α levels (Avgeropoulou et al. 2005).

Levosimendan sensitizes troponin C to calcium in the myocardium. Moreover, it acts as an inhibitor of phosphodiesterases (Szilagyi et al. 2005) and opens K_{ATP} channels (Yokoshiki et al. 1997). Levosimendan has been shown to have about 10-fold affinity to myocardial troponin C compared to its stereoisomer dextrosimendan (Sorsa et al. 2004). Accordingly, levosimendan was found to be a more potent calcium sensitizer than dextrosimendan (levosimendan was 76 times more potent) and the difference was even greater when the inhibitory effects on PDE III were studied (levosimendan was 427 times more potent) (Kaheinen et al. 2006). In the present study, the potencies of dextrosimendan and levosimendan were similar when their inhibitory effects on iNOS expression and NO production were studied. Therefore it is unlikely that binding to troponin C or PDE inhibition is involved in the mechanism by which simendans inhibit iNOS expression. Levo- and dextrosimendan have been shown to act as K_{ATP} channel openers at similar concentrations (Haikala et al. 2005). K_{ATP} channel openers diazoxide and iptakalim have been reported to decrease

iNOS expression in BV-2 cells (Liu et al. 2005) and in rats (Yang et al. 2006) exposed to rotenone. Therefore it is possible that opening of K_{ATP} channels contributes to the mechanism by which simendans decreased iNOS expression in the present study.

Comparison of the mechanisms of JAK inhibitors, orazipones and simendans in the inhibition of iNOS expression

The pharmacological inhibitors tested, AG-490, WHI-P154, orazipone, orazipone derivative OR-1958 together with both levo- and dextrosimendan and their analog OR-1560, were able to inhibit iNOS expression and NO production in activated macrophages. The mechanisms by which the inhibitors caused lowered iNOS protein levels were diverse. JAK inhibitors AG-490 and WHI-P154 are known inhibitors of kinases upstream of STAT1 and therefore decreased the activation of STAT1, which is an important transcription factor for iNOS. These JAK inhibitors were unable to inhibit the activation of NF- κ B, which is usually considered the most important transcription factor in inflammation related regulation of gene transcription including iNOS (Barnes and Karin 1997, Karin 2005).

Orazipone and its derivative were potent inhibitors of NF- κ B, while their inhibitory effect on STAT1 activation was moderate. The negative control compound of orazipone, OR-2149, was unable to inhibit either NF- κ B or STAT1. In addition, iNOS expression was not affected by this derivative, which, unlike orazipone, is not a sulfhydryl modifying compound. The effects of orazipone on iNOS expression were eliminated in the presence of exogenously added thiols. These results suggest that sulfhydryl groups are involved in the regulation of iNOS expression and in the mechanisms of action of orazipone.

Levo- and dextrosimendans inhibited iNOS expression whereas they did not alter the activation or DNA binding of NF- κ B or STAT1, the important transcription factors in iNOS expression. However, simendans decreased the activities of the NF- κ B responsive promoter and iNOS promoter when studied in genetically modified cell lines. Simendans did not seem to increase the decay of

iNOS mRNA. These results imply that simedans affected iNOS expression at transcriptional level by inhibiting the transcriptional activity of NF- κ B. It is possible that simendans may additionally alter the transcriptional activity of the other transcription factors involved in iNOS expression. Other proteins in the transcription complex are also possible targets of simendans even though simendans did not seem to be general transcriptional inhibitors.

Taken together, JAK inhibitors, orazipone and its derivative OR-1958 and levo- and dextrosimendan inhibited iNOS expression at the level of transcription, which is a well known regulation step in iNOS expression. Regulation of mRNA stability is also a common mechanism by which iNOS expression is regulated (Kleinert et al. 2004, Korhonen et al. 2005). However, none of the drugs were found to affect iNOS mRNA decay when determined by actinomycin D assay.

Nitric oxide generation as a target of drug treatment in inflammatory diseases

Even though NO has several regulatory functions and participates in host defence mechanisms, overproduction of NO is associated with various diseases including arthritis (Cuzzocrea 2006, Vuolteenaho et al. 2007), asthma (Ricciardolo et al. 2004), inflammatory bowel disease (Kolios et al. 2004), cardiac disorders (Rastaldo et al. 2007) and septic shock (Kirkebøen and Strand 1999). In the present study, a novel drug candidate orazipone and a calcium sensitizer levosimendan (used in the treatment of decompensated heart failure) were found to inhibit iNOS expression and NO production in cells activated by inflammatory stimuli.

At least some of the beneficial effects of orazipone in colitis (Wroblewski et al. 1998) could be explained by lowered iNOS expression and NO production, since selective inhibition of iNOS has been shown to relieve inflammation in a 2,4,6-trinitrobenzenesulfonic acid induced model of colitis (Kankuri et al. 2001, Menchen et al. 2001) and in dextran sodium sulphate induced colitis in rats (Rumi et al. 2004) and in mice (Di Paola et al. 2005). Inhibition of iNOS has also been reported to inhibit the production of inflammatory mediators COX-2, TNF- α and IL-1 β and to attenuate inflammation-related large bowel

carcinogenesis in a dextran sodium sulphate induced model of colitis in mice (Kohno et al. 2007). Orazipone has previously been shown to inhibit IL-1 β , TNF- α and IL-6 production in human monocytes (Wroblewski et al. 1998) and TNF- α production in human mast cell line HMC-1 (Vendelin et al. 2005). The inhibitory effects of orazipone on the activation of NF- κ B and STAT1 observed in the present study are likely involved in the general anti-inflammatory mechanisms of orazipone because those transcription factors regulate the expression of various inflammatory factors in addition to iNOS. Moreover, suppression of NF- κ B-mediated transcription is involved in the anti-inflammatory effects of glucocorticoids (Adcock et al. 2004).

Orazipone has been shown to have beneficial effects in an animal model of asthma (Ruotsalainen et al. 2000) and to induce apoptosis in human eosinophils (Kankaanranta et al. 2006). The iNOS inhibitor 1400W was shown to attenuate bronchoconstriction and inflammatory and remodelling processes in a guinea pig model of chronic allergic pulmonary inflammation (Prado et al. 2006). In contrast, selective iNOS inhibitor GW271540 did not affect airway hyperreactivity or airway inflammatory cell numbers after allergen challenge in asthmatic patients (Singh et al. 2007). In light of these observations, iNOS inhibition by orazipone may possibly have an influence on the beneficial effects of orazipone in animal models of asthma but the effect of iNOS inhibition in asthmatic patients remains to be ascertained.

In the present study, levosimendan, a calcium sensitizer used in the treatment of decompensated heart failure, was found to inhibit iNOS expression and NO production in response to proinflammatory stimuli. Levosimendan has been reported to possess anti-inflammatory properties (Paraskevaidis et al. 2005). It was shown to decrease the concentrations of proinflammatory cytokines, TNF- α receptors and soluble FAS in serum from patients with severe heart failure (Trikas et al. 2006). Levosimendan has also been shown to reduce plasma levels of TNF- α , IL-6, soluble FAS, soluble FAS ligand and N-terminal pro-B-type natriuretic peptide in patients with heart failure (Parissis et al. 2004, Kyrzopoulos et al. 2005). In addition, simendans reduced swelling when carrageenan-induced paw edema was used as a model of acute inflammation in rats (Haikala et al. 2005). Simendans were also shown to reduce lung eosinophilia in a mouse model

of allergic inflammation and to induce apoptosis in human eosinophils (Kankaanranta et al. 2007).

It has been demonstrated that NO, at least partly, mediates the negative inotropic effect of cytokines on the heart (Finkel et al. 1992) and that overproduction of NO is harmful in the heart in the systemic inflammation response syndrome (Ungureanu-Longrois et al. 1995). Heart failure is a complex state caused by several mediators. Excessive NO production by iNOS contributes to the decreased cardiac contractility in relation to decompensated heart failure and cardiodepression in septic shock (Rastaldo et al. 2007). In addition, simultaneous production of superoxide and NO by macrophages may result in the formation of peroxynitrite. Peroxynitrite is a highly reactive molecule that causes cellular damage by oxidation and nitration reactions, which may contribute to the development of heart failure (Rastaldo et al. 2007, Pacher et al. 2007). Therefore, it is possible that iNOS inhibition by levosimendan may contribute to the beneficial effects of levosimendan in the treatment of decompensated heart failure.

Besides its use in the treatment of contractile dysfunction in patients with decompensated heart failure, levosimendan has been reported to have beneficial effects in experimental models of septic shock (Oldner et al. 2001, Faivre et al. 2005) and was recently reported to ameliorate myocardial depression in a patient with pneumococcal septic shock (Ramaswamykanive et al. 2007). Because excessive NO production is associated with sepsis, it is possible that iNOS inhibition by levosimendan may partly explain the positive results obtained in the treatment of septic shock. Even though overproduction of NO in sepsis causes vasodilation, hypotension and shock, NO at moderate concentrations has a critical role in maintaining microvascular perfusion and in mediating the host defence against pathogens. Therefore excessive inhibition of iNOS may be harmful and lead to unwanted side-effects in patients. Even though specific iNOS inhibitors were first found useful in animal models of septic shock, their usefulness in the therapy of clinical septic shock has subsequently been considered questionable (Li and Förstermann 2000, Cobb 2001). Nevertheless, a prospective, randomized, placebo-controlled trial is ongoing to investigate the use of levosimendan as rescue therapy in refractory septic shock as an adjunct to

conventional therapy (Powell and De Keulenaer 2007) which may constitute a new pharmacological indication for levosimendan. In addition, simendans may have anti-inflammatory effects in other conditions and diseases which are complicated by increased NO production through iNOS pathway.

In conclusion, compounds that inhibit iNOS expression or iNOS activity have anti-inflammatory properties and may be useful in the treatment of inflammatory diseases which are complicated by excessive NO production through iNOS pathway.

Summary and conclusions

The aim of the study was to investigate the pharmacological regulation of iNOS expression and NO production in response to proinflammatory stimuli and to ascertain the involvement of JAK2 and JAK3 pathways in the regulation of iNOS by pharmacological means. The main objective of the study was to characterize novel drug compounds, orazipones and simendans, as inhibitors of iNOS pathway and to investigate the mechanisms leading to reduced iNOS expression and NO production.

The major findings and conclusions were:

1. JAK2-inhibitor AG-490 and JAK3-inhibitor WHI-P154 were potent inhibitors of iNOS expression and NO production in macrophages that were activated by either IFN γ or bacterial LPS. These inhibitors decreased the activation of transcription factor STAT1 which could well explain the inhibitory effects observed in iNOS expression. (I–II)
2. Orazipone, a novel thiol modulating compound, inhibited iNOS expression and NO production in murine macrophages and in human alveolar epithelial cells and the effect was reversed in the presence of excessive amounts of exogenously added thiols. Orazipone inhibited the activation of transcription factors NF- κ B and STAT1, which are important transcription factors for iNOS expression. Inhibition of these transcription factors may explain the inhibitory effects of orazipone on iNOS expression. The thiol modulating property was found to contribute to the mechanism by which orazipone inhibited LPS-induced NO production. (III)
3. Levosimendan (a calcium sensitizer used in the treatment of decompensated heart failure) and its stereoisomer dextrosimendan were potent inhibitors of iNOS expression and NO production in macrophages and fibroblasts. Simendans inhibited iNOS promoter

activity and the transcriptional activity of NF- κ B, an important transcription factor for iNOS expression. Inhibition of iNOS expression may contribute to the beneficial effects of levosimendan in the treatment of heart failure and to its recently found positive effects in experimentally-induced septic shock. (IV)

In light of cellular and animal models, compounds that inhibit iNOS expression or iNOS activity have anti-inflammatory properties and may be useful in the treatment of inflammatory diseases which are complicated by excessive NO production through iNOS pathway. In the present study, novel compounds and mechanisms to inhibit iNOS expression and NO production were identified. This information can be utilised in the development of novel anti-inflammatory drugs.

Acknowledgements

The work presented in this thesis was accomplished in the Immunopharmacology Research Group, Medical School, University of Tampere, Finland.

I want to express my deepest gratitude to my supervisor, Professor Eeva Moilanen, for the opportunity to do this, for your broad scientific knowledge, enthusiasm for science and support over the years. The co-authors Riku Korhonen, MD, PhD, Riina Nieminen, MSc (Pharm), Outi Kärpäniemi, MSc (Pharm), Adjunct Professor Hannu Kankaanranta, Mari Hämäläinen, MSc, Adjunct Professor Erkki Nissinen and Hanna Auvinen, MSc, are gratefully acknowledged for their able work and profound discussions on scientific or non-scientific matters. My sincere thanks to all the members of the immunopharmacology research group, all working as a real team.

I thank Adjunct Professor Kari Eklund and Professor Hartmut Kleinert for reviewing this work and for their valuable comments.

My warmest thanks go to my friends and family. Anna-Leena has reminded me what is important in life. Especially my sister Hanna and her husband Jaakko together with Marja-Leena, Ulla, Katri, Kiti and Henna are appreciated for their companionship and for providing a place to sleep when I had to stay overnight in Tampere. I wish to thank all the orienteering friends together with whom I had an opportunity to refresh my brains in training and competitions. Ulla, Sanna, Heidi, Jenni and Paula, my student friends, thank you for being excellent listeners and friends to discuss with. My mother Aila and my father Pertti are appreciated for understanding my decisions, and for the endless support and love. Last but definitely not least, my loving thanks to my husband, Olli, for his endless support and encouragement, all the way.

The Finnish Cultural Foundation and the Satakunta Regional Fund of the Finnish Cultural Foundation together with the TEKES Drug 2000 Program and

Clinical Drug Research Graduate School are acknowledged for the grants that made it possible for me to work as a full-time researcher. The grants for the international congresses in Beijing, China and Stockholm, Sweden were kindly provided by Satakunta Regional Fund of the Finnish Cultural Foundation, Days of Molecular Medicine Foundation, The Hilda Kauhanen Foundation and Tampere Graduate School in Biomedicine and Biotechnology.

Tampere, 28.3.2008

Outi Sareila

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Original communications

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Research Communication

JAK Inhibitors AG-490 and WHI-P154 Decrease IFN- γ -Induced iNOS Expression and NO Production in Macrophages

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Received 10 January 2006; Accepted 28 January 2006

In inflammation, inducible nitric oxide synthase (iNOS) produces nitric oxide (NO), which modulates inflammatory processes. We investigated the effects of Janus kinase (JAK) inhibitors, AG-490 and WHI-P154, on iNOS expression and NO production in J774 murine macrophages stimulated with interferon- γ (IFN- γ). JAK inhibitors AG-490 and WHI-P154 decreased IFN- γ -induced nuclear levels of signal transducer and activator of transcription 1 α (STAT1 α). JAK inhibitors AG-490 and WHI-P154 decreased also iNOS protein and mRNA expression and NO production in a concentration-dependent manner. Neither of the JAK inhibitors affected the decay of iNOS mRNA when determined by actinomycin D assay. Our results suggest that the inhibition of JAK-STAT1-pathway by AG-490 or WHI-P154 leads to the attenuation of iNOS expression and NO production in IFN- γ -stimulated macrophages.

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INTRODUCTION

Nitric oxide (NO) is a small gaseous signalling molecule that is synthesized from amino acid L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). In mammalian cells, there are three isoforms of the enzyme: neuronal nNOS and endothelial eNOS are constitutively expressed and the third isoform, iNOS, is induced in response to proinflammatory cytokines and bacterial products in inflammatory and tissue cells [4, 8, 13]. Once iNOS is expressed, it produces high amounts of NO for prolonged periods. NO production through iNOS pathway is regulated mainly at the level of iNOS expression [8, 10]. In inflammation, NO modulates immune responses and inflammatory process [10, 16], and is associated with the pathophysiology of various inflammatory diseases such as asthma [18] and arthritis [23]. Compounds that inhibit iNOS expression or iNOS activity have a promise as antiinflammatory drugs based on their effects in various forms of experimentally-induced inflammation [22].

One of the central cytokines involved in the induction of iNOS expression and NO production in macrophages is interferon- γ (IFN- γ). IFN- γ regulates iNOS expression at transcriptional and post-transcriptional level [8, 10]. One of the intracellular signal transduction pathways that are

activated by IFN- γ is Janus kinase (JAK)—signal transducer and activator of transcription (STAT) -pathway [17]. In the present study, we investigated the effects of two JAK inhibitors, AG-490 and WHI-P154, on the IFN- γ -induced iNOS expression and NO production in cultured macrophages. Both compounds inhibited iNOS expression and NO production in IFN- γ -treated macrophages along with their inhibitory effect on activation of STAT1.

MATERIALS AND METHODS

Materials

JAK inhibitors AG-490 (tyrphostin B42) and WHI-P154 (Calbiochem, La Jolla, Calif, USA), rabbit polyclonal mouse iNOS and STAT1 α p91 antibodies and goat anti-rabbit HRP-conjugated polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif, USA), rabbit polyclonal phospho-STAT1 (Tyr701) antibody (Cell Signaling Technology Inc, Beverly, Mass, USA) and recombinant mouse γ -interferon (R&D systems, Minneapolis, Minn, USA) were obtained as indicated. All other reagents were from Sigma Chemical Co (St Louis, Mo, USA).

Cell culture

J774 macrophages (ATCC, Manassas, Virginia, USA) were cultured at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium with Glutamax-I (Cambrex BioScience, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum (Cambrex BioScience), 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (all from Gibco, Paisley, UK). Cells were seeded on 24-well plates for nitrite measurement and RT-PCR, on 6-well plates for Western blot and on 10 cm dishes for nuclear extract preparation, and were grown for 72 h to confluence before the commencement of the experiments.

Toxicity of the tested compounds was ruled out by measuring cell viability using Cell Proliferation Kit II (XTT) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Preparation of cell lysates

At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) containing 2 mM sodiumorthovanadate. For pSTAT1 Western blot, the cells were solubilized in cold lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM sodiumorthovanadate, 80 µM leupeptin, 1 µg/mL aprotinin, 1 mM NaF, 1 µg/mL pepstatin, 2 mM sodiumpyrophosphate, 0.25% sodiumdeoxycholate and 10 µM N-octyl-β-D-glucopyranoside). After incubation for 15 min on ice, lysates were centrifuged (13 500 g, 5 min). The protein content of the supernatants was measured by the Coomassie blue method.

For iNOS Western blot, the cells were resuspended in lysis buffer containing 1% Triton X, 50 mM NaCl, 10 mM Tris-base pH 7.4, 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 1 mM sodiumorthovanadate, 40 µM leupeptin, 50 µg/mL aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate, 10 µM N-octyl-β-D-glucopyranoside. Otherwise the lysis was performed as described above.

Preparation of nuclear extracts

At indicated time points, the cells were rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/mL leupeptin, 25 µg/mL aprotinin, 0.1 mM EGTA, 1 mM sodiumorthovanadate, 1 mM NaF). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei separated by centrifugation at 4°C, 21 000 g for 10 s. The pellet was resuspended in buffer C (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/mL leupeptin, 25 µg/mL aprotinin, 0.1 mM EGTA, 1 mM sodiumorthovanadate, 1 mM NaF) and incubated on ice for 20 min. Nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4°C, 21 000 g for 2 min. The protein content of the super-

natant was measured by the Coomassie blue method. The samples were boiled in SDS sample buffer and stored at -20°C.

Western blotting

Protein (20 µg of lysates or nuclear extracts) was loaded on 8% SDS-polyacrylamide electrophoresis gel and was electrophoresed for 2 h at 120 V in buffer containing 25 mM Tris base, 250 mM glycine and 0.1% SDS. After electrophoresis, the proteins were electrically transferred to Hybond ECLTM nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.005% SDS. After transfer, the membrane was blocked in TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% skimmed milk for 1 h at room temperature. The membrane was incubated with anti-STAT1α or anti-iNOS in the blocking solution for 1 h at room temperature or with anti-pSTAT1 in TBST containing 5% bovine serum albumin at 4°C overnight. Thereafter the membrane was washed three times with TBST for 5 min, incubated with secondary antibody in the blocking solution for 50 min at room temperature, and washed three times with TBST for 5 min. Bound antibody was detected using Super Signal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, Ill, USA) and FluorChemTM 8800 imaging system (Alpha Innotech Corporation, San Leandro, Calif, USA). The quantitation of the chemiluminescent signal was carried out with the use of FluorChemTM software version 3.1.

RNA extractions and quantitative PCR

Cell homogenization, RNA extraction, reverse transcription, and quantitative PCR were performed as described in [11]. Mouse iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probes [6-FAM (6-carboxyfluorescein) as 5'-reporter dye and TAMRA (6-carboxytetramethyl-rhodamine) as 3'-quencher] were designed using Express Software (Applied Biosystems, Foster City, Calif, USA) and were 5'-CCTGGTACGGGCATTGCT-3' (miNOS forward), 5'-GCTCATGCGGCCTCCTT-3' (miNOS reverse), 5'-CAGCAGCGGCTCCATGACTCCC-3' (miNOS probe), 5'-GCATGGCCTTCCGTGTTTC-3' (GAPDH forward), 5'-GATGTCATCATACTTGGCAGGTTT-3' (GAPDH reverse), and 5'-TCGTGGATCTGACGTGCCCGCC-3' (the GAPDH probe). The primers were used at 300 nM and the probes at 150 nM concentrations. All primers and probes were purchased from Metabion Planegg-Martinsried, Germany. Thermal cycling conditions were: incubation at 50°C for 2 min, 95°C for 10 min, thereafter 40 cycles of denaturation at 92°C for 15 s, and annealing/extension at 60°C for 1 min. The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin #2. Each sample was determined in duplicate.

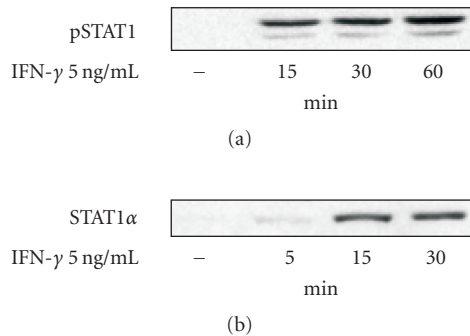


FIGURE 1: (a) Time-dependent activation (Tyr701 phosphorylation) of signal transducer and activator of transcription 1 (STAT1) by interferon- γ (IFN- γ) in J774 macrophages. Cells were treated with IFN- γ (5 ng/mL) for different times as indicated. Proteins were extracted with modified RIPA-buffer, and the protein contents were measured. Equal amounts of lysates (20 μ g protein) were subjected to immunoblot analysis with antibody specific for STAT1 phosphorylated at the tyrosine residue 701. Similar results were obtained in three independent experiments. (b) Time-dependent nuclear translocation of STAT1 α in IFN- γ -stimulated J774 macrophages. Cells were treated with IFN- γ (5 ng/mL) for different times as indicated. The nuclear proteins were extracted as described in materials and methods. The protein content of the samples was measured, and equal amounts of proteins (20 μ g) were subjected to immunoblot analysis with antibody against STAT1 α . Similar results were obtained in two independent experiments.

Nitrite assays

After 24 h incubation, the culture medium was collected for the nitrite measurement, which was used as a measure of NO production. Culture medium (100 μ L) was incubated with 100 μ L of Griess reagent (0.1% naphthaethylenediamine dihydrochloride, 1% sulfanilamine, 2.4% H₃PO₄) and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard [5].

Statistics

Results are expressed as mean \pm standard error of mean (SEM). When indicated, statistical analysis was carried out by analysis of variances supported by Dunnett adjusted significance levels. Differences were considered significant at $P < .05$.

RESULTS

Activation of STAT1 by IFN- γ

Activation of the JAK-STAT signalling pathway in J774 mouse macrophages was studied by measuring STAT1 phosphorylation and nuclear translocation of STAT1 α after IFN- γ -treatment. In cells treated with IFN- γ , tyrosine (Tyr701) phosphorylation of STAT1 was detected 15 min after addition of IFN- γ and it was further enhanced up to 60 minutes (Figure 1(a)). Phosphorylated STATs dimerize and

diffuse into the nucleus to initiate transcription [6]. Therefore we investigated the nuclear translocation of STAT1 α in IFN- γ -stimulated J774 macrophages. The presence of STAT1 α in nuclear extracts was measured by Western blot. The level of STAT1 α in the nucleus increased in a time-dependent manner after addition of IFN- γ into the culture. In nuclei, low levels of STAT1 α were detected already 5 min after exposure to IFN- γ and it was increased up to 30 minutes (Figure 1(b)).

Effects of JAK inhibitors AG-490 and WHI-P154 on STAT1 activation

The action of JAK inhibitors AG-490 and WHI-P154 on STAT1 activation was studied by measuring their effects on nuclear translocation of STAT1 α in IFN- γ -stimulated cells. Both AG-490 and WHI-P154 decreased the nuclear translocation of STAT1 α in a concentration-dependent manner (Figure 2). WHI-P154 was somewhat more potent than AG-490, and at 10 μ M drug concentration, WHI-P154 decreased the IFN- γ -induced nuclear translocation of STAT1 α by approximately 50% when measured after 30 min incubation with IFN- γ .

Effects of JAK inhibitors AG-490 and WHI-P154 on NO production in J774 macrophages

To investigate the effects of JAK inhibitors on NO production in J774 macrophages, the cells were treated with IFN- γ in the absence or in the presence of increasing concentrations (3, 10, and 30 μ M) of JAK inhibitors AG-490 and WHI-P154, and NO production was detected as nitrite accumulation in the culture medium. IFN- γ induced NO production in J774 macrophages and it was inhibited in a concentration-dependent manner by AG-490 and WHI-P154 (Figure 3). WHI-P154 was somewhat more potent inhibitor of NO production than AG-490. Cytotoxicity as a contributing factor was ruled out by XTT test. When the compounds were added to cells 6 h after IFN- γ stimulation, no effect on NO production was seen. This suggests that the compounds do not inhibit iNOS activity but rather suppress iNOS expression.

Effects of JAK inhibitors AG-490 and WHI-P154 on iNOS protein expression

The effects of JAK inhibitors, AG-490 and WHI-P154, on iNOS protein expression were investigated by Western blot analysis. IFN- γ induced iNOS protein expression in J774 macrophages, and it was reduced in a concentration-dependent manner by AG-490 or WHI-P154 (Figure 4).

Effects of JAK inhibitors AG-490 and WHI-P154 on iNOS mRNA expression and decay

The effects of JAK inhibitors, AG-490 and WHI-P154, on iNOS mRNA expression in IFN- γ treated cells were measured by quantitative PCR. Both AG-490 (10 μ M) and WHI-P154 (10 μ M) reduced iNOS mRNA levels by 60%

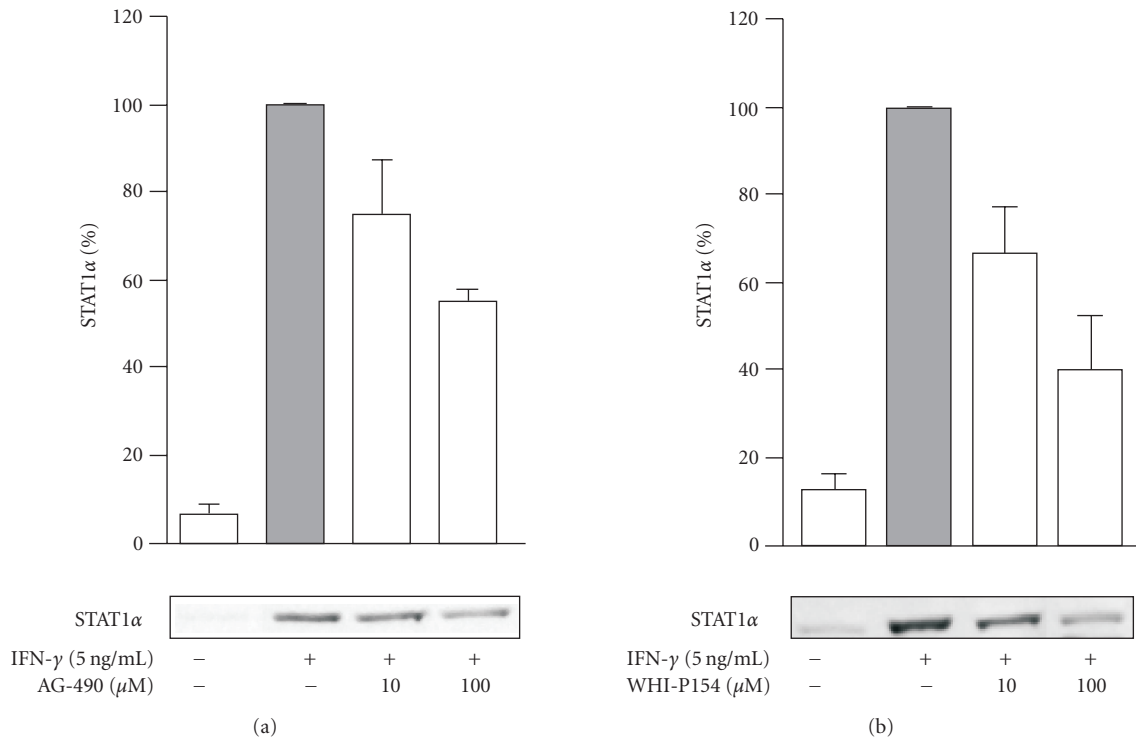


FIGURE 2: Effects of AG-490 and WHI-P154 on nuclear translocation of STAT1 α in IFN- γ -stimulated J774 macrophages. The cells were pretreated with (a) AG-490 or (b) WHI-P154 for 30 minutes. Thereafter, the medium was replaced with fresh medium containing the combination of the inhibitor and IFN- γ (5 ng/mL). The cells were incubated for another 30 minutes, and the nuclear proteins were extracted as described in materials and methods. The protein content of the samples was measured and equal amounts (20 μ g) were subjected to immunoblot analysis with antibody against STAT1 α . The results are expressed as mean \pm SEM ($n = 2-3$ for AG-490 and $n = 4$ for WHI-P154).

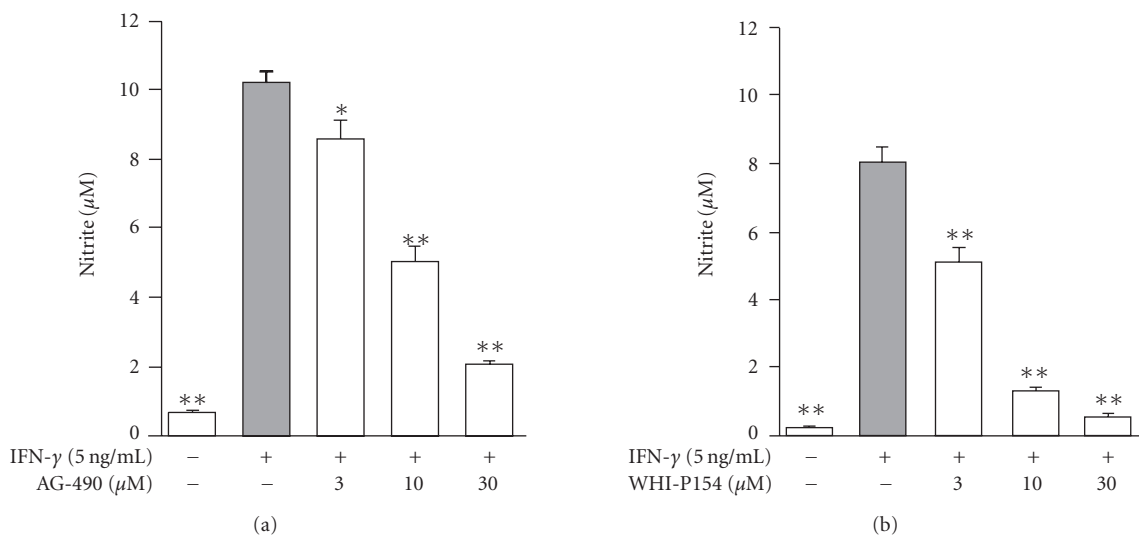


FIGURE 3: Effects of (a) AG-490 and (b) WHI-P154 on IFN- γ -induced nitric oxide (NO) generation in J774 macrophages. After 24-hour incubation with IFN- γ (5 ng/mL), the supernatants were collected and nitrite was measured in the culture medium as an indicator of NO production by Griess reaction. The values are mean \pm SEM ($n = 6$), * $P < .05$, and ** $P < .01$ when compared to cells treated with IFN- γ alone.

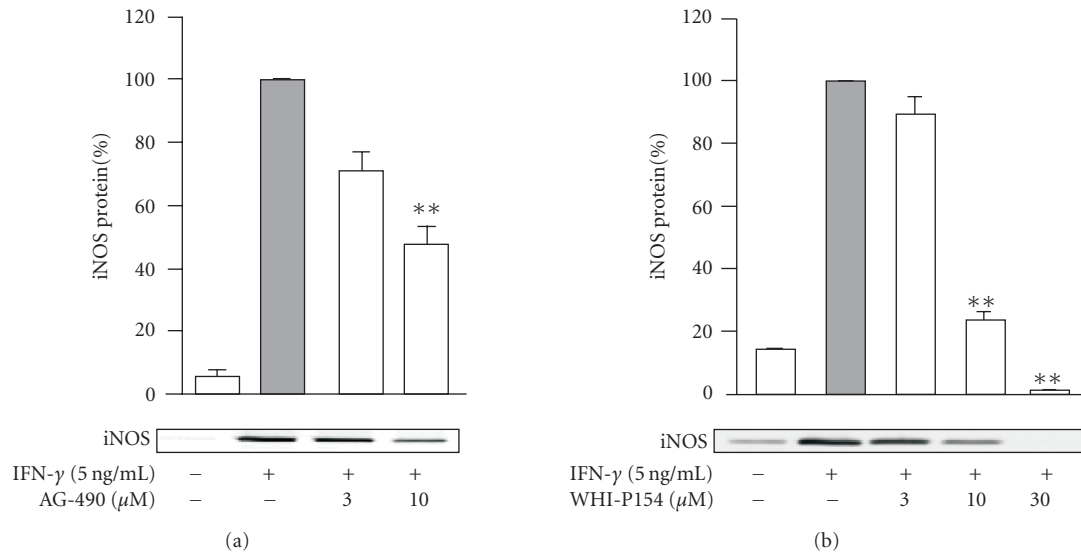


FIGURE 4: Effects of (a) AG-490 and (b) WHI-P154 on iNOS protein expression in J774 macrophages. Cells were incubated with IFN- γ (5 ng/mL) in the presence or in the absence of the tested compound for 24 hours. Cells were lysed and the protein content of the lysates was measured, and equal amounts of proteins (20 μ g) were subjected to immunoblot analysis with an antibody against iNOS. The results are shown as mean \pm SEM ($n = 6$ for AG-490 and $n = 4$ for WHI-P154), ** $P < .01$ when compared to cells treated with IFN- γ alone.

when measured after 4 h incubation (Figure 5(a)). To study whether the JAK inhibitors affect the rate of iNOS mRNA degradation, actinomycin D assay was applied. An inhibitor of transcription, actinomycin D (0.1 μ g/mL), was added into the culture after 6 h incubation with IFN- γ or a combination of IFN- γ and the drugs tested. Cells were harvested at time points 0, 1, 2, 3, 4, and 6 h after the addition of actinomycin D. Neither AG-490 nor WHI-P154 affected the decay of iNOS mRNA (Figure). The results suggest that AG-490 and WHI-P154 suppress iNOS expression at the level of transcription rather than at the level of regulation of the stability of iNOS mRNA.

DISCUSSION

In the present study, we tested the effects of two JAK inhibitors, AG-490 and WHI-P154, on the activation of JAK-STAT1-signalling pathway, iNOS expression, and NO production in IFN- γ -treated macrophages. JAK inhibitors AG-490 and WHI-P154 decreased IFN- γ -induced iNOS expression and NO production along with inhibition of STAT1 activation. To our knowledge, down-regulation of iNOS expression and NO production by JAK inhibitor WHI-P154 has not been reported previously. The inhibitors did not affect the decay of iNOS mRNA.

Typically, cytokine stimulation involves the ligation of two different receptor subunits, and this results in the formation of JAK heterodimers and their subsequent autophosphorylation. IFN- γ signalling preferentially leads to activation of STAT1 [6], which is phosphorylated on Tyr701 by JAK [12]. Phosphorylation of STAT1 induces STAT1 dimerization, nuclear translocation, and initiation of transcription of

gamma activated site (GAS) -driven genes [7]. In our study, we followed STAT1 activation by detecting STAT1 (Tyr701) phosphorylation and by probing nuclear lysates for STAT1 α at different time points after IFN- γ activation. The results show that STAT1 was activated in 15 minutes after IFN- γ -stimulation in J774 cells. Similar results have been reported recently when whole cell and nuclear lysates of J774 cells were immunoblotted for phosphorylated STAT1 [3].

STAT1 has been reported to act as a key transcription factor in IFN- γ -dependent mouse iNOS expression [1], whereas NF- κ B, another important transcription factor in the induction of iNOS, is merely involved in lipopolysaccharide (LPS)-induced iNOS expression and has a minor role following IFN- γ stimulation [1, 24]. An IFN- γ -activated site (GAS) is necessary for full expression of iNOS in response to IFN- γ and LPS [2, 15]. In addition, macrophages derived from STAT1-deficient mice displayed severely impaired NO production in response to a combination of IFN- γ and LPS [15].

In the present study, stimulation of J774 macrophages by IFN- γ led to the phosphorylation and nuclear translocation of STAT1, which was inhibited by AG-490 and WHI-P154. On molar basis, WHI-P154 was somewhat more potent inhibitor than AG-490. Similarly to our results, AG-490 has previously been shown to prevent JAK2 phosphorylation and to decrease STAT1 phosphorylation in J774 cells [1] and to decrease activation of STAT1 pathway in B-cell chronic lymphocytic leukemia (B-CLL) cells [14]. WHI-P154 was designed to specifically inhibit JAK3, and it has been shown to inhibit IL-2-triggered JAK3-dependent STAT activation in 32Dc11-IL-2R β -cells [20]. WHI-P131 (another WHI-P154-related JAK inhibitor) has been shown to inhibit STAT1 activation in B-CLL cells, in platelets, and in mesenchymal stem

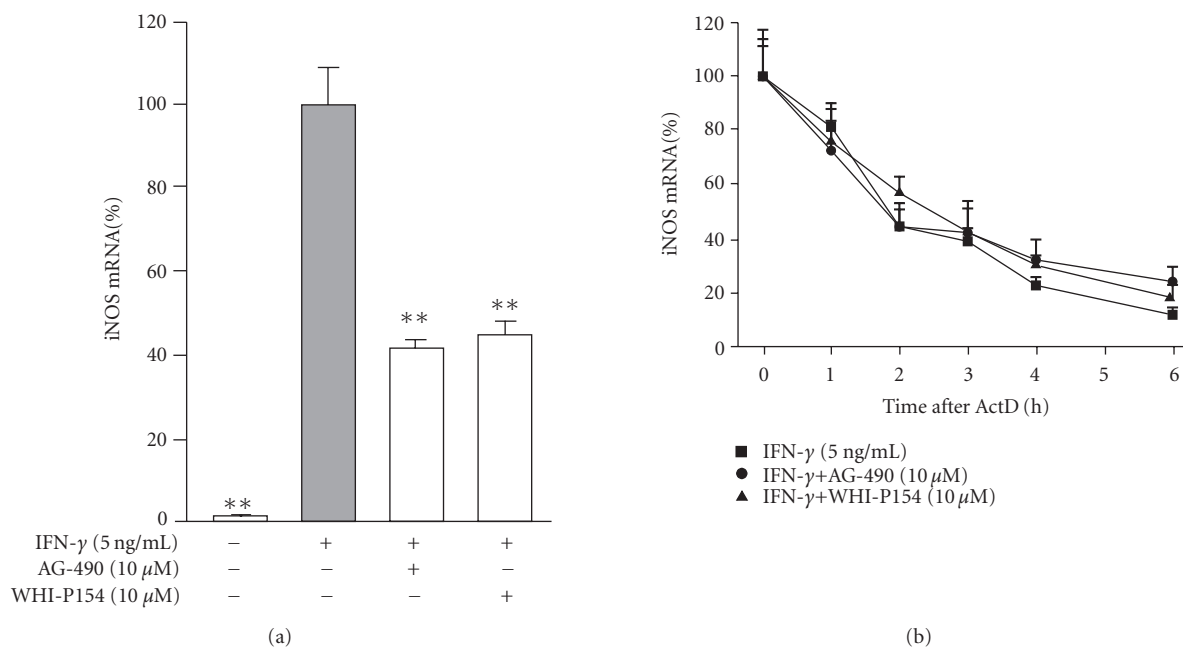


FIGURE 5: Effects of JAK inhibitors, AG-490 and WHI-P154, on IFN- γ -induced iNOS mRNA expression and degradation in J774 macrophages. (a) Cells were incubated with IFN- γ (5 ng/mL) in the absence or in the presence of AG-490 and WHI-P154 (10 μ M). The cells were disrupted after 4-hour incubation and total RNA was collected. Isolated RNA was converted to cDNA. iNOS and GAPDH mRNAs were measured by quantitative PCR, and iNOS mRNA levels were normalized against GAPDH mRNA. The results are expressed as mean \pm SEM, $n = 3$. ** $P < .01$ when compared to cells treated with IFN- γ alone. (b) Cells were incubated as in (a) except that actinomycin D (actD) (0.1 μ g/mL) was added after 6-hour incubation to stop transcription. Incubations were terminated at the indicated time points after addition of actD into the culture medium. Total RNA was isolated and converted to cDNA. iNOS and GAPDH mRNA were measured by quantitative PCR. iNOS mRNA levels were normalized against GAPDH mRNA. The results are expressed as mean \pm SEM, $n = 3$.

cells [14, 19, 21]. Here we extend the earlier data by showing that WHI-P154 inhibits STAT1 activation also in IFN- γ -treated macrophages.

In the present study, IFN- γ induced iNOS expression and NO production in J774 macrophages, and it was inhibited by JAK inhibitors, AG-490 and WHI-P154, in a dose-dependent manner along with their inhibitory action on STAT1 activation. When the drugs were added to the culture 6 h after IFN- γ , no effect on NO production was detected suggesting that the compounds do not inhibit iNOS activity. The results confirm the earlier studies showing that AG-490 inhibits IFN- γ -induced iNOS expression in macrophages [1]. To our knowledge, down-regulation of iNOS expression and NO production by JAK inhibitor WHI-P154 has not been reported previously.

The regulation of iNOS expression is controlled at the level of mRNA stability in addition to the transcriptional regulation [8, 10]. In murine macrophages, dexamethasone, and SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), reduced LPS-induced iNOS expression by destabilizing the mRNA [9, 11]. In contrast, IFN- γ has been shown to retard iNOS mRNA degradation when compared to iNOS mRNA induced by LPS alone [9]. In the present study, the effects of AG-490 and WHI-P154 on iNOS mRNA decay were tested by actinomycin D assay. JAK inhibitors, AG-490 and

WHI-P154 did not affect the rate of degradation of iNOS mRNA in cells treated with IFN- γ . This suggests that AG-490 and WHI-P154 inhibit iNOS expression at transcriptional level and they do not regulate mechanisms involved in the iNOS mRNA stabilization.

In conclusion, we have shown that JAK inhibitors, AG-490 and WHI-P154 down-regulate STAT1 activation, iNOS expression, and NO production in IFN- γ -treated macrophages. A better understanding of the mechanisms regulating iNOS expression and NO production in inflammation could facilitate the development of novel anti-inflammatory drugs acting through iNOS pathway.

ABBREVIATIONS

AG-490, α -cyano-(3,4-dihydroxy)-N-benzylcinnamide; B-CLL, B-cell chronic lymphocytic leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, gamma activated site; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NO, nitric oxide; STAT, signal transducer and activator of transcription; WHI-P154, 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline.

ACKNOWLEDGMENTS

We thank Mrs Niina Ikonen for the skillful technical assistance and Mrs Heli Määttä for secretarial help. This work was supported by The National Technology Agency (Tekes), Finnish Cultural Foundation, and the Medical Research Fund of Tampere University Hospital.

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Janus kinase 3 inhibitor WHI-P154 in macrophages activated by bacterial endotoxin: Differential effects on the expression of iNOS, COX-2 and TNF- α

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Received 5 February 2007; received in revised form 19 October 2007; accepted 19 October 2007

KEYWORDS

Inflammation;
JAK-STAT pathway;
iNOS;
COX-2;
TNF- α ;
JAK3;
LPS

Abstract

Bacterial endotoxin is a potent inducer of inflammatory response, including the induction of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production, and the expression of cyclo-oxygenase (COX)-2 and tumor necrosis factor (TNF)- α in inflammatory cells. In the present study, we investigated the effects of pharmacological inhibition of Janus kinase (JAK) 3 on the production of these proinflammatory molecules in macrophages exposed to bacterial endotoxin (lipopolysaccharide; LPS). JAK3 inhibitors WHI-P154 (4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline) and its derivative WHI-P131 inhibited LPS-induced iNOS expression and NO production in a dose-dependent manner. WHI-P154 inhibited the activation of signal transducer and activator of transcription (STAT) 1 and the expression of iNOS mRNA but it had no effect on iNOS mRNA decay when determined by actinomycin D assay. The JAK3 inhibitor had no effect on COX-2 expression, and TNF- α production was slightly inhibited only at higher drug concentrations (30 μ M). In addition, WHI-P154 inhibited iNOS expression and NO production also in human epithelial cells. Our results suggest that JAK3 inhibition modulates human and murine iNOS expression and NO production in response to inflammatory stimuli.

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Abbreviations: AP, activator protein; AG-490, α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; C/EBP, CCAAT enhancer binding protein; CM, cytokine mixture; COX-2, cyclo-oxygenase 2; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IRF1, interferon regulatory factor-1; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NF- κ B, nuclear factor κ B; NO, nitric oxide; PG, prostaglandin; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; TNF, tumor necrosis factor; WHI-P131, 4-(4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P154, 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline.

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1. Introduction

Lipopolysaccharide (LPS), a bacterial endotoxin, is a potent activator of innate immune response. It induces the activation of nuclear factor κ B (NF- κ B) through Toll-like receptor 4 (TLR4) leading to the expression of several inflammatory factors [1]. NF- κ B is an important transcription factor in the expressional regulation of inducible nitric oxide synthase (iNOS) in macrophages. Another signaling pathway involved in the regulation of iNOS expression is Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway [2]. Inhibitors of JAK2 have been shown to inhibit LPS-induced iNOS expression [3,4], whereas less is known about the effects of the inhibitors of other members of the JAK family.

Nitric oxide (NO) production in response to LPS is mediated by enzyme iNOS. NO production through iNOS pathway is mainly regulated at the level of iNOS expression. Once expressed, iNOS synthesizes large amounts of NO, which has both regulatory and detrimental effects [5–7]. Elevated NO levels are involved in the pathogenesis of inflammatory diseases such as asthma and arthritis in addition to their involvement in septic shock. Compounds that inhibit iNOS expression or iNOS activity have anti-inflammatory effects [8]. Other inflammatory factors induced by LPS include cyclo-oxygenase (COX)-2 and tumor necrosis factor (TNF)- α . Prostaglandins (PGs) are of central importance in the regulation of inflammation. They are synthesized from arachidonic acid by prostaglandin synthases or COX-enzymes [9,10]. The inducible isoform COX-2 is expressed in response to bacterial endotoxin and proinflammatory cytokines, and it is responsible for excessive PG production and subsequent inflammatory symptoms like swelling and pain [9,11]. TNF- α is a proinflammatory cytokine that is produced by macrophages in response to bacterial endotoxin [12,13]. Its role in the inflammatory diseases, such as rheumatoid arthritis, has been established [14].

We have recently found, that JAK inhibitors decreased iNOS expression and NO production in murine macrophages stimulated by interferon γ (IFN- γ) which preferably activated the cells via JAK-STAT pathway [15]. In the present study, we further studied the effects of pharmacological inhibition of JAK3 in the inflammatory cellular responses by investigating the effects of JAK3 inhibitor WHI-P154 (4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline) on the expression of pro-inflammatory enzymes iNOS and COX-2, and on TNF- α production in macrophages exposed to bacterial endotoxin.

2. Materials and methods

2.1. Materials

JAK inhibitors WHI-P154, WHI-P131 and AG-490 (tyrphostin B42) (Merck Chemicals Ltd., Nottingham, UK), rabbit anti-iNOS, anti-COX-2, anti-STAT1 α p91, anti-NF- κ B p65, anti-actin (I-19) and anti-lamin A/C and goat anti-rabbit HRP-conjugated and donkey anti-goat HRP-conjugated polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-phospho-STAT1 (Tyr701) and anti-STAT1 antibodies (Cell Signaling Technology Inc., Danvers, MA, USA) were obtained as indicated. Lipopolysaccharide from *Escherichia*

coli 0111:B4 and all other reagents were from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Cell culture

J774 macrophages (ATCC, Manassas, VA, USA) were cultured as reported previously [15].

A549 (ATCC, Manassas, VA, USA) human alveolar epithelial cells were cultured at 37 °C in 5% CO₂ atmosphere in Ham's F12K (Kaighn's modification) medium (Invitrogen, Paisley, UK) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK). Cells were seeded on 24-well plates for nitrite measurement, RT-PCR and Western blot. The cells were then grown for 48 h to confluence before the commencement of the experiments.

Toxicity of the tested compounds was ruled out by measuring cell viability using Cell Proliferation Kit II (XTT) (Roche Diagnostics

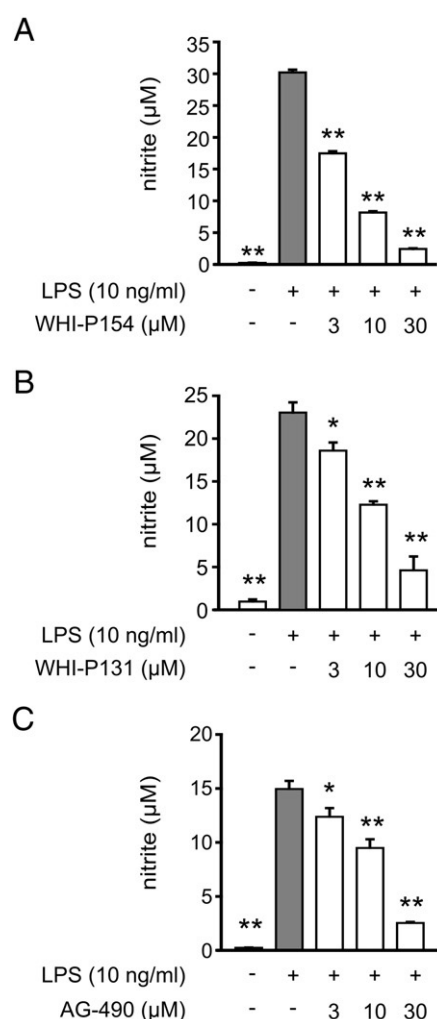


Figure 1 Effects of JAK inhibitors WHI-P154 (A), WHI-P131 (B) and AG-490 (C) on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in J774 macrophages. After 24 h incubation with indicated combinations of LPS (10 ng/ml) and the inhibitor, the supernatants were collected and nitrite was measured in the culture medium as an indicator of NO production by Griess reaction. The values are mean \pm SEM, $n=6$. * $P<0.05$ and ** $P<0.01$ when compared to cells treated with LPS alone.

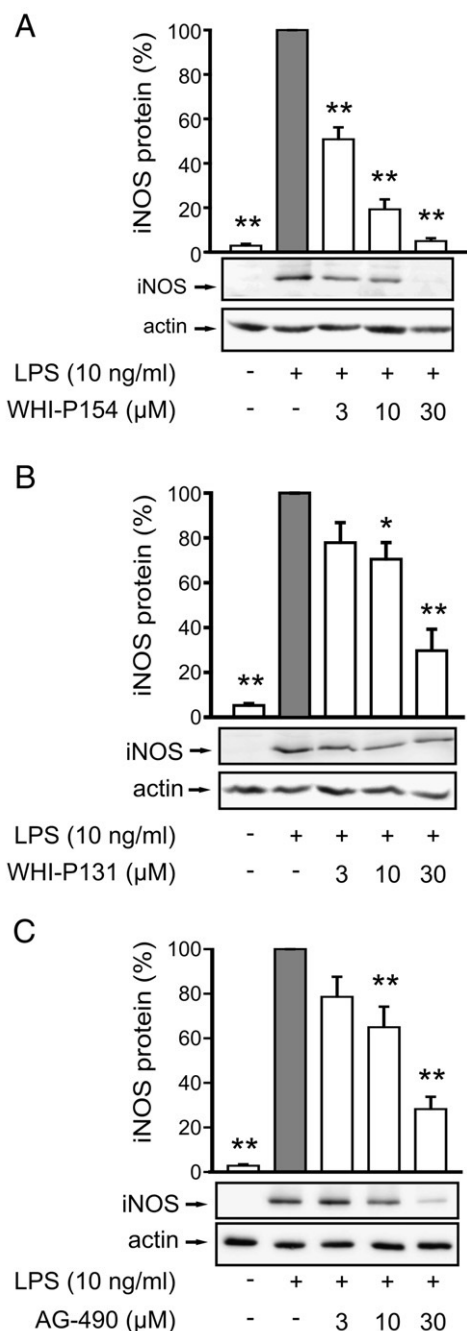


Figure 2 Effects of JAK inhibitors WHI-P154 (A), WHI-P131 (B) and AG-490 (C) on inducible nitric oxide synthase (iNOS) protein expression in J774 macrophages. Cells were incubated with lipopolysaccharide (LPS, 10 ng/ml) in the presence or in the absence of the tested compounds for 24 h. Cells were lysed and equal amounts of protein (20 μg/lane) were subjected to immunoblot analysis with antibody against iNOS. LPS-induced iNOS protein expression was set as 100%, and the other values were related to it. Actin was used as a loading control. The results were calculated as mean ± SEM ($n=7$ in A, $n=4$ in B and $n=12$ in C). ** $P<0.01$ when compared to cells treated with LPS alone.

GmbH, Mannheim, Germany) according to the manufacturer's instructions. Compounds were considered cytotoxic if the response was affected by more than 25%.

2.3. Preparation of cell lysates for Western blotting

At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) containing 2 mM sodium orthovanadate. For pSTAT1 Western blot, the cells were solubilized in cold lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, 0.25% sodiumdeoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM sodium orthovanadate, 80 μM leupeptin, 1 μg/ml aprotinin, 1 mM NaF, 1 μg/ml pepstatin, 2 mM sodiumpyrophosphate, 10 μM *N*-octyl-β-D-glucopyranoside). After incubation on ice for 15 min, lysates were centrifuged (13 500 g, 5 min). The protein content of the supernatants was measured by the Coomassie blue method [16].

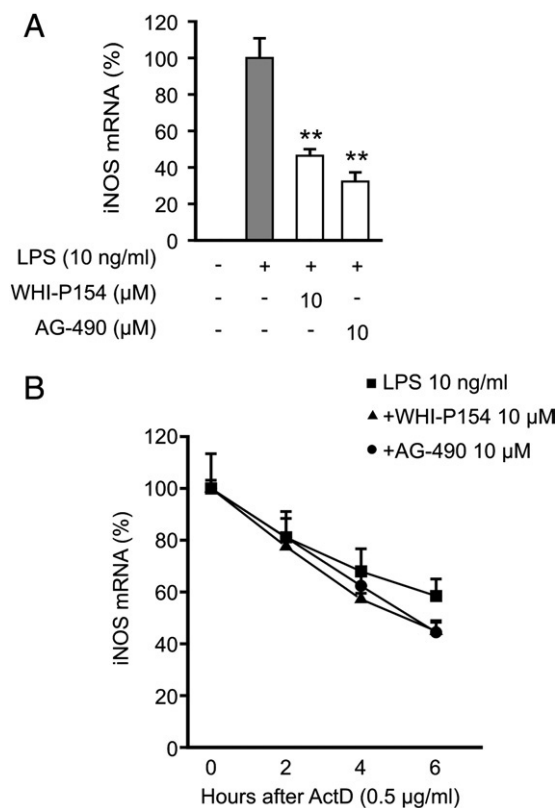


Figure 3 Effects of JAK inhibitors WHI-P154 and AG-490 on inducible nitric oxide synthase (iNOS) mRNA expression and degradation. (A) Cells were incubated with lipopolysaccharide (LPS, 10 ng/ml) in the absence or in the presence of WHI-P154 or AG-490 (10 μM) as indicated. After 6 h, the cells were lysed. Total RNA was isolated and converted to cDNA. iNOS mRNA levels were measured by quantitative RT-PCR, and normalized against GAPDH mRNA. LPS-induced iNOS mRNA expression was set as 100%, and the other values were related to it. The results are expressed as mean ± SEM, $n=3$. ** $P<0.01$ when compared to cells treated with LPS alone. (B) Degradation of iNOS mRNA as measured by actinomycin D-assay. Cells were incubated as in (A) except that actinomycin D (ActD, 0.5 μg/ml) was added after 6 h incubation to stop transcription. Incubations were terminated at the indicated time points after addition of ActD. Total RNA was isolated and converted to cDNA. iNOS mRNA was measured by quantitative RT-PCR, and normalized against GAPDH mRNA. In every treatment, iNOS mRNA at the time of addition of ActD was set as 100%, and the amount of iNOS mRNA left at the subsequent time points was related to that. The results are expressed as mean ± SEM, $n=3$.

For iNOS Western blot, the cells were resuspended in lysis buffer containing 1% Triton-X, 50 mM NaCl, 10 mM Tris base pH 7.4, 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, 40 μ M leupeptin, 50 μ g/ml aprotinin, 5 mM NaF, 1 μ g/ml pepstatin, 2 mM sodiumpyrophosphate, 10 μ M *N*-octyl- β -D-glucopyranoside. Otherwise the lysis was performed as described above. The extracts were boiled in SDS sample buffer and stored at -20°C .

2.4. Preparation of nuclear extracts for Western blotting and EMSA

Nuclear proteins were extracted as described in [15]. The protein content of the extracts was measured by the Coomassie blue method [16] and the extracts were stored at -70°C for EMSA. For Western blot, the extracts were boiled in SDS sample buffer and stored at -20°C .

2.5. Western blotting

Protein (20 μ g of lysates and nuclear extracts unless otherwise stated) was analyzed according to standard Western blotting procedure as previously described [15]. The membrane was incubated with the primary antibodies anti-iNOS, anti-pSTAT1, anti-STAT1 α , anti-NF- κ B p65, anti-lamin A/C or anti-actin antibody in the blocking solution (bovine serum albumin for pSTAT1 and STAT1 antibodies, skimmed milk for other antibodies) at 4°C overnight. The quantification of the chemiluminescent signal was carried out with the use of FluorChem™ (Alpha Innotech Corporation, San Leandro, CA, USA) software version 3.1.

2.6. Electrophoretic mobility shift assay (EMSA)

Transcription factor consensus oligonucleotides for NF- κ B (Promega, Madison, WI, USA) were 5'-end-labeled with γ - ^{32}P -ATP (GE Healthcare Europe GmbH, Munich, Germany) using T4 polynucleotide kinase (Promega, Madison, WI, USA). For binding reactions, 5 μ g of nuclear extracts were incubated in a 20 μ l reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM dithiothreitol, 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 DNA probe by electrophoresis on a native 4% polyacrylamide gel. The gel was dried and autoradiographed using intensifying screen at -70°C .

2.7. RNA extraction and quantitative reverse transcriptase/Real Time-PCR

RNA extractions and quantitative reverse transcriptase/Real Time-PCR (RT-PCR) were performed as described previously [15]. Briefly, total RNA was extracted and converted to cDNA. iNOS and GAPDH mRNA were measured by quantitative RT-PCR. iNOS mRNA levels were normalized to GAPDH, which was considered as a housekeeping gene. Each sample was determined in duplicate. Mouse iNOS and GAPDH primers and probes [15] have been described earlier. Human iNOS and GAPDH primers and probes were: 5'-GCAG-GTCGAGGACTATTTCTTTCA-3' (human iNOS forward), 5'-TCCTTCTTCGCTCGTAAGGA-3' (human iNOS reverse), 5'-TCAAGAGC-CAGAAGCGCTATCACGAAGATA-3' (human iNOS probe, 6-FAM (6-carboxy-fluorescein) as 5'-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3'-quencher), 5'-AAGGTCGG-AGTCAACGGATTT-3' (human GAPDH forward), 5'-GCAACAATATC-CACTTTACCAGAGTTAA-3' (human GAPDH reverse), 5'-CGCCTGGTACCAGGGCTGC-3' (human GAPDH probe, 5'-6-FAM and 3'-TAMRA).

2.8. Nitrite assays

After 24 h incubation the culture medium was collected for the nitrite measurement, which was used as a measure of NO production. Culture medium (100 μ l) was incubated with 100 μ l of Griess reagent (0.1% naphthaethylenediamine dihydrochloride, 1% sulfanilamine, 2.4% H_3PO_4) and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard [17].

2.9. TNF- α ELISA

TNF- α was measured in the culture medium by enzyme linked immunosorbent assay (ELISA) using reagents from R & D Systems Europe, Abingdon, UK.

2.10. Statistics

Results are expressed as mean \pm standard error of mean (SEM). When indicated, statistical analysis was carried out by analysis of variances followed by Dunnett multiple comparisons test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of JAK3 inhibitors WHI-P154 and WHI-P131 on NO production in J774 macrophages

The effects of JAK inhibitors on LPS-induced iNOS expression and NO production were investigated in J774 macrophages. Two structurally related JAK3 inhibitors, namely WHI-P154 and WHI-P131, and a JAK2 inhibitor AG-490 as a reference compound, were used. NO production was measured as nitrite accumulation in the culture medium. LPS induced NO production in J774 macrophages and it was inhibited by WHI-P154 and WHI-P131 in a concentration-dependent manner WHI-P154 being somewhat more potent than WHI-P131 (Fig. 1A–B). AG-490 was also found to inhibit NO production in a dose-dependent manner (Fig. 1C). When the inhibitors WHI-P154 and AG-490 were added to cells 6 h after LPS, NO production was comparable to that in cells treated with LPS only. This suggests that the compounds did not act as direct iNOS enzyme inhibitors. Cytotoxicity as a contributing factor was tested by XTT-test. None of the tested JAK inhibitors with or without LPS at concentrations up to 100 μ M were toxic to the cells during the 24 h incubation.

3.2. Effects of JAK3 inhibitors WHI-P154 and WHI-P131 on iNOS protein expression in J774 macrophages

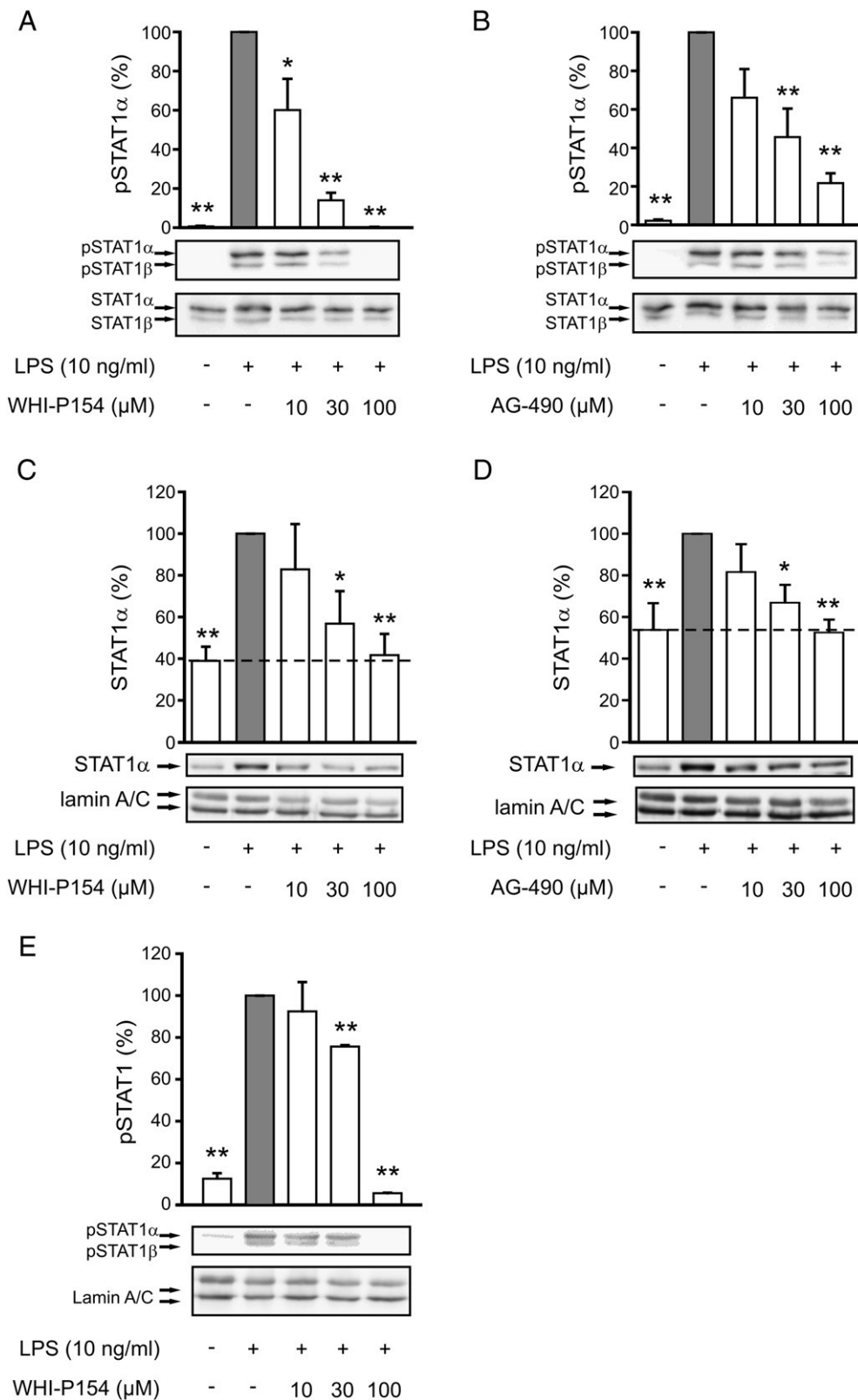
The effects of JAK3 inhibitors WHI-P154 and WHI-P131 and JAK2 inhibitor AG-490 on LPS-induced iNOS protein expression were investigated by Western blot analysis. Both WHI-P154 and WHI-P131 inhibited LPS induced iNOS protein expression in a concentration-dependent manner WHI-P154 being more potent than WHI-P131 (Fig. 2A, B). Also AG-490 inhibited iNOS expression, and its effect was comparable to that seen with WHI-P131 (Fig. 2C).

3.3. Effects of JAK3 inhibitor WHI-P154 on iNOS mRNA expression and decay in J774 macrophages

From the two JAK3 inhibitors, WHI-P154 was found to be more potent inhibitor of iNOS protein expression and NO production than

WHI-P131. Therefore, WHI-P154 was used in the further experiments. We investigated the effects of JAK3 inhibitor WHI-P154 on iNOS mRNA expression in LPS-treated cells by quantitative RT-PCR.

WHI-P154 (10 μ M) clearly reduced iNOS mRNA expression when measured after 6 h incubation (Fig. 3A). Similarly, JAK2 inhibitor AG-490 (10 μ M) decreased LPS-induced iNOS mRNA levels.



To study whether the JAK inhibitors affect the rate of iNOS mRNA degradation, actinomycin D assay was carried out. In the beginning of the experiment, cells were stimulated with LPS in the presence or in the absence of the compounds of interest, and actinomycin D (an inhibitor of transcription; 0.5 $\mu\text{g}/\text{ml}$) was added into the culture medium after 6 h incubation. After that, cells were further incubated and harvested 2, 4 and 6 h after the addition of actinomycin D. Neither WHI-P154 nor AG-490 affected the iNOS mRNA decay (Fig. 3B). The results suggest that JAK3 inhibitor WHI-P154 and JAK2 inhibitor AG-490 suppress iNOS mRNA expression at the level of transcription and not at the level of iNOS mRNA stability.

3.4. Effects of JAK3 inhibitor WHI-P154 on LPS-induced STAT1 activation in J774 macrophages

STAT1 has been reported to be an important transcription factor for iNOS [18]. Activation of the JAK-STAT1 signaling pathway in response to LPS in J774 mouse macrophages was first studied by measuring STAT1 α phosphorylation (Tyr701) by Western blot. In untreated cells, pSTAT1 α was not detected in total cell lysates. In cells treated with LPS (10 ng/ml), STAT1 α phosphorylation at the tyrosine residue (Tyr701) was detected after 2 h incubation, reached its maximum at 4 h, and remained elevated up to the 6 h follow-up (data not shown). The effects of JAK3 inhibitor WHI-P154 on STAT1 α phosphorylation in LPS-stimulated cells was studied after 4 h incubation. WHI-P154 decreased the levels of pSTAT1 α in a dose-dependent manner (Fig. 4A). Similar effect was obtained with JAK2 inhibitor AG-490 (Fig. 4B).

Phosphorylated STATs dimerize and translocate into the nucleus to initiate transcription [19]. Therefore we investigated the levels of STAT1 α in nuclear extracts from LPS (10 ng/ml) – stimulated J774 macrophages by Western blot. Slightly increased levels of STAT1 α were detected in nuclei 1.5 h after the exposure to LPS and the presence of STAT1 α in the nuclear preparations was further increased during 4 h incubation (data not shown). WHI-P154 decreased the nuclear translocation of STAT1 α when the nuclear proteins were extracted 4 h after the exposure to LPS (10 ng/ml), and JAK2 inhibitor AG-490 inhibited STAT1 α translocation similarly (Fig. 4C, D). We also measured the nuclear levels of phosphorylated STAT1 by Western blot. As expected, WHI-P154 was found to decrease pSTAT1 levels in the nuclei of J774 cells stimulated by LPS (Fig. 4E).

3.5. Effects of JAK3 inhibitor WHI-P154 on LPS-induced NF- κ B activation in J774 macrophages

Another important transcription factor in LPS-induced iNOS expression is NF- κ B [20]. The effect of JAK3 inhibitor WHI-P154 on NF- κ B activation was studied by measuring nuclear translocation

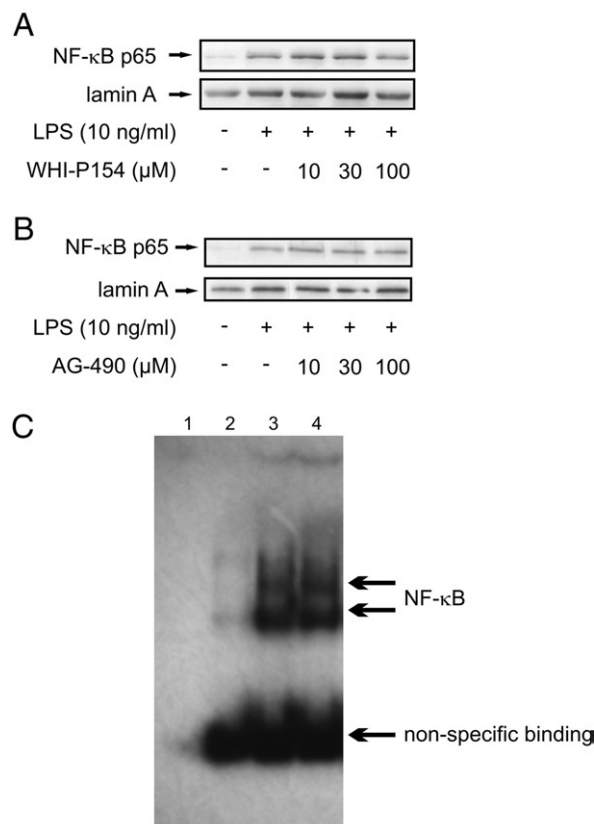


Figure 5 Effects of JAK inhibitors WHI-P154 and AG-490 on the activation of nuclear factor κ B (NF- κ B) in lipopolysaccharide (LPS)-stimulated J774 macrophages. Cells were incubated with LPS (10 ng/ml) in the absence or in the presence of WHI-P154 (A) or AG-490 (B) as indicated. After 30 min incubation, the nuclear proteins were extracted. Equal amounts of protein (10 $\mu\text{g}/\text{lane}$) were subjected to immunoblot analysis with antibody against NF- κ B p65. Lamin A was used as a loading control. The blots are representatives of three experiments with similar results. (C) The effect of WHI-P154 on NF- κ B activation in J774 macrophages as measured by EMSA. The cells were incubated and the nuclear extracts were prepared as in A–B. The extracts were incubated with a radio-labeled NF- κ B consensus oligonucleotide and separated from the free probe by electrophoresis. Lane 1: probe, lane 2: untreated cells, lane 3: LPS 10 ng/ml, lane 4: LPS 10 ng/ml + WHI-P154 30 μM . The bands were detected by autoradiography. The picture is a representative of three experiments with similar results.

of NF- κ B subunit p65 in LPS-stimulated cells, or by EMSA. LPS caused a rapid translocation of NF- κ B p65, which peaked at 30 min after addition of LPS (data not shown). Neither WHI-P154

Figure 4 Effects of JAK inhibitors WHI-P154 and AG-490 on the activation of signal transducer and activator of transcription 1 (STAT1) in lipopolysaccharide (LPS)-stimulated J774 macrophages. (A–B) The cells were treated with LPS (10 ng/ml) in the absence or in the presence of WHI-P154 or AG-490 for 4 h, and whole cell lysates were extracted. Equal amounts of protein (20 $\mu\text{g}/\text{lane}$) were subjected to immunoblot analysis with an antibody against STAT1 phosphorylated at the tyrosine residue 701 (pSTAT1) and against total STAT1. LPS-induced STAT1 α phosphorylation was set as 100% and the other values were related to that. The values are mean \pm SEM, $n=3$ in A and $n=6$ in B. * $P<0.05$ and ** $P<0.01$ when compared to cells treated with LPS only. (C–E) The cells were treated with LPS (10 ng/ml) in the absence or in the presence of WHI-P154 or AG-490 for 4 h, and the nuclear proteins were extracted. Equal amounts of protein (20 $\mu\text{g}/\text{lane}$) were subjected to immunoblot analysis with an antibody against STAT1 α (C–D) or pSTAT1 (E). Lamin A/C was used as a loading control. The nuclear level of STAT1 α (C–D) or pSTAT1 (E) in LPS-treated cells was set as 100% and the other values were related to it. The values are mean \pm SEM, $n=6$ in C–D and $n=3$ in E. The dash line (C–D) indicates the basal level of STAT1 α in the nuclear preparations from unstimulated cells. * $P<0.05$ and ** $P<0.01$ when compared to cells treated with LPS only.

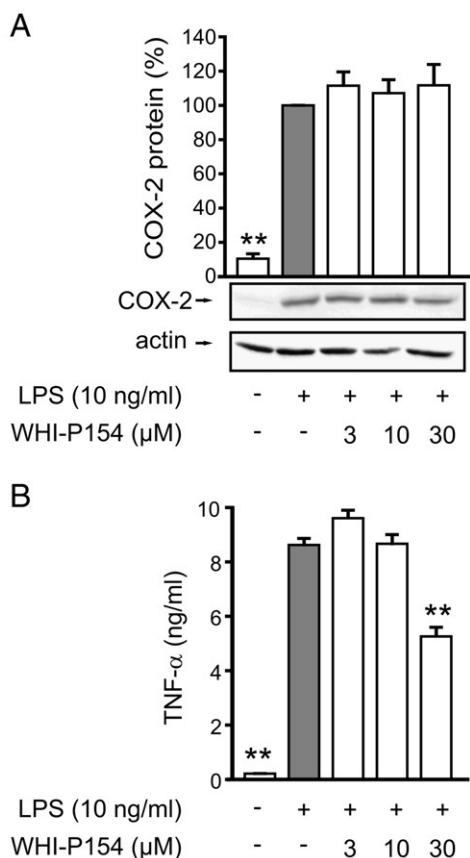


Figure 6 Effects of JAK3 inhibitor WHI-P154 on inducible cyclo-oxygenase (COX-2) protein expression (A) and on tumor necrosis factor α (TNF- α) production (B) in J774 macrophages exposed to bacterial lipopolysaccharide (LPS). Cells were incubated with LPS (10 ng/ml) in the presence or in the absence of WHI-P154. After 24 h incubation, the culture media were collected and TNF- α was measured by ELISA. Cells were lysed and equal amounts of protein (20 μ g/lane) were subjected to immunoblot analysis with an antibody against COX-2. LPS-induced COX-2 protein expression was set as 100%, and the other values were related to it. Actin was used as a loading control. The results are expressed as mean \pm SEM, $n=4$ in A and $n=4-8$ in B. $**P<0.01$ when compared to cells treated with LPS only.

nor AG-490 inhibited the nuclear translocation of NF- κ B p65 when the proteins were extracted 30 min after the exposure to LPS (10 ng/ml) (Fig. 5A-B). WHI-P154 did neither alter LPS-induced NF- κ B nuclear binding activity as measured by EMSA (Fig. 5C).

3.6. Effects of JAK3 inhibitor WHI-P154 on LPS-induced COX-2 expression and TNF- α production in J774 macrophages

To test the effect of WHI-P154 on other inflammatory factors in these cells, the expression of COX-2 and TNF- α were analyzed. COX-2 expression was studied by Western blot. LPS induced COX-2 expression and it was not affected by WHI-P154 when used at 3–30 μ M drug concentrations (Fig. 6A). TNF- α production into the

culture medium was measured by ELISA. WHI-P154 (30 μ M) decreased LPS-induced TNF- α production by about 40%, but the lower concentrations (3 and 10 μ M) did not inhibit TNF- α production (Fig. 6B).

3.7. Effects of JAK3 inhibitor WHI-P154 on NO production and iNOS expression in human A549 epithelial cells activated by proinflammatory cytokines

The induction of the expression of iNOS in human cells requires usually stimulation with a combination of proinflammatory cytokines instead of LPS [5]. JAK3 inhibitors attenuated iNOS expression and NO production in LPS treated murine macrophages. Hence, we

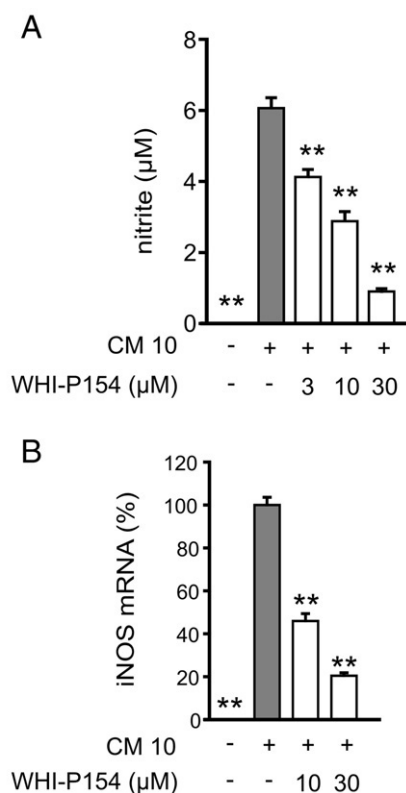


Figure 7 Effects of JAK3 inhibitor WHI-P154 on nitric oxide (NO) production and on iNOS mRNA expression in A549 human epithelial cells treated with a combination of proinflammatory cytokines. (A) After 24 h stimulation with cytokine mixture (CM) containing TNF- α , IFN- γ and IL-1 β (10 ng/ml each) in the absence or in the presence of WHI-P154, accumulated nitrite was measured in the culture medium as a marker of NO production. Values are mean \pm SEM, $n=4$. $**P<0.01$ when compared to cells treated with cytokine mixture only. (B) Cells were incubated with cytokine mixture (CM) containing TNF- α , IFN- γ and IL-1 β (10 ng/ml each) in the absence or in the presence of WHI-P154 as indicated. After 6 h, the cells were lysed. Total RNA was isolated and converted to cDNA. iNOS mRNA levels were measured by quantitative RT-PCR, and normalized against GAPDH mRNA. iNOS mRNA expression in cytokine-stimulated cells was set as 100%, and the other values were related to it. The results are expressed as mean \pm SEM, $n=4$. $**P<0.01$ when compared to cells treated with cytokine mixture only.

wanted to test, whether JAK3 inhibitor decreases NO production also in human cells in response to inflammatory stimuli. Stimulation with a combination of proinflammatory cytokines (cytokine mixture (CM) containing TNF- α , IFN- γ and IL-1 β ; 10 ng/ml each) resulted in NO production in human A549 epithelial cells (Fig. 7A). NO production was inhibited by WHI-P154 in a dose-dependent manner and the WHI-P154 concentration of 10 μ M decreased NO production by about 50%. Accordingly, WHI-P154 decreased also iNOS mRNA expression in A549 epithelial cells exposed to the proinflammatory cytokines when measured after 6 h incubation (Fig. 7B).

4. Discussion

In the present study, we found that JAK3 inhibitors WHI-P154 and WHI-P131 attenuated iNOS expression and NO production in a dose-dependent manner. However, pharmacological inhibition of JAK3 had no effect on COX-2 expression, and TNF- α production was inhibited only slightly with higher drug concentrations (30 μ M). JAK3 inhibition attenuated iNOS mRNA expression and STAT1 activation but did not affect iNOS mRNA stability suggesting that JAK3 is involved in the transcriptional regulation of iNOS expression.

LPS is a strong activator of the innate immune response and it activates NF- κ B through Toll-like receptor pathway [1]. NF- κ B is an important transcription factor in iNOS expression [20]. In the present study, LPS was found to activate NF- κ B and induce iNOS expression in macrophages. JAK3 inhibitor WHI-P154 and JAK2 inhibitor AG-490 reduced iNOS expression without decreasing NF- κ B activation. Concomitantly, LPS was found to activate STAT1 and that was markedly attenuated with the two tested JAK inhibitors. Inhibition of STAT1 activation may well explain the suppressive effect of the JAK inhibitors on iNOS levels, since LPS-induced iNOS expression was severely impaired in macrophages prepared from STAT1^{-/-} mice [18]. Similarly, LPS/IFN- γ -induced iNOS expression and NO production was reduced in fibroblasts obtained from STAT1^{-/-} mice [21].

In the present study, we report that JAK3 inhibitors WHI-P154 and WHI-P131 decreased iNOS expression and NO production in cells exposed to LPS, which is to our knowledge a novel finding. In addition, WHI-P154 inhibited NO production and iNOS expression in cytokine-treated human A549 epithelial cells indicating that it affects the up-regulation of both human and murine iNOS. WHI-P154 and WHI-P131 have been shown to be rather specific for JAK3 and not to inhibit significantly JAK1, JAK2 or protein tyrosine kinases including SYK (spleen tyrosine kinase), LYN (protein tyrosine kinase related to LCK and YES), BTK (Bruton's tyrosine kinase) and IRK (insulin receptor kinase) within the concentration range used [22,23].

To rule out cytotoxicity, the JAK inhibitors were tested by XTT test. Neither of the compounds (at even 100 μ M) affected cell viability when compared to LPS-activated cells. Therefore, we concluded that the observed inhibitory effects were not a result of cytotoxicity of the drugs. This is supported by the observation that nuclear lamin (the fragmentation of which is widely used as a marker of apoptosis) remained intact in the nuclei of cells challenged with up to 100 μ M concentrations of the inhibitors (Fig. 4C–D).

The results suggest that JAK3 plays a role in the regulation of iNOS expression. Our results confirm recent observations

that JAK2 inhibitor AG-490 inhibits LPS-induced iNOS expression in murine macrophages [24]. Similar inhibition of iNOS expression by AG-490 has been reported in a mouse fetal skin dendritic cell line [4].

In addition to transcriptional regulation, iNOS expression is critically regulated at post-transcriptional level [5,6]. In murine J774 macrophages, dexamethasone and SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), have been shown to reduce LPS-induced iNOS expression by destabilizing the mRNA [25,26]. In the present study, the effects of WHI-P154 and AG-490 on iNOS mRNA decay were tested with actinomycin D assay. Degradation of LPS-induced iNOS mRNA was not altered in the presence of JAK inhibitors. However, both WHI-P154 and AG-490 decreased iNOS mRNA levels significantly. Accordingly, WHI-P154 and AG-490 down-regulated LPS-induced iNOS protein expression and NO production. When the inhibitors were added to the culture 6 h after LPS, NO production was comparable to that induced by LPS alone. These data together suggest that the tested JAK inhibitors decrease iNOS transcription but do not regulate the mRNA degradation or affect iNOS enzyme activity.

JAK3 inhibitor WHI-P154 attenuated TNF- α production in LPS treated macrophages by about 30% when used at 30 μ M concentrations, but the lower drug concentrations were ineffective. Previously, it has been shown that IFN- γ augments the expression of TNF- α in LPS-stimulated macrophages [27–29] suggesting that JAK-STAT pathway is involved in the induction of TNF- α expression. In the present study, 30 μ M WHI-P154 decreased the production of TNF- α , whereas NO production was decreased by WHI-P154 at 3 μ M concentration. WHI-P131 has also been shown to inhibit TNF- α expression in human chondrocytes following *Borrelia burgdorferi* infection [30]. In contrast, JAK2 inhibitor AG-490 did not inhibit TNF- α expression in LPS-stimulated murine macrophages [31]. This suggests that JAK3, and not JAK2, would be a regulatory factor in TNF- α production.

In COX-2 expression, the most important transcription factors include C/EBP (CCAAT enhancer binding protein), NF- κ B and AP-1 (activator protein 1) [11], whereas JAK-STAT pathway has a minor role [32,33]. In our studies, JAK3 inhibitor WHI-P154 did not affect COX-2 expression in macrophages exposed to bacterial LPS. These results suggest that in these cells, JAK3 is not an important factor in the regulation of LPS-induced COX-2 expression.

In summary, we have shown that JAK3 inhibitor WHI-P154 down-regulated iNOS expression and NO production along with its inhibitory effect on STAT1 activation in macrophages exposed to bacterial endotoxin LPS. WHI-P154 had no effect on COX-2 expression, and TNF- α expression was slightly inhibited only at higher drug concentrations. In addition, WHI-P154 suppressed iNOS expression and NO production in cytokine-treated human epithelial cells. The results suggest that JAK3 is involved in the regulation of iNOS expression in human and murine cells.

Acknowledgements

We thank Mrs. Salla Hietakangas, Mrs. Niina Ikonen and Mrs. Marja-Leena Lampen for their excellent technical assistance and Mrs. Heli Määttä for skillful secretarial help. This work was supported by the Medical Research Fund of Tampere

University Hospital, Tampere, Finland, by the Satakunta and Pirkanmaa Regional Funds of the Finnish Cultural Foundation, Finland, and by the Finnish Funding Agency for Technology and Innovation (TEKES), Finland.

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Orazipone Inhibits Activation of Inflammatory Transcription Factors Nuclear Factor- κ B and Signal Transducer and Activator of Transcription 1 and Decreases Inducible Nitric-Oxide Synthase Expression and Nitric Oxide Production in Response to Inflammatory Stimuli

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Received July 24, 2007; accepted November 21, 2007

ABSTRACT

Orazipone [OR-1384; 3-[4-(methylsulfonyl)benzylidene]pentane-2,4-dione] is a novel sulfhydryl-modulating compound that has anti-inflammatory properties in experimental models of asthma and inflammatory bowel disease. In inflammation, inducible nitric-oxide synthase (iNOS) generates NO, which modulates the immune response. Compounds that inhibit iNOS expression or iNOS activity possess anti-inflammatory effects. In the present study, we examined the effects of orazipone and its derivative OR-1958 [3-[3-chloro-4-(methylsulfonyl)benzylidene]pentane-2,4-dione] on iNOS expression and NO production in J774 macrophages stimulated by bacterial lipopolysaccharide (LPS) and in human alveolar epithelial cells activated by proinflammatory cytokines. Protein expression and nuclear translocation of transcription factors were measured by Western blot. iNOS mRNA expression was determined by quantitative reverse transcription-polymerase chain reaction and iNOS mRNA stability by ac-

tinomycin D assay. iNOS promoter activity was studied in a cell line expressing luciferase under the control of iNOS promoter. Orazipone and its derivative OR-1958 but not its nonthiol-modulating analog inhibited iNOS expression and NO production in a concentration-dependent manner. Orazipone decreased LPS-induced iNOS mRNA expression, but the decay of iNOS mRNA was not affected. Orazipone extensively prevented LPS-induced activation of nuclear factor κ B (NF- κ B) and signal transducer and activator of transcription (STAT) 1, which are important transcription factors for iNOS. In agreement, human iNOS promoter activity was inhibited by orazipone. In conclusion, orazipone decreased activation of inflammatory transcription factors NF- κ B and STAT1, and expression of iNOS in cells exposed to inflammatory stimuli. The thiol-modulating property seems to be critical in mediating the anti-inflammatory effects of orazipone.

This work was supported by the Medical Research Fund of Tampere University Hospital, Tampere, Finland; The Satakunta Regional Fund of the Finnish Cultural Foundation, Finland; Orion Pharma Research, Finland; and The Academy of Finland. E.N. is an employee of Orion Pharma, which provided orazipone, its derivative OR-1958, and the nonthiol-modulating control compound OR-2149.

Parts of the results have been published as a meeting abstract as follows: Sareila O, Hämäläinen M, Kankaanranta H, Nissinen E, and Moilanen E (2007) Orazipone decreases inducible nitric oxide synthase expression and nitric oxide production in activated macrophages, in *2007 37th Scandinavian Society for Immunology Meeting*; 2007 Jun 6-9; Turku, Finland. *J Immunol* 65:589-622.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.107.129114.

Orazipone (OR-1384) and its derivative OR-1958 are novel thiol-modulating compounds that exert their effects most likely by forming reversible conjugates with the thiol groups of glutathione and proteins (Wroblewski et al., 1998; Aho et al., 2001). The reaction with glutathione is readily reversible, which makes orazipone and OR-1958 unique among thiol-modulating compounds. Orazipone has been shown to express anti-inflammatory properties in various models of experimentally induced colitis (Wroblewski et al., 1998; Boerma et al., 2006). In addition, orazipone was shown to inhibit eosinophil accumulation in animal models of asthma (Ruot-

ABBREVIATIONS: OR-1384, orazipone, 3-[4-(methylsulfonyl)benzylidene]pentane-2,4-dione; OR-1958, 3-[3-chloro-4-(methylsulfonyl)benzylidene]pentane-2,4-dione; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; TLR, Toll-like receptor; NF- κ B, nuclear factor- κ B; STAT, signal transducer and activator of transcription; JAK, Janus tyrosine kinase; OR-2149, 3-[4-(methylsulfonyl)benzylidene]pentane-2,4-dione; AG-490, α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDTC, pyrrolidine dithiocarbamate; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-one; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; NAC, *N*-acetyl-L-cysteine; GSH, glutathione.

salainen et al., 2000) and to possess antieosinophilic activity (Kankaanranta et al., 2006). However, the cellular and molecular mechanisms of the anti-inflammatory actions of orazipone remain mostly unknown.

Increased NO production is associated with inflammatory diseases such as inflammatory bowel disease (Kolios et al., 2004), arthritis (Cuzzocrea, 2006), and asthma (Ricciardolo et al., 2004). NO is a small gaseous molecule synthesized in inflammatory and tissue cells by nitric-oxide synthase enzymes. In mammalian cells, two isoforms of the enzyme are constitutively expressed, but the third isoform, iNOS, is induced in response to bacterial products and proinflammatory cytokines (Alderton et al., 2001; Kleinert et al., 2004; Korhonen et al., 2005). NO production through iNOS pathway is regulated mainly at the level of iNOS expression (Kleinert et al., 2004; Korhonen et al., 2005). In inflammation, NO has regulatory and proinflammatory effects (Coleman, 2001; Korhonen et al., 2005). Compounds that inhibit iNOS expression or iNOS activity possess anti-inflammatory properties (Vallance and Leiper, 2002).

Bacterial lipopolysaccharide (LPS) induces the expression of iNOS in various cells, including murine macrophages. LPS activates toll-like receptor (TLR) 4 (Poltorak et al., 1998). TLR4 activation leads to the activation of nuclear factor κ B (NF- κ B) (Zhang and Ghosh, 2000), which is an important transcription factor for iNOS. Another transcription factor involved in LPS-induced iNOS expression is the signal transducer and activator of transcription (STAT) 1, which is activated through the Janus kinase (JAK)-STAT pathway (Gao et al., 1998; Jacobs and Ignarro, 2001). In addition, post-transcriptional mechanisms are important regulators of iNOS expression (Kleinert et al., 2004; Korhonen et al., 2005).

In the present study, we investigated the anti-inflammatory properties of orazipone by studying its effects and mechanisms of action on iNOS expression and NO production in activated macrophages and epithelial cells.

Materials and Methods

Reagents. Orazipone (OR-1384), its derivative OR-1958, and the nonthiol-modulating control compound OR-2149 were provided by Orion Pharma (Espoo, Finland). OR compounds were dissolved in dimethyl sulfoxide just before the experiments. They were further diluted into culture medium 1:1000 to give the final concentration of 0.1% dimethyl sulfoxide. Rabbit anti-iNOS (sc-650), STAT1 α p91 (sc-345), NF- κ B p65 (sc-7151), actin (sc-1616-R), lamin A/C (sc-20681), and goat anti-rabbit horseradish peroxidase-conjugated polyclonal (sc-2004) antibodies were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). JAK inhibitor AG-490 (tyrphostin B42) was from Calbiochem (San Diego, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cell Culture. J774 macrophages (TIB-67; American Type Culture Collection, Manassas, VA) were cultured at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium with GlutaMAX-I (Lonza Verviers SPRL, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum (Lonza Verviers SPRL), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B (all obtained from Invitrogen, Paisley, UK). Cells were seeded on 24-well plates for nitrite measurement, Western blot analysis, and reverse transcription (RT)-PCR, and on 10-cm dishes for nuclear extract preparation. Cells were grown for 72 h to confluence before experiments.

To study the regulation of human iNOS promoter, pools of stably

transfected A549/8 cells containing the full-length (16-kilobase) human iNOS promoter (GenBank accession number AC005697) cloned in front of a luciferase reporter gene (Hausding et al., 2000) were used. These A549/8-pNOS2(16)Luc cells were kindly provided by Prof. Hartmut Kleinert (Johannes Gutenberg University, Mainz, Germany), and they were cultured at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium with 1 mM sodium pyruvate (Lonza Verviers SPRL) containing 5% heat-inactivated fetal bovine serum (Lonza Verviers SPRL), 2.5 μ g/ml polymyxin B, and 0.5 mg/ml G-418 disulfate salt (Sigma-Aldrich; for selection of cells stably transfected with the vector containing iNOS promoter construct and neomycin resistance gene). Cells were seeded on 24-well plates for nitrite measurement and RT-PCR. They were then grown for 48 h to confluence before experiments unless otherwise stated.

Cell viability after treatment with combinations of LPS or cytokine mixture and the tested compounds was assessed using modified 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide test (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Compounds were considered cytotoxic if the response was affected by more than 20%.

Preparation of Cell Lysates. At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline. Cells were solubilized in ice-cold lysis buffer containing 1% Triton X, 50 mM NaCl, 10 mM Tris-base, pH 7.4, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 40 μ M leupeptin, 50 μ g/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate, and 10 μ M *N*-octyl- β -D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged at 13,500g for 5 min. The protein content of the supernatants was measured by the Coomassie Blue method (Bradford, 1976). The extracts were boiled in SDS sample buffer, and they were stored at -20°C.

Preparation of Nuclear Extracts. Nuclear proteins were extracted as described previously (Sareila et al., 2006). The protein content of the extracts was measured by the Coomassie Blue method (Bradford, 1976). The extracts were boiled in SDS sample buffer, and they were stored at -20°C.

Western Blotting. Protein (20 μ g of lysates and nuclear extracts unless otherwise stated) was analyzed according to standard Western blotting procedure as described previously (Sareila et al., 2006). The membrane was incubated with the primary antibodies anti-iNOS, anti-STAT1 α , anti-NF- κ B p65, anti-lamin A/C, or anti-actin antibody in the blocking solution at 4°C overnight. The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1 (Alpha Innotech, San Leandro, CA).

RNA Extractions and Quantitative Reverse Transcription-PCR. RNA extractions and quantitative RT-PCR were performed as described previously (Sareila et al., 2006). In brief, total RNA was extracted and converted to cDNA. iNOS, luciferase and GAPDH mRNA were measured by quantitative RT-PCR. iNOS and luciferase mRNA levels were normalized to GAPDH, which was considered as a housekeeping gene. Each sample was determined in duplicate.

Luciferase, and human and murine iNOS and GAPDH primer and probe (6-carboxyfluorescein as 5'-reporter dye and 6-carboxytetramethylrhodamine as 3'-quencher) sequences are described in Table 1.

Nitrite Assays. For nitrite assays, J774 cells were treated with the compounds of interest in cell culture media volume of 1 ml and A549/8-pNOS2(16)Luc cells in a volume 0.5 ml. After 24-h incubation, the culture medium was collected for the nitrite measurement, which was used as a measure of NO production. Culture medium (100 μ l) was incubated with 100 μ l of Griess reagent (0.1% naphthalenediamine dihydrochloride, 1% sulfanilamine, and 2.4% H₃PO₄), and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard (Green et al., 1982).

Statistics. Results are expressed as mean + S.E.M. When indicated, statistical analysis was carried out by analysis of variances

TABLE 1
Primer and probe sequences

Gene	Oligonucleotide	Sequence 5'→3'
Human iNOS	Forward primer	GCAGGTCGAGGACTATTCTTTCA
	Reverse primer	TCCTTCTTCGCCTCGTAAGGA
	Probe	TCAAGAGCCAGAAGCGCTATCACGAAGATA
Human GAPDH	Forward primer	AAGGTCGGAGTCAACGGATTT
	Reverse primer	GCAACAATATCCACTTTACCAGAGTTAA
	Probe	CGCTGGTCACCAGGGCTGC
Murine iNOS	Forward primer	CCTGGTACGGGCATTGCT
	Reverse primer	GCTCATGCGCCTCTCTT
	Probe	CAGCAGCGGCTCCATGACTCCC
Murine GAPDH	Forward primer	GCATGGCCTTCCGTGTTT
	Reverse primer	GATGTCATCATACTTGGCAGGTTT
	Probe	TCGTGGATCTGACGTGCCGCC
Luciferase	Forward primer	AAAAAGTTGCGCGGAGGAG
	Reverse primer	TTTTTCTTGCCTCGAGTTTTC
	Probe	TGTGTTTTGTGGACGAAGTACCGAAAGGTCTTAC

followed by Dunnett's multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

Effect of Orazipone on LPS-Induced iNOS mRNA Expression in J774 Macrophages. We studied the effects of orazipone and its derivatives (Fig. 1) on LPS-induced iNOS mRNA expression in J774 macrophages. iNOS mRNA was measured by quantitative RT-PCR after 3-h incubation with the combination of LPS and the drugs (Fig. 2). LPS (10 ng/ml) induced iNOS mRNA expression, which was dose-dependently inhibited by orazipone (OR-1384), and a significant reduction was obtained also with its derivative OR-1958 (60 μ M). iNOS mRNA expression was not affected by the nonthiol-modulating control compound OR-2149 (60 μ M).

Effect of Orazipone on LPS-Induced NF- κ B p65 Activation in J774 Macrophages. Because iNOS mRNA expression was inhibited by orazipone, we investigated the effects of orazipone on the activation of NF- κ B, which is an important transcription factor in iNOS expression (Xie et al., 1994). NF- κ B activation in LPS-stimulated J774 cells was studied by measuring the nuclear translocation of NF- κ B subunit p65 by Western blot. LPS caused nuclear translocation of NF- κ B p65, which peaked at 30 min. Both orazipone (OR-1384; 60 μ M) and its derivative OR-1958 (60 μ M) decreased significantly the nuclear levels of NF- κ B p65 in LPS-activated macrophages (Fig. 3a). The nonthiol-modulating control compound OR-2149 (60 μ M) did not alter LPS-induced NF- κ B activation. In a similar manner to orazipone and OR-1958, an NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC; 100 μ M) inhibited LPS-induced nuclear translocation of NF- κ B by approximately 50% (Fig. 3b) and iNOS mRNA expression by approximately 75% (Fig. 3c).

Effect of Orazipone on LPS-Induced STAT1 Activation in J774 Macrophages. In addition to NF- κ B, transcription factor STAT1 is involved in LPS-induced iNOS expression (Gao et al., 1998). We studied the effects of orazipone on LPS-induced

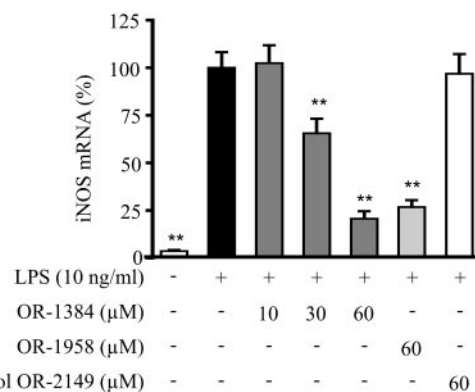


Fig. 2. Effects of orazipone (OR-1384), its derivative OR-1958, and its nonthiol-modulating control compound OR-2149 on iNOS mRNA levels in LPS-stimulated J774 macrophages. Macrophages were incubated with LPS (10 ng/ml) in the presence or in the absence of the tested compounds. After 3 h, the cells were lysed, and total RNA was extracted and converted to cDNA. iNOS mRNA levels were measured by quantitative RT-PCR, and they were normalized against GAPDH mRNA. LPS-induced iNOS mRNA expression was set as 100%, and the other values were related to that value. The results are expressed as mean + S.E.M., $n = 3$ to 7. **, $P < 0.01$ compared with cells treated with LPS alone.

STAT1 activation in J774 cells by measuring nuclear translocation of STAT1 α by Western blot. LPS caused nuclear translocation of STAT1 α , which increased up to the 6-h follow-up after addition of LPS. Orazipone (OR-1384; 60 μ M) and its derivative OR-1958 (60 μ M) inhibited LPS-induced nuclear translocation of STAT1 α , whereas the nonthiol-modulating control compound OR-2149 (60 μ M) had no effect (Fig. 4a). The effects of orazipone and OR-1958 on LPS-induced STAT1 α activation were similar to the inhibitory effect of JAK inhibitor AG-490, which inhibited STAT1 α activation (Fig. 4b) and iNOS mRNA expression (Fig. 4c).

Effect of Orazipone on LPS-Induced iNOS mRNA Decay in J774 Macrophages. In addition to transcriptional regulation, iNOS expression is regulated at the level of iNOS mRNA degradation (Kleinert et al., 2004; Korhonen et al.,

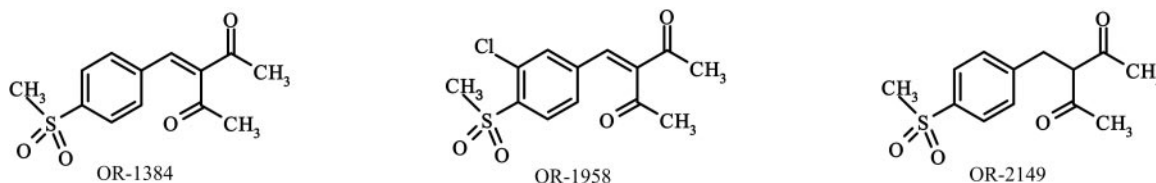


Fig. 1. Chemical structures of orazipone (OR-1384), orazipone derivative OR-1958, and the nonthiol-modulating control compound OR-2149.

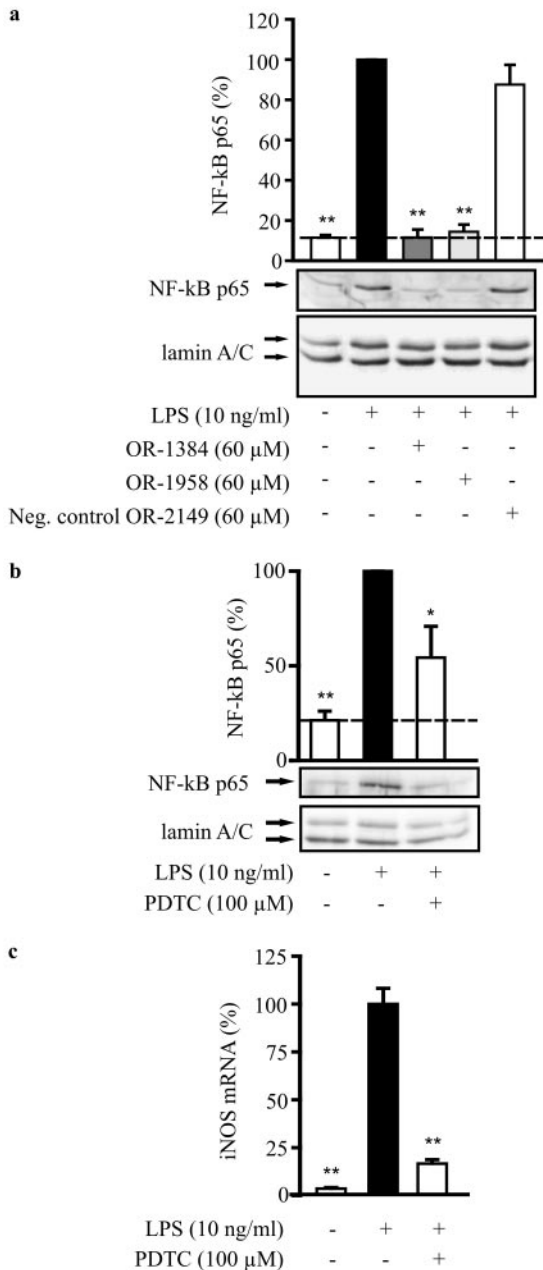


Fig. 3. a, effects of orazipone (OR-1384), its derivative OR-1958, and nonthiol-modulating control compound OR-2149 on nuclear translocation of NF- κ B in LPS-stimulated J774 macrophages. Cells were incubated with LPS (10 ng/ml) in the absence or in the presence of the tested compounds (60 μ M). After 30 min, the nuclear proteins were extracted. Equal amounts of protein (30 μ g/lane) were subjected to immunoblot analysis with an antibody against NF- κ B p65. Lamin A/C was used as a loading control. Nuclear levels of NF- κ B p65 in LPS-treated cells were set as 100%, and the other values were related to that. The results are expressed as mean \pm S.E.M. ($n = 3$). The dashed line represents the nuclear level of NF- κ B p65 in resting cells. **, $P < 0.01$ compared with cells treated with LPS alone. b, effect of the NF- κ B inhibitor PDTC on nuclear translocation of NF- κ B in LPS-stimulated J774 macrophages. The cells were incubated with LPS (10 ng/ml) in the absence or in the presence of PDTC (100 μ M). After 30 min, the nuclear proteins were extracted. Equal amounts of protein were subjected to immunoblot analysis with an antibody against NF- κ B p65. Lamin A/C was used as a loading control. Nuclear levels of NF- κ B p65 in LPS-treated cells were set as 100%, and the other values were related to that value. The results are expressed as mean \pm S.E.M. ($n = 3$). The dashed line represents the nuclear level of NF- κ B p65 in resting cells. **, $P < 0.01$ and *, $P < 0.05$ compared with cells treated with LPS alone. c, effect of an NF- κ B inhibitor PDTC on LPS-induced iNOS mRNA expression in J774 macrophages.

2005). In J774 macrophages, dexamethasone and SP600125, an inhibitor of c-Jun NH₂-terminal kinase (Bennett et al., 2001), have been shown to reduce LPS-induced iNOS expression by destabilizing the mRNA (Korhonen et al., 2002; Lahti et al., 2003). To study whether orazipone enhances iNOS mRNA degradation, the actinomycin D assay was applied. Cells were stimulated with LPS (10 ng/ml) in the presence and in the absence of orazipone (60 μ M). After 6 h, actinomycin D (0.5 μ g/ml) was added into the culture medium to stop transcription. The decay of iNOS mRNA was followed by measuring iNOS mRNA levels at 2-h intervals. Orazipone did not affect iNOS mRNA decay (Fig. 5), when measured by quantitative RT-PCR.

Effect of Orazipone on LPS-Induced iNOS Protein Expression in J774 Macrophages. To study whether the reduction in iNOS mRNA levels by orazipone results in decreased iNOS protein expression, we measured iNOS protein levels in J774 cells by Western blot after 24-h LPS treatment. LPS (10 ng/ml) induced iNOS protein expression, which was inhibited by orazipone (OR-1384) in a concentration-dependent manner (Fig. 6). The nonthiol-modulating control compound OR-2149 (60 μ M) did not affect LPS-induced iNOS expression. Orazipone derivative OR-1958 (60 μ M) reduced iNOS protein levels, but it was somewhat less potent than orazipone.

Effect of Orazipone on LPS-Induced NO Production in J774 Macrophages. Because iNOS protein levels were decreased by the drugs, we investigated the effects of orazipone (OR-1384), its derivative OR-1958, and the nonthiol-modulating control compound OR-2149 on LPS-induced NO production in J774 macrophages. LPS (10 ng/ml) induced NO production, which was inhibited by OR-1384 in a concentration-dependent manner (Fig. 7a). Orazipone was somewhat more potent than its derivative OR-1958, whereas the nonthiol-modulating control compound OR-2149 had no effect.

When orazipone was added to the culture 6 h after LPS, NO production was not altered (Fig. 7b). These data suggest that orazipone does not inhibit iNOS enzyme activity, but the effect is due to its suppressive action on iNOS expression (see above).

Because orazipone has been described to be labile in aqueous solutions (Vendelin et al., 2005), we incubated the cells with LPS and orazipone for only 4 h (which is needed for induction of iNOS), changed fresh medium thereafter, and measured accumulated nitrite after an additional 20-h incubation in medium free of LPS and orazipone. In these conditions, orazipone was more potent as an inhibitor of NO production than when LPS and orazipone were present in the culture medium for the whole 24-h incubation time (Fig. 7, a and c). That result supports the idea that the labile action of orazipone is partly overtaken by LPS in longer incubations.

Cytotoxicity as a contributing factor was ruled out by 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide test. None of the tested compounds at highest

The cells were stimulated with LPS (10 ng/ml) in the presence or in the absence of PDTC (100 μ M). After 3 h, the cells were lysed, and total RNA was extracted and converted to cDNA. iNOS mRNA levels were measured by quantitative RT-PCR, and they were normalized against GAPDH mRNA. LPS-induced iNOS mRNA expression was set as 100%, and the other values were related to that value. The results are expressed as mean \pm S.E.M. ($n = 3$). **, $P < 0.01$ compared with cells treated with LPS alone.

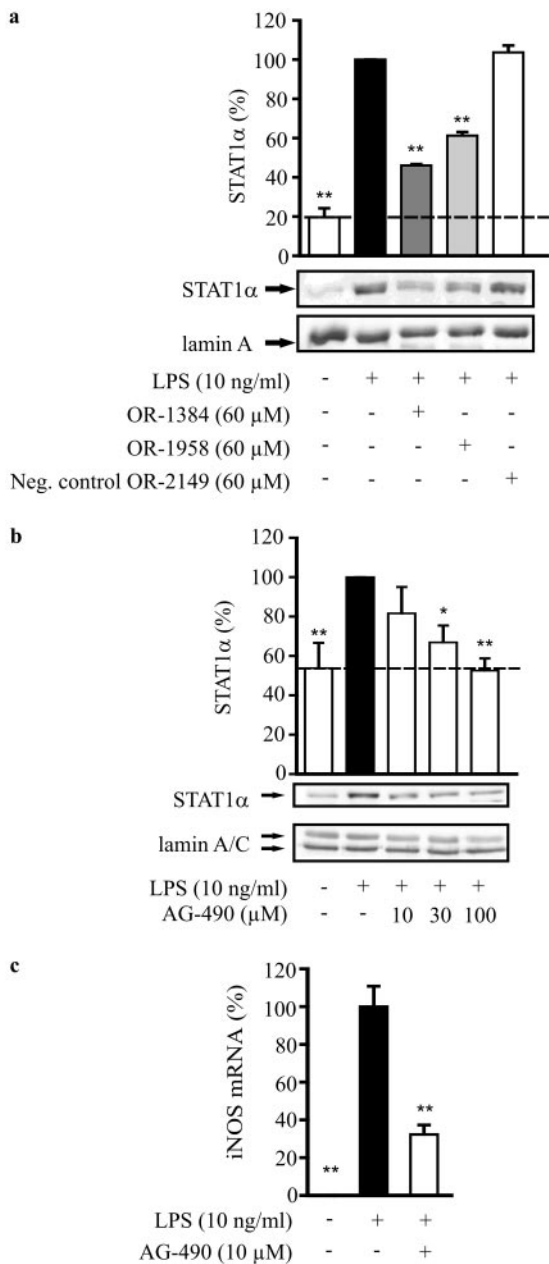


Fig. 4. a, effects of orazipone (OR-1384), its derivative OR-1958, and nonthiol-modulating control compound OR-2149 on the activation of STAT1 in LPS-stimulated J774 macrophages. The cells were incubated with LPS (10 ng/ml) in the absence or in the presence of the tested compounds (60 μM). After 6 h, the nuclear proteins were extracted, and equal amounts of protein (25 μg/lane) were subjected to immunoblot analysis with an antibody against STAT1α. Lamin A was used as a loading control. Nuclear levels of STAT1α in LPS-treated cells were set as 100%, and the other values were related to that value. The results are expressed as mean + S.E.M. (n = 3). The dashed line represents the nuclear level of STAT1α in resting cells. **, P < 0.01 and *, P < 0.05 compared with cells treated with LPS alone. b, effects of JAK inhibitor AG-490 on the activation of STAT1 in J774 macrophages stimulated by LPS. Cells were incubated with LPS (10 ng/ml) in the absence or in the presence of the tested compound. After 4 h, the nuclear proteins were extracted, and equal amounts of protein were subjected to immunoblot analysis with an antibody against STAT1α. Lamin A/C was used as a loading control. Nuclear levels of STAT1α in LPS-treated cells were set as 100%, and the other values were related to that value. The results are expressed as mean + S.E.M. (n = 6). The dashed line represents the nuclear level of STAT1α in resting cells. **, P < 0.01 and *, P < 0.05 compared with cells treated with LPS alone. c, effects of JAK inhibitor AG-490 on iNOS mRNA expression in J774 macrophages. The cells were incubated with

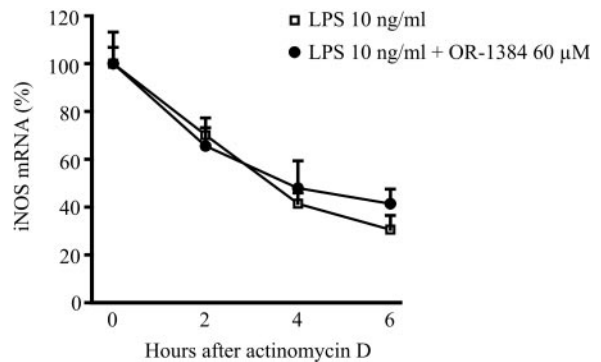


Fig. 5. Effect of orazipone (OR-1384) on iNOS mRNA decay in LPS-stimulated J774 macrophages. The cells were incubated with LPS (10 ng/ml) with or without orazipone (60 μM). After 6 h, actinomycin D (0.5 μg/ml) was added to stop transcription. Incubations were terminated at the indicated time points after addition of actinomycin D. Total RNA was extracted and converted to cDNA. iNOS mRNA was measured by quantitative RT-PCR, and it was normalized against GAPDH mRNA. iNOS mRNA level at the time of addition of actinomycin D was set as 100%, and the amount of iNOS mRNA left at the subsequent time points was related to that value. The results are expressed as mean + S.E.M. (n = 3).

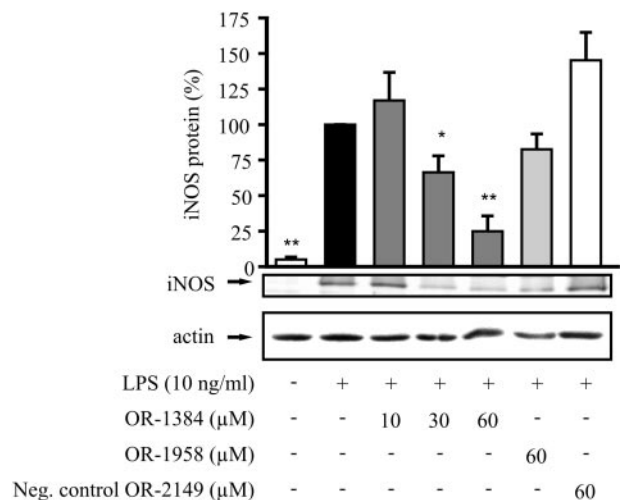


Fig. 6. Effects of orazipone (OR-1384), its derivative OR-1958, and its nonthiol-modulating control compound OR-2149 on iNOS protein expression in LPS-stimulated J774 macrophages. The cells were incubated with LPS (10 ng/ml) and the tested drugs for 24 h. Cells were lysed, and equal amounts of protein were subjected to immunoblot analysis with an antibody against iNOS. LPS-induced iNOS protein expression was set as 100%, and the other values were related to that value. Actin was used as a loading control. The results are expressed as mean + S.E.M. (n = 3–7). **, P < 0.01 and *, P < 0.05 compared with cells treated with LPS alone.

concentrations used (60 μM) showed cytotoxicity in combination with LPS in 24-h incubation.

Effect of Orazipone on iNOS mRNA Expression and iNOS Promoter Activity in Human Alveolar Epithelial Cells. The human alveolar epithelial A549/8-pNOS2(16)Luc cells are genetically modified to express luciferase reporter gene under the control of full-length human iNOS promoter (16 kilobases) (Hausding et al., 2000).

LPS (10 ng/ml) in the absence or in the presence of AG-490 (10 μM) as indicated. After 6 h, the cells were lysed. Total RNA was extracted and converted to cDNA. iNOS mRNA levels were measured by quantitative RT-PCR, and they were normalized against GAPDH mRNA. LPS-induced iNOS mRNA expression was set as 100%, and the other values were related to that value. The results are expressed as mean + S.E.M. (n = 3). **, P < 0.01 compared with cells treated with LPS alone.

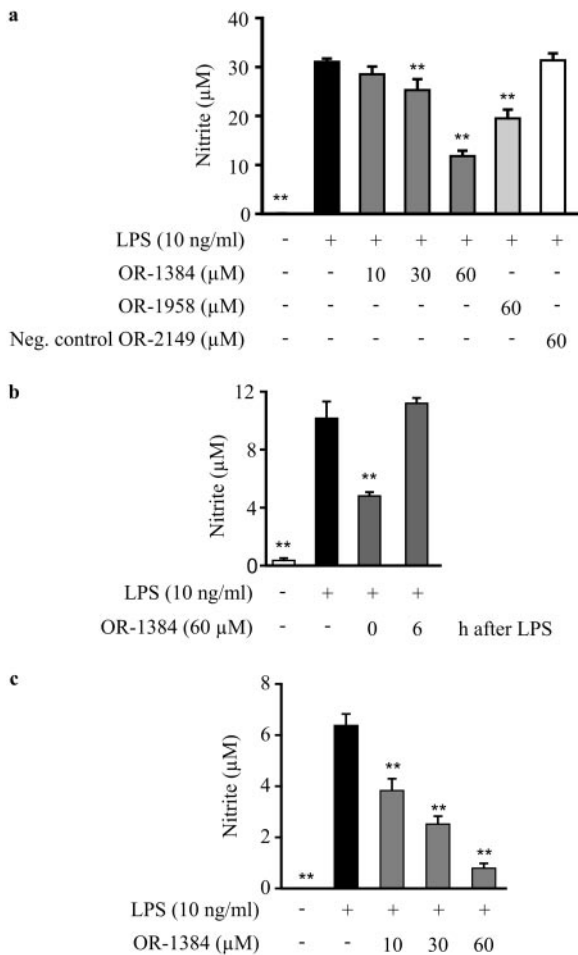


Fig. 7. a, effects of orazipone (OR-1384), its derivative OR-1958, and its nonthiol-modulating control compound OR-2149 on LPS-induced NO production in J774 macrophages. After 24-h incubation with indicated combinations of LPS (10 ng/ml) and the drugs, the supernatants were collected. Accumulated nitrite was measured in the culture medium by Griess reaction as an indicator of NO production. The values are mean \pm S.E.M. ($n = 12-31$). **, $P < 0.01$ compared with cells treated with LPS alone. b, effect of orazipone (OR-1384; 60 μ M) on LPS-induced NO production in J774 cells, when orazipone was added to the cells simultaneously or 6 h after LPS (10 ng/ml). The cells were incubated for 24 h, and nitrite was measured in the culture medium by Griess reaction as an indicator of NO production. The values are mean \pm S.E.M. ($n = 6$). **, $P < 0.01$ compared with cells treated with LPS alone. c, effect of orazipone (OR-1384) on LPS-induced NO production in J774 cells, when the cells were exposed to LPS and orazipone for 4 h. Thereafter, the culture medium was replaced with fresh medium that did not contain LPS or orazipone. The incubation was continued for another 20 h before the samples were collected, and nitrite was measured by Griess reaction as an indicator of NO production. The values are mean \pm S.E.M. ($n = 6$). **, $P < 0.01$ compared with cells treated with LPS alone.

To investigate the mechanism how orazipone lowered iNOS expression and NO production, we studied the effects of orazipone on iNOS and luciferase mRNA expression induced by a combination of proinflammatory cytokines [cytokine mixture of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and interleukin (IL)-1 β ; 10 ng/ml each] in A549/8-pNOS2(16)Luc cells. The combination of proinflammatory cytokines was used to induce NO production in these cells, which produce only low amounts of NO when exposed to LPS. The effects of orazipone were compared with those of PDTC, which prevents the activation of NF- κ B, an important transcription factor in human iNOS expression, and thereby inhibits iNOS expression at the level of transcription.

iNOS mRNA and luciferase mRNA were measured by quantitative RT-PCR after 6-h incubation with the combination of cytokine mixture and the drugs. Cytokine mixture induced iNOS mRNA expression, which was inhibited by orazipone (OR-1384; 60 μ M) and PDTC (100 μ M) (Fig. 8a).

In A549/8-pNOS2(16)Luc cells, luciferase gene expression is controlled by full-length human iNOS promoter. In this experiment, luciferase mRNA was used as a reporter of iNOS promoter activity. Unstimulated cells expressed a basal level of luciferase mRNA as a marker of some iNOS promoter activity in resting cells, which was expected according to previous data (Kleinert et al., 2004). To examine cytokine-induced portion of luciferase mRNA expression, the basal expression was subtracted from the luciferase expression in cells stimulated by cytokine mixture. Cytokine mixture induced luciferase mRNA expression, which was decreased by both orazipone and PDTC (Fig. 8a). The inhibitory effects of orazipone and PDTC on both iNOS mRNA and luciferase mRNA were about equal compared with mRNA expression induced by cytokine mixture. These results suggest that orazipone inhibits iNOS expression mostly at transcriptional level rather than by regulating mRNA expression through 3'-untranslated region of iNOS mRNA or by other post-transcriptional mechanisms.

In A549/8-pNOS2(16)Luc cells, also NO production was inhibited by orazipone (60 μ M) and PDTC (100 μ M), but the nonthiol-modulating control compound OR-2149 (60 μ M) was ineffective (Fig. 8b). The effects of the drug compounds on A549/8-pNOS2(16)Luc cell viability were tested. Orazipone and its derivatives (60 μ M) did not affect cell viability in combination with the cytokine mixture in 24-h incubation.

Effect of Orazipone on iNOS mRNA Expression and NO Production in the Presence of *N*-Acetyl-L-cysteine or Glutathione. To evaluate whether the effects of orazipone are dependent on the thiol-modulating property of orazipone, we tested the effects of orazipone in the presence of exogenously added thiols, i.e., *N*-acetyl-L-cysteine (NAC) and glutathione (GSH). J774 macrophages were preincubated with NAC or GSH for 1 h before they were stimulated with LPS to induce iNOS expression and NO production. iNOS mRNA levels (Fig. 9a) and NO production (Fig. 9b) were decreased by orazipone, but when NAC or GSH were present, the effects of orazipone were abolished. Likewise, the effect of orazipone on NO production was abolished in the presence of NAC or GSH also in A549/8-pNOS2(16)Luc human epithelial cells stimulated by proinflammatory cytokines (data not shown). These results support the assumption that thiol-modulating properties of orazipone are involved in the down-regulation of LPS or cytokine-induced iNOS expression and NO production by orazipone.

Discussion

Orazipone is a novel anti-inflammatory sulfhydryl-modulating compound that forms reversible conjugates with thiol groups in proteins and glutathione (Wroblewski et al., 1998). In the present study, we showed that the thiol-modulating compounds orazipone and its derivative OR-1958 inhibited activation of transcription factors NF- κ B and STAT1, and iNOS expression in response to inflammatory stimuli. The latter effect was related to the ability of orazipone to inhibit

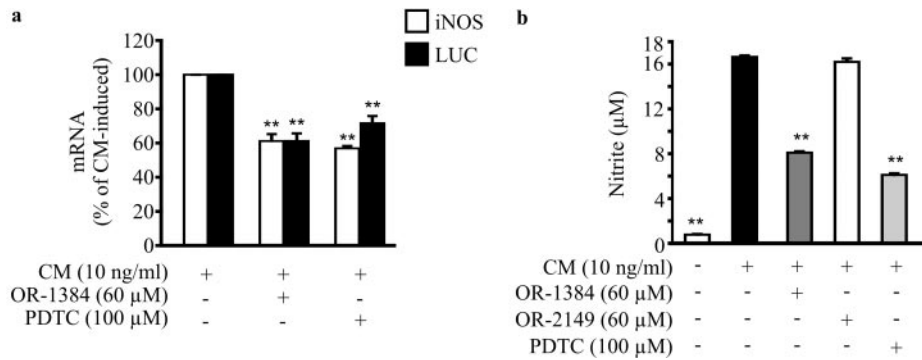


Fig. 8. a, effects of orazipone (OR-1384) and the NF-κB inhibitor PDTC on iNOS and luciferase mRNA accumulation in human alveolar epithelial A549/8-pNOS2(16)Luc cells. A549/8-pNOS2(16)Luc cells were stimulated with cytokine mixture (CM) containing IL-1β, IFN-γ, and TNF-α (10 ng/ml each) in the presence or in the absence of the tested compounds. After 6-h incubation, the cells were lysed, and total RNA was extracted and converted to cDNA. iNOS and luciferase mRNA levels were measured by quantitative RT-PCR, and they were normalized against GAPDH mRNA. iNOS mRNA was not detected in unstimulated cells. The basal luciferase mRNA expression in unstimulated cells was subtracted from all values to obtain the values that stand for cytokine-induced luciferase expression. Cytokine-induced mRNA expression was set as 100%, and the other values were related to that value. The results are expressed as mean + S.E.M. (n = 3). **, P < 0.01 compared with cells treated with cytokine mixture alone. b, effect of orazipone (OR-1384), its nonthiol-modulating control compound OR-2149, and an NF-κB inhibitor PDTC on NO production in A549/8-pNOS2(16)Luc cells stimulated with a combination of proinflammatory cytokines. After an 18-h period of serum starvation, the cells were stimulated with CM containing IL-1β, IFN-γ, and TNF-α (10 ng/ml each) in the presence or in the absence of the tested compounds in a serum-free media. After 24-h incubation, the culture medium was collected for nitrite measurement. Accumulated nitrite was measured in the culture medium by Griess reaction as an indicator of NO production. The values are mean + S.E.M. (n = 6). **, P < 0.01 compared with cells treated with cytokine mixture alone.

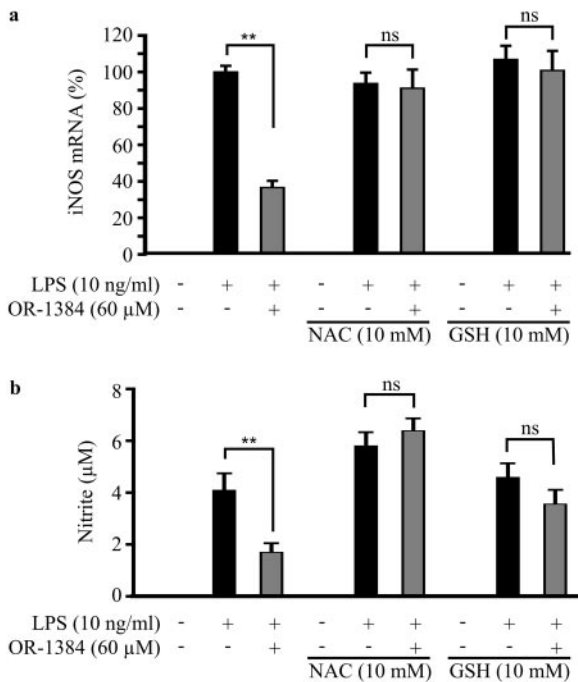


Fig. 9. a, effect of orazipone (OR-1384) on LPS-induced iNOS mRNA levels in J774 macrophages in the presence of exogenous thiols. Macrophages were preincubated with NAC or GSH for 1 h, and thereafter LPS (10 ng/ml) and orazipone (60 μM) were added. After 3-h incubation, the cells were lysed, and total RNA was extracted and converted to cDNA. iNOS mRNA levels were measured by quantitative RT-PCR and normalized against GAPDH mRNA. LPS-induced iNOS mRNA expression was set as 100%, and the other values were related to that value. The results are expressed as mean + S.E.M. (n = 3). **, P < 0.01; ns, nonsignificant. b, effect of orazipone (OR-1384) on LPS-induced NO production in J774 macrophages in the presence of exogenous thiols. The cells were first incubated with NAC or GSH for 1 h before LPS and orazipone were added, and the cells were incubated for another 4 h. Thereafter, the culture medium was replaced with fresh medium that did not contain LPS or orazipone, but it did contain NAC and GSH but not LPS or orazipone. The incubation was continued for additional 20 h before the samples were collected, and nitrite was measured by Griess reaction as an indicator of NO production. The values are mean + S.E.M. (n = 4). **, P < 0.01; ns, nonsignificant.

activation of iNOS promoter, which may well be explained by its ability to inhibit transcription factors NF-κB and STAT1.

LPS-induced NO production has been shown to be decreased in murine RAW264.7 macrophages in which glutathione was depleted by inhibiting its synthesis (Srisook and Cha, 2005). Because orazipone reacts with thiols in, e.g., glutathione, at least part of its effects on NO production could be mediated through inactivation of glutathione. In the present study, the effects of orazipone were abolished when an excess of glutathione or N-acetyl-L-cysteine was present in the culture, supporting the assumption that the effects of orazipone are mediated through its reactions with glutathione and/or thiol groups in intracellular signal transduction proteins. This idea is further supported by the present results showing that the nonthiol-modulating derivative of orazipone (OR-2149) did not affect NF-κB or STAT1 activation, iNOS expression, or NO production. Recently, protein glutathionylation has become of interest as an important post-translational modification that serves to transduce redox signals (Ghezzi, 2005; Gallogly and Mieyal, 2007). Therefore, we studied the existing literature to find out whether glutathionylation of transcription factors NF-κB or STAT1 has been documented to have functional consequences that could explain the present results. We did not find reports on glutathionylation of STAT1. Glutathionylation of p50 subunit of NF-κB has been reported to inhibit its DNA binding (Pineda-Molina et al., 2001). However, in the present study, we found that orazipone inhibited nuclear translocation of NF-κB. Another explanation is that orazipone targets some of the TLR4-receptor-activated signaling mechanisms upstream of NF-κB and STAT1 either by a mechanism related or unrelated to glutathionylation.

In the present study, we found that orazipone decreased iNOS expression and NO production in a concentration-dependent manner in activated macrophages. LPS-induced NF-κB activation and iNOS mRNA accumulation were inhibited by orazipone, when measured after 30-min and 3-h incubation, respectively. For comparison, effects of an NF-κB inhibitor PDTC were tested, and it was shown to inhibit

nuclear translocation of NF- κ B, along with its inhibitory effect on iNOS mRNA expression. PDTC has also previously been demonstrated to inhibit LPS-induced NO production in J774 macrophages (Ruetten et al., 1999). In the present study, we found that orazipone inhibited nuclear translocation of NF- κ B. In further experiments, we studied the effects of orazipone on iNOS promoter activity in A549/8-pNOS2(16)Luc human epithelial cells. In these cells, orazipone and NF- κ B inhibitor PDTC inhibited the expression of a reporter gene that was under the control of human iNOS promoter similarly as the expression of iNOS and the production of NO. NF- κ B is known to be an important transcription factor in human and murine iNOS expression (Kleinert et al., 2004; Korhonen et al., 2005), and inhibition of NF- κ B by orazipone or PDTC resulted in decreased iNOS mRNA expression along with inhibition of iNOS promoter activity. These data together suggest that orazipone inhibits NF- κ B activation and iNOS transcription, which leads to lowered levels of iNOS expression and NO production.

In addition to NF- κ B, another important transcription factor in iNOS expression is STAT1 (Kleinert et al., 2004; Korhonen et al., 2005). Orazipone and OR-1958 inhibited STAT1 activation and iNOS expression. Likewise, JAK2 inhibitor AG-490 inhibited both STAT1 activation and iNOS mRNA expression in J774 macrophages. AG-490 has also previously been shown to inhibit LPS-induced NO production in J774 macrophages (Salonen et al., 2006) and other cell lines (Cruz et al., 1999, 2001). JAK2 is regarded to be the kinase primarily responsible for phosphorylation and activation of STAT1 (Leonard and O'Shea, 1998), and JAK2 inhibition leads to lowered STAT1 activity. These results suggest that the decrease in iNOS expression and in NO production caused by orazipone may be partly mediated through the inhibition of STAT1 activation.

The reaction between glutathione and the thiol-modulating compounds orazipone and OR-1958 has been shown to be reversible (Nissinen et al., 1997). In addition, orazipone has been described to be labile in aqueous solutions (Vendelin et al., 2005). Therefore, orazipone and OR-1958 might be partly degraded during prolonged incubation periods (e.g., 12–24 h, which we used in the present study). Orazipone extensively reduced NF- κ B activation in J774 macrophages when measured after 30-min incubation and iNOS mRNA expression when measured after 3-h incubation, whereas it was less potent as an inhibitor of iNOS protein expression and NO production, which were measured after 24-h incubation. When the cells were incubated with LPS and orazipone for only 4 h (which is enough to induce iNOS) and then cultured in fresh medium (without LPS and orazipone) for another 20 h, orazipone was more potent as an inhibitor of NO production than in experiments where the cells were incubated with LPS and orazipone for 24 h. Therefore, we concluded that orazipone might have been partly degraded or its effects deteriorated during time in the culture, and while LPS was present its effects overcame those of orazipone. Therefore, it is possible that our experimental conditions (24-h incubations) may underestimate the potency of orazipone. This is in line with the studies in colitis models, where orazipone seemed to be more potent when it was given locally (intracolonic administration) than systemically (Wroblewski et al., 1998).

Orazipone has been tested in models of inflammatory bowel disease, with favorable results (Wroblewski et al., 1998), and it

has been reported to ameliorate intestinal radiation injury in a rat model (Boerma et al., 2006). Orazipone also has been shown to have beneficial effects in an animal model of asthma (Ruotsalainen et al., 2000) and to have antieosinophilic activity in human cells (Kankaanranta et al., 2006). Orazipone has been previously shown to inhibit IL-1 β , TNF- α , and IL-6 production in human monocytes (Wroblewski et al., 1998) and TNF- α production in human mast cell line HMC-1 (Vendelin et al., 2005). The present study extends the previous data by showing that orazipone inhibits activation of inflammatory transcription factors NF- κ B and STAT1, and iNOS expression in activated macrophages and epithelial cells. At least part of the beneficial effects of orazipone in colitis could be explained by lowered iNOS expression and NO production, because selective inhibition of iNOS has been shown to ameliorate inflammation in 2,4,6-trinitrobenzenesulfonic acid-induced model of colitis (Kankuri et al., 2001; Menchén et al., 2001) and in dextran sodium sulfate-induced colitis in rats (Rumi et al., 2004). More importantly, the observed inhibitory effects on the activation of NF- κ B and STAT1 are probably involved in the general anti-inflammatory mechanisms of orazipone because those transcription factors regulate the expression of various inflammatory factors in addition to iNOS. Moreover, the anti-inflammatory effects of glucocorticoids are mainly transmitted through suppressed NF- κ B-mediated transcription (Clark, 2007).

In conclusion, we have shown that orazipone and its derivative OR-1958 inhibited activation of NF- κ B and STAT1, and iNOS expression and NO production in response to inflammatory stimuli. Therefore, they are candidates for the treatment of inflammatory diseases whose pathogenesis is related to the activation of those inflammatory factors.

Acknowledgments

We thank Niina Ikonen, Salla Hietakangas, and Eeva Lahtinen for excellent technical assistance and Heli Määttä for skillful secretarial help. We thank Orion Pharma for providing orazipone, its derivative OR-1958, and the nonthiol-modulating control compound OR-2149 and Prof. Hartmut Kleinert for providing A549/8-pNOS2(16)Luc cells.

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