ALEKSI LAHTI

Regulation of Inducible Nitric Oxide Synthase Expression and Nitric Oxide Production by Mitogen-Activated Protein Kinase Pathways

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ACADEMIC DISSERTATION

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ABSTRACT

The inflammatory response is regulated by mediators produced by cells at the site of inflammation. A complex network of intracellular signaling pathways, activated in response to these mediators, regulates the activity and function of the inflammatory cells. Mitogen-activated protein kinases (MAPKs) are an evolutionally conserved family of protein kinases which form part of the signaling pathways connecting the the extracellular signal to intracellular regulatory proteins. Three better characterized MAPK pathways are extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 pathways.

One of the genes expressed by activated inflammatory cells is inducible nitric oxide synthase (iNOS). Nitric oxide (NO) produced by iNOS is a signaling molecule which modulates inflammatory responses. It is important in defence against bacteria and other pathogens but in chronic inflammation is considered to be harmful.

In this study, the role of MAPK pathways in the regulation of iNOS expression and NO production was investigated in macrophages activated by lipopolysaccharide (LPS). Small molecular inhibitors of the MAPKs were used as pharmacological tools to block the LPS-induced activity of these pathways. Total inhibition of ERK1/2 activity only partially inhibited iNOS expression and NO production. Inhibition of the JNK pathway inhibited iNOS expression and NO production by facilitating iNOS mRNA degradation. An unexpected finding was that inhibition of the p38 pathway augmented LPS-induced JNK activity, which resulted in increased iNOS expression and NO production.

These results show that MAPK pathways regulate iNOS expression and NO production. They also suggest that regulation of mRNA stability is an important means to regulate iNOS expression. Present finding together with characterization of the protein targets of MAPK pathways responsible for the regulation of mRNA stability could provide interesting targets for the development of novel anti-inflammatory drugs.

TIIVISTELMÄ

Tulehduspesäkkeen solut tuottavat välittäjäaineita, jotka säätelevät tulehduksen kulkua. Tulehdussolujen monimutkainen solunsisäisten signaalivälitysreittien verkosto aktivoituu tulehdusvälittäjäaineiden vaikutuksesta ja säätelee solujen toimintaa. Mitogeeneilla aktivoituvat proteiinikinaasit (MAP-kinaasit) on evoluution aikana säilynyt proteiinikinaasien perhe. MAP-kinaasit muodostavat signaalireittejä, jotka yhdistävät solunulkoiset signaalit solunsisäisiin säätelijäproteiineihin. Kolme parhaiten tunnettua MAP-kinaasireittiä ovat ERK1/2, p38 ja JNK reitit.

Yksi monista aktivoituneissa tulehdussoluissa ilmentyvistä geeneistä on indusoituva typpioksidisyntaasi (iNOS). iNOS:n tuottama typpioksidi (NO) on signaalimolekyyli, joka muuntelee tulehdusvastetta. NO on tärkeä taistelussa bakteereita ja muita taudinaiheuttajia vastaan, mutta NO:lla on haitallisia vaikutuksia kroonisessa tulehduksessa.

Tässä tutkimuksessa selvitettiin MAP-kinaasien roolia iNOS:n ilmentymisen ja NO:n tuoton säätelyssä lipopolysakkaridilla (LPS) aktivoiduissa makrofageissa. Pienimolekulaarisia MAP-kinaasiestäjiä käytettiin farmakologisina työkaluina estämään LPS:lla aktivoitujen MAP-kinaasireittien toimintaa. ERK1/2 reitin täydellinen esto aiheutti osittaisen iNOS:n ilmentymisen ja NO:n tuoton vähenemisen. JNK reitin esto nopeutti iNOS:n lähetti RNA:n hajoamista, mikä johti vähentyneeseen iNOS:n ilmentymiseen ja NO:n tuottoon. Yllättävä tulos oli, että p38 reitin esto lisäsi JNK reitin aktiivisuutta, joka johti lisääntyneeseen iNOS:n ilmentymiseen ja NO:n tuottoon.

Nämä tulokset osoittavat, että MAP-kinaasit säätelevät iNOS:n ilmentymistä ja NO:n tuottoa. Tulokset myös viittaavat siihen, että lähetti RNA:n stabiiliuden säätely on tärkeä keino säädellä iNOS:n ilmentymistä. Tämän tutkimuksen tuloksia voidaan hyödyntää uusien tulehdusta vaimentavien lääkkeiden kehitystyössä.

ABBREVIATIONS

AP-1	activator protein-1
ARE	AU-rich element
ATF	activating transcription factor
AUF1	ARE/poly(U)-binding/degradation factor-1
BH_4	(6R)-tetrahydrobiopterin
cAMP	3',5'-cyclic adenosine monophosphate
$[Ca^{2+}]_i$	intracellular free calcium concentration
C/EBPβ	CAAT/enhancer binding protein beta
cGMP	3',5'-cyclic guanosine monophosphate
COX-2	cyclooxygenase-2
EC_{50}	concentration required to achieve 50% of the maximal effect
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase 1 and 2
hnRNP	heterogenous nuclear ribonucleoprotein
IC_{50}	concentration required to inhibit 50% of the enzyme activity
IFN	interferon
IκB	inhibitor κB
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF-1	interferon regulatory factor-1
JAK	Janus-activated kinase
JNK	c-Jun N-terminal kinase
L-NMMA	N^G-monomethyl–L-arginine
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MK	MAPK-activated protein kinase (also called MAPKAPK)
MKK	MAPK kinase

MKK1/2	MAPK/ERK kinase 1 and 2 (also called MEK1/2)
MKKK	MAPK kinase kinase
MEKK	MAPK/ERK kinase kinase
MKP	MAPK phosphatase
MLK	mixed lineage kinase
MSK1	mitogen- and stress-activated protein kinase 1
NF-κB	nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PDTC	ammonium pyrrolidinedithiocarbamate
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
PTP	protein tyrosine phosphatase
RSK	p90 ribosomal S6 kinase
SB202190	4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4 pyridyl)-1H-imidazole
SB202474	4-ethyl-2-(4-methoxyphenyl)-5-(4-hydroxyphenyl)-5-(4pyridyl)-imidazole
SB203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4 pyridyl)-1H-imidazole
SB220025	5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)-imidazole
SC68376	2-methyl-4-phenyl-5-(4-pyridyl)oxazole
sGC	soluble guanylyl cyclase
SOCS	suppressor of cytokine signaling
SP600125	anthra(1,9-cd)pyrazol-6(2H)-one
STAT	signal transducer and activator of transcription
TAB-1	TAK1-binding protein 1
TAK1	TGF-β-activated protein kinase 1
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
UTR	untranslated region

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their Roman numerals (I-IV). In addition, some unpublished data are presented.

I. Lahti A, Lähde M, Kankaanranta H and Moilanen E (2000). Inhibition of extracellular signal regulated kinase suppresses endotoxin-induced nitric oxide synthesis in mouse macrophages and in human colon epithelial cells. *Journal of Pharmacology and Experimental Therapeutics*, 294: 1188-1194.

II. Lahti A, Jalonen U, Kankaanranta H and Moilanen E (2003). c-Jun NH_2-terminal kinase inhibitor anthra(1,9-cd)pyrazol-6(2H)-one reduces inducible nitric-oxide synthase expression by destabilizing mRNA in activated macrophages. *Molecular Pharmacology*, 64:308-315.

III. Lahti A, Kankaanranta H and Moilanen E (2002). P38 mitogen-activated protein kinase inhibitor SB203580 has a bidirectional effect on iNOS expression and NO production. *European Journal of Pharmacology*, 454:115-123.

IV. Lahti A, Kankaanranta H and Moilanen E. Inhibition of p38 mitogen-activated protein kinase regulates c-Jun N-terminal kinase activity: Implication in inducible nitric oxide synthase expression. Submitted for publication.

INTRODUCTION

Inflammation is the body's reaction to invasion by an infectious agent, to antigen challenge or to tissue damage. It is characterized by increased capillary permeability and migration of leukocytes to the site of inflammation. Leukocytes are activated by inflammatory mediators produced by cells at the site of inflammation or directly through recognition of foreign molecular structures. Many pathogenic bacteria contain conserved molecular structures which are recognized and bound by pattern recognition receptors. Binding of inflammatory mediators or foreign molecular structures to their receptors activates signal transduction pathways, which connect the extracellular signal from the cell surface receptors to intracellular regulatory proteins. These proteins then regulate the activity of the cell e.g. by modulating gene expression. Signaling pathways are not only passive messengers, but amplify and integrate signals from different receptors and thereby help to coordinate cellular responses.

Mitogen-activated protein kinases (MAPKs) form an evolutionarily conserved family of protein kinases which are part of the signaling pathways regulating various cellular responses, e.g. cytokine production, cell proliferation and apoptosis. Although the core MAPK pathways and the activating extracellular signals are well characterized, their downstream targets and the physiological roles are only partially known (Kumar et al. 2003).

A macrophage is often the activator cell of the inflammatory response. One of the genes expressed in activated macrophages is inducible nitric oxide synthase (iNOS). Nitric oxide (NO) generated by iNOS has both pro- and anti-inflammatory effects, depending on the stage of inflammation, the amount of NO produced and the site of NO production. Since the discovery of iNOS in 1992, regulation of its expression has been intensively investigated. Several transcription factors which regulate iNOS expression, have been characterized. However, the signaling pathways which regulate iNOS expression are poorly characterized and little is known of the post-transcriptional regulatory mechanisms (Kleinert et al. 2003).

Although the inflammatory response is usually beneficial and required for survival, it can sometimes be unnecessary and harmful to the host. This is the case in chronic inflammatory diseases such as rheumatoid arthritis, where chronic inflammation leads to joint destruction and functional disability. Antirheumatic drugs are used to alleviate the inflammation in these diseases. However, many of these drugs are of limited efficacy, or their use is limited by adverse effects. New drugs are therefore constantly being developed for the treatment of these diseases. The iNOS and MAPK pathways are seen as potential targets for antiinflammatory drug development (Kumar et al. 2003, Vallance and Leiper 2002).

REVIEW OF THE LITERATURE

1. Nitric Oxide

NO is a water- and lipid-soluble gas containing an unpaired electron, which makes it a free radical and a highly reactive molecule. Its role as a biological messenger was discovered when two research groups showed in 1987 that the endothelium-derived relaxing factor was NO (Palmer et al. 1987, Ignarro et al. 1987, Ignarro et al. 1987). Since then knowledge of the biology of NO has increased enormously. It has many important physiological functions, e.g. it regulates the inflammatory response, controls vascular tone and serves as a neurotransmitter in the brain and in nonadrenergic noncholinergic nerves in the periphery (Mashimo and Goyal 1999).

1.1 Biosynthesis of NO

NO is synthetised from L-arginine in a reaction catalysed by a family of nitric oxide synthase (NOS) enzymes. Active NOS is a tetramer formed by two NOS proteins and two calmodulins. Conversion of L-arginine to NO and L-citrulline also requires nicotineamide adenine dinucleotide phosphate and O_2 as cosubstrates and (6R)-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide, flavin mononucleotide and iron protoporphyrin IX (haem) as cofactors (Alderton et al. 2001).

Three different NOS isoforms have been isolated. The neuronal NOS (nNOS, NOS I) is expressed predominantly in neurons in the brain and peripheral nervous system, but also in other cell types e.g. skeletal muscle (Bredt et al. 1991, Boissel et al. 1998). Endothelial NOS (eNOS, NOS III) is expressed in endothelial cells of various tissues e.g. vascular wall, airways and kidneys and other cell types such as cardiac myocytes and platelets (Janssens et al. 1992, Shaul 2002). Both nNOS and eNOS are considered to be mainly constitutively expressed and are inactive in resting cells. An increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) stabilizes the binding of calmodulin to eNOS and nNOS, and by this mechanism switches on NO production. Thus, any stimuli which increase the $[Ca^{2+}]_i$, e.g. acetylcholine in endothelial

cells, triggers the production of NO. As the increases in $[Ca^{2+}]_i$ are usually transient and short-lasting, so is the NO production of the constitutively expressed NOSs (Alderton et al. 2001).

The third isoform of the NOS family is inducible NOS (iNOS, NOS II). This was first cloned from murine macrophages (Lowenstein et al. 1992, Lyons et al. 1992, Xie et al. 1992) and shortly theseafter from human hepatocytes (Geller et al. 1993) and chondrocytes (Charles et al. 1993). Mouse and human iNOS genes code proteins with 80 % amino acid sequence identity and a calculated molecular mass of ~131 kDa. Human iNOS has 51 % and 53 % amino acid sequence identity to human eNOS and nNOS, respectively. No or only low-level expression of iNOS is generally observed in normal resting cells. Exposure of cells to microbial products (e.g. lipopolysaccharide (LPS), double-stranded RNA) or cytokines (e.g. interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1)) induces the expression of the iNOS gene in a number of cell types (Ganster and Geller 2000). Binding of calmodulin to iNOS is tight even at low $[Ca^{2+}]_i$. Thus, once expressed, iNOS produces NO constantly for several hours, even days. Expression of iNOS and nNOS splice variants has been reported, but their physiological significance is uncertain (Alderton et al. 2001).

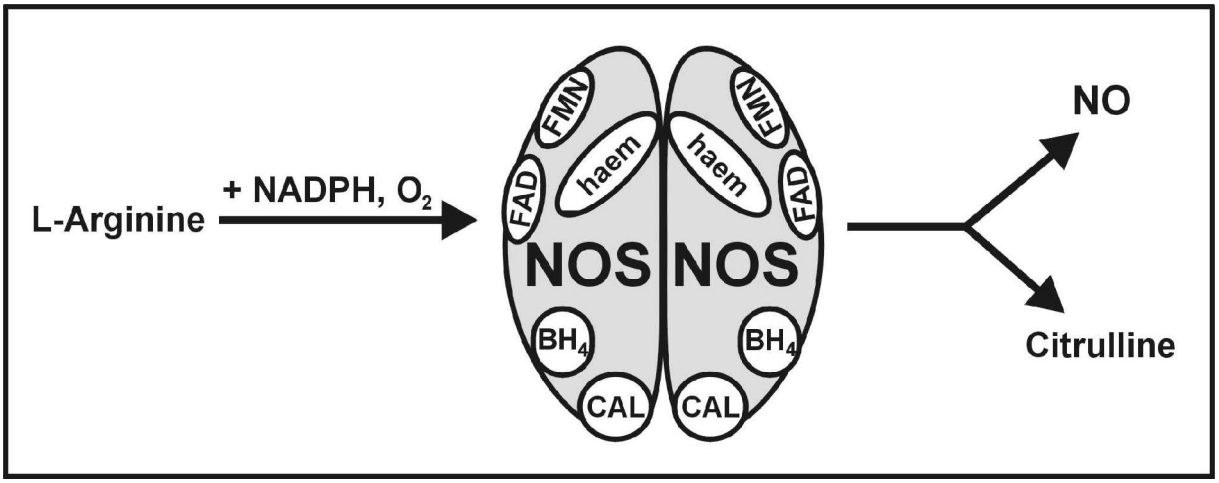


Figure 1. The NOS pathway. For enzymatic activity, NOS must dimerize and bind the cofactors flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), haem, tetrahydobopterin (BH_4) and calmodulin (CAL). Nicotineamide adenine dinucleotide phosphate (NADPH). (Modified from Vallance et al., 2002)

1.2 NO as a signaling molecule

NO has been implicated in the regulation of a number of physiological processes. It has however, been difficult to understand how specificity in signaling can be achieved with a molecule which is diatomic, short-lived and readily diffusible. As a free radical, NO easily interacts with transition metals of enzymes, which is an important mechanism whereby NO modifies cellular functions. The first enzyme known to be regulated in this manner is soluble guanylate cyclase (sGC) (Ignarro 1990). NO binds to ferrous iron in the haem moiety of sGC, which increases its activity 400-fold. Activated sGC catalyses the formation of 3',5'-cyclic guanosine monophosphate (cGMP), which is involved in the activation of a variety of effectors and physiological responses such as vasodilatation (Hanafy et al. 2001). Organic nitrates such as nitroglycerin are widely used drugs which donate NO after enzymatic conversion. Upon NO release, sGC is activated and cGMP produced. An increased cGMP concentration promotes vasodilatation, an effect of nitroglycerin which is observed clinically (Fung 2004). Phosphodiesterases, especially phosphodiesterase 5, catalyse the inactivation of cGMP to 5'-GMP, thus acting as a negative inhibitory loop. Another drug acting through NO-activated pathways is sildenafil, which is used to treat erectile dysfunction. Sildenafil selectively inhibits phosphodiesterase 5 expressed in the corpus cavernosum. Inhibition of phosphodiesterase 5 results in an increase in cGMP concentration, which leads to vasodilatation of the penile vessels and erection (Corbin and Francis 1999).

NO controls cellular functions also by S-nitrosylation of critical cysteine residues of proteins. S-nitrosylation causes allosteric changes, this leading to activation (Lander et al. 1997) or inactivation (Choi et al. 2000, Park et al. 2000) of the target proteins. Localization of NOSs and target proteins to the same subcellular compartments increases the specificity of S-nitrosylation signaling. Further specificity is achieved through scaffolding and anchoring proteins, which bring NOSs and targets into close proximity (Stamler et al. 2001).

A third proposed mechanism for NO signaling is nitration of tyrosine residues. Although tyrosine nitration has been associated with reduced activity of manganese superoxide dismutase (MacMillan-Crow et al. 1996) and prostacyclin synthase (Zou et al. 1997), little is

known regarding the effect of tyrosine nitration of proteins. It is thought that nitration of tyrosine residues modulates their phosphorylation and thus affects the function of the target proteins (Li et al. 1998). However, the role of tyrosine nitration as a signal transduction mechanism has yet to be elucidated (Hanafy et al. 2001).

1.3 Role of NO in inflammation

Markers of increased NO production have been observed in various inflammatory diseases. For instance, increased amounts of NO have been detected in air exhaled by patients with asthma or alveolitis compared with healthy controls (Lehtimaki et al. 2001b, Lehtimaki et al. 2001a). Increased amounts of the NO metabolites nitrite, nitrotyrosine and S-nitrosoproteins have been observed in serum and synovial fluid from patients with rheumatoid arthritis (Kaur and Halliwell 1994, Farrell et al. 1992, Hilliquin et al. 1997). The inducible isoform of NOS is mainly thought to be responsible for increased production of NO in sites of inflammation. This conception is supported by reports in which expression of iNOS has been shown by immunohistochemistry and in situ hybridization at the site of inflammation, e.g. the synovium of patients with rheumatoid arthritis (Sakurai et al. 1995), temporal artery biopsies from patients with giant cell arteritis (Weyand et al. 1996), in interface membrane tissue around loosened joint replacement implants (Moilanen et al. 1997), in osteoarthritic cartilage (Vuolteenaho et al. 2001), bronchial biopsies from asthmatic patients (Redington et al. 2001, Saleh et al. 1998), lung biopsies from patients with interstitial pneumonia or granulomatous lung disease (Lakari et al. 2002) and in tissue samples of inflamed colon from patients with ulcerative colitis, Crohn's disease and diverticulitis (Singer et al. 1996, Godkin et al. 1996, Kankuri et al. 2003). In patients with septic shock, increased NOS activity has been measured in putrescent tissue samples, compared with normal tissue from the same patients (Annane et al. 2000).

The role of NO in inflammation is complex. NO has both pro- and anti-inflammatory effects, which depend on the stage of inflammation, the amount of NO produced and the site of NO production (Bogdan 2001). Proinflammatory effects of NO include increased local blood flow and vascular leakage, which promote edema (Warren et al. 1992, Laszlo and Whittle 1997,

Kajekar et al. 1995) and recruitment of inflammatory cells to sites of inflammation through increased chemokine expression (Trifilieff et al. 2000, Isenberg 2003). Efficient activation of natural killer cells by IL-12 requires NO (Bogdan et al. 2000). NO can augment tissue destruction by activating metalloproteinase enzymes (Murrell et al. 1995), suppressing the formation of novel matrix components (Taskiran et al. 1994, Studer et al. 2000) and inducing necrosis and apoptosis (Brown and Borutaite 2002). Many of the cytotoxic effects of NO are considered to be mediated through peroxynitrite, which is formed in a reaction between NO and superoxide anion. Peroxynitrite is a highly reactive molecule which can initiate lipid peroxidation, trigger DNA breakage and cause protein oxidation and nitration (Virag et al. 2003). Anti-inflammatory properties of NO include inhibition of leukocyte adhesion to the endothelium due to reduced expression of adhesion molecules (Kubes et al. 1991, Kosonen et al. 2000) and reduced proliferation of leukocytes (Albina et al. 1991, Bobe et al. 1999, Kosonen et al. 1998). NO induces apoptosis in eosinophils, which might limit eosinophilia in allergic diseases (Zhang et al. 2003), and NO has also been shown to be involved in the negative selection of self-reactive T cells in thymus (Aiello et al. 2000).

It is unclear to what extent the NO production of each of the NOS isoforms is responsible for the effects observed in inflammation. It was previously thought that high amounts of NO produced by iNOS are responsible for the detrimental effects and low amounts of NO produced by nNOS and eNOS for the protective effects of NO. This is now known to be an over simplification (Bogdan 2001). Evidence on the role of iNOS in the inflammatory response has been obtained comparing responses of iNOS knockout mice to wild-type mice with the same genetic background in different disease models. In infectious diseases iNOS seems to be an important component in defense mechanisms against various pathogens, as iNOS knockout mice are more susceptible to infections by mycobacterium tuberculosis, leishmania major and listeria monocytogenes (Wei et al. 1995, MacMicking et al. 1995, MacMicking et al. 1997). However, the survival rate of iNOS knockout mice is increased after influenza A virus infection (Karupiah et al. 1998) and these mice are also more resistant to septic shock (Wei et al. 1995, MacMicking et al. 1995). Impaired tumor suppression (Scott et al. 2001) and wound healing (Yamasaki et al. 1998), reduced ischemic brain injury

(Iadecola et al. 1997) and a reduced number of disease manifestations in autoimmune disease model (Gilkeson et al. 1997) have also been reported.

NOSs are seen as promising targets for drug development. Especially iNOS inhibitors have been considered to be potential anti-inflammatory drugs in a number of indications. The first inhibitors described were L-arginine analogues (e.g. N^G-monomethyl–L-arginine (L-NMMA) and N^G-nitro–L-arginine methyl ester (L-NAME)) and they were unspecific in NOS isoform selectivity. L-arginine analogues, like most iNOS inhibitors identified so far, compete with L-arginine for the substrate binding site in the enzyme. Selective iNOS inhibition over other NOSs has been difficult to achieve, but recently selective iNOS inhibitors have been developed (e.g. 1400W, GW274150) (Alderton et al. 2001). Inhibition of NOS dimerization is another way of inhibiting NOS activity and some isoform selectivity has been achieved with these inhibitors (Vallance and Leiper 2002). Selective iNOS inhibitors have been shown to be protective in ischemia reperfusion injury models of different organs (Cuzzocrea et al. 2002a, Chatterjee et al. 2003), to protect against organ injury in hemorrhagic shock models (McDonald et al. 2002), and to reduce bone resorption in a mouse arthritis model (Cuzzocrea et al. 2002b). There are few studies on the effects of NOS inhibitors in humans. Non-selective NOS inhibitors L-NMMA and L-NAME have been used in patients with septic shock. NOS inhibitors improve hemodynamics in these patients and the early clinical data suggested that they could be effective in the treatment of septic shock. However, in one prospective, double-blind, placebo-controlled trial, L-NMMA was reported to increase mortality (Feihl et al. 2001). L-NMMA would also appear to have an analgesic effect in patients with chronic tension-type headache (Ashina et al. 1999).

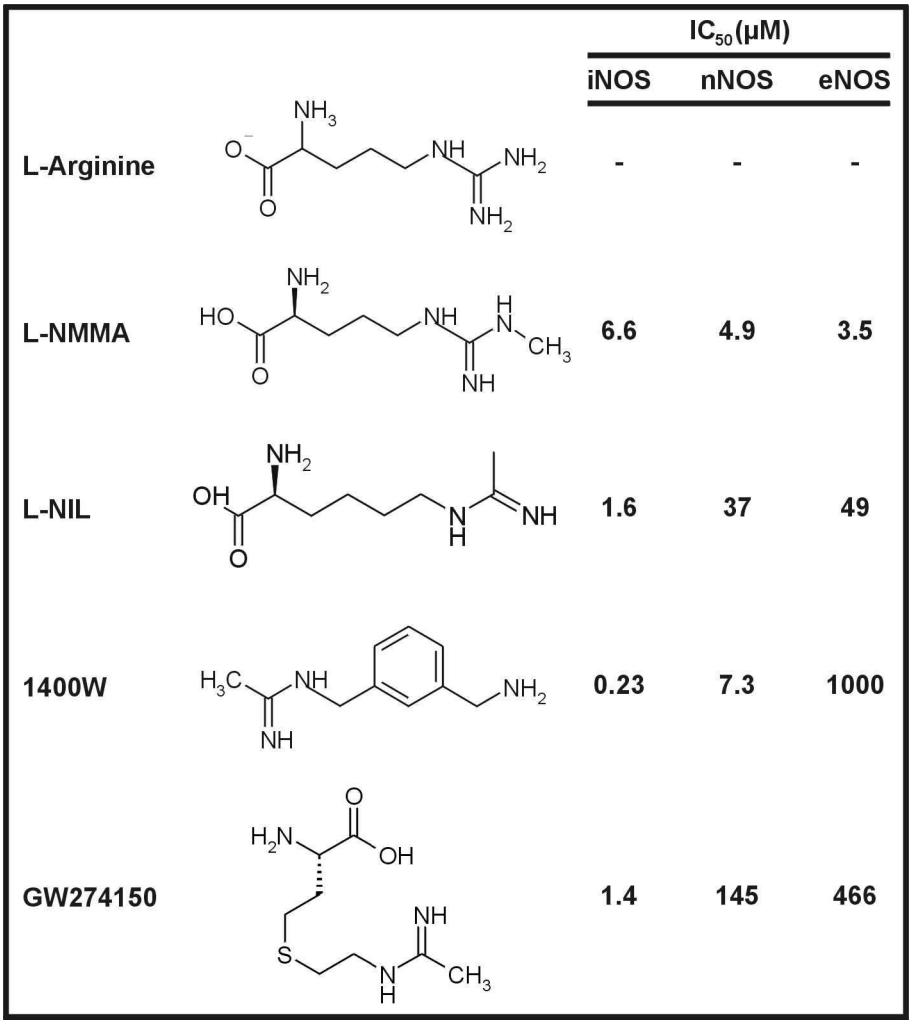


Table 1. Structures of NOS inhibitors and the concentrations required for 50 % inhibition of the enzyme activity (IC_{50} values) of NOS isoforms. (Modified from Alderton et al., 2001)

2. Regulation of iNOS expression and NO production

iNOS expression is inducible in many mouse and human cell types. However, there are marked cell type- and species-specific differences in the induction of iNOS expression in response to different stimuli. Many mouse cell types readily express iNOS in response to one cytokine or LPS, whereas human cells usually require a combination of different cytokines for detectable iNOS expression and NO synthesis. Furthermore, iNOS is expressed at high levels in activated mouse macrophages, but it has been difficult to induce iNOS expression in human

monocytes and macrophages in vitro, although expression can be shown in human macrophages in vivo (Sakurai et al. 1995, Weyand et al. 1996, Moilanen et al. 1997). Research into the molecular mechanisms which regulate iNOS expression, has mostly focused on transcriptional regulation, but it is increasingly apparent that post-transcriptional regulation is also important (Kleinert et al. 2003, Taylor and Geller 2000).

2.1 Transcriptional regulation

Transcription of the mouse iNOS gene is regulated by a ~1 kb-long promoter region (Xie et al. 1993, Lowenstein et al. 1993) which contains several putative binding sites for transcription factors. An in vivo footprinting assay revealed LPS-induced binding to sequences similar to Octamer, E-box, nuclear factor kappa B (NF-κB) and IFN-stimulated response element consensus sequences (Goldring et al. 1996). The mouse iNOS promoter contains two NF-κB elements which bind the homodimer p50/p50 and heterodimers p50/p65 and p50/c-Rel (Table 2). NF-κB is essential for iNOS expression. as site-directed mutations of both of the sites completely abolish promoter activity and chemical inhibitors of NF-κB prevent iNOS expression and NO production (Diaz-Guerra et al. 1996b, Kleinert et al. 1996).

IFN-γ is an efficient enhancer of iNOS expression in most cells. It activates signal transducer and activator of transcription 1 (STAT1), which binds to a gamma-activated site in the iNOS promoter. Activation of STAT1 also leads to expression of IFN consensus sequence binding protein and IFN regulatory factor-1 (IRF-1), which form a complex and bind to the IFN-stimulated response element in the iNOS promoter (Table 2). The expression of iNOS is seriously impaired in macrophages isolated from mice deficient in STAT1 (Meraz et al. 1996), IRF-1 (Kamijo et al. 1994) or IFN consensus sequence binding protein (Contursi et al. 2000). Furthermore, IRF-1 and NF-κB have been shown to interact and promote change in iNOS promoter conformation, which is held to enhance assembly of the pre-initiation complex critical for induction of transcription (Saura et al. 1999).

An octamer site is also required for iNOS promoter activity, as site directed mutation of this site completely abolishes promoter activity. The octamer site binds members of the POU

family of transcription factors (Table 2) and high mobility group protein-I(Y). High mobility group protein-I(Y) alone cannot promote transcription, but it augments and stabilizes the p50/p65 binding to the NF-κB site (Perrella et al. 1999). Epithelium-specific Ets is an inducible transcription factor which is not essential but can augment iNOS promoter activity possibly by interacting with p50 (Rudders et al. 2001). An in vivo footprinting assay has shown binding to CAAT/enhancer binding protein beta (C/EBPβ) element in iNOS promoter (Goldring et al. 1996) and site-directed mutation of this site reduces iNOS promoter activity (Dlaska and Weiss 1999). However, macrophages from C/EBPβ knockout mice produce NO normally in response to LPS and IFN-γ (Tanaka et al. 1995), suggesting that this factor is not essential for iNOS transcription or that it can be replaced by other factors of the C/EBP family. The promoter also contains a functional hypoxia-responsive enhancer sequence, which binds hypoxia-inducible factor 1 (Melillo et al. 1997). Activator protein-1 (AP-1) transcription factors are composed of dimers of proteins belonging to the Jun, Fos, Maf and activating transcription factor (ATF) subfamilies (Shaulian and Karin 2002). The role of AP-1 in the regulation of iNOS transcription is controversial. The existence of both positive and negative regulatory AP-1 sites has been reported (Table 2), but the composition of the AP-1 which bind to these sites is not known. Overexpression of c-Fos, FosB and c-Jun suppresses iNOS promoter activity (Okada et al. 2003).

Negative regulators of iNOS transcription have also been found. Overexpression of STAT3 inhibits IL-1β- and LPS+IFN-γ-induced iNOS promoter activity and NO production. STAT3 interacts with p65 and inhibits NF-κB activity, but this effect does not require binding of STAT3 to a gamma-activated site (Yu et al. 2002). Elk-3 is a member of the Ets family of transcription factors which is able to repress transcription. Overexpression of Elk-3 suppresses LPS-induced promoter activity and iNOS expression. However, the repressive effect does not require binding to an Ets site in the iNOS promoter (Chen et al. 2003). Site-directed mutation of the E-box element increases iNOS promoter activity. E-box binds homo- or heterodimers formed by upstream stimulatory factors 1 and 2. In agreement with the data obtained by site-directed mutation, overexpression of upstream stimulatory factors 1 and 2 suppresses iNOS promoter activity, whereas the dominant negative mutant of upstream stimulatory factor 1 augments promoter activity (Gupta and Kone 2002).

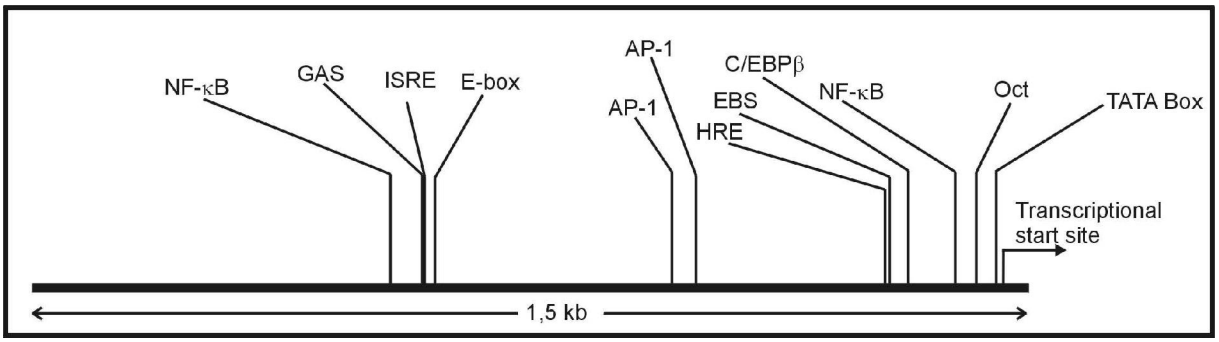


Figure 2. Schematic representation of mouse iNOS promoter showing the relative positions of the regulatory elements. Gamma activated site (GAS), IFN-stimulated response element (ISRE), Octamer (Oct), hypoxia responsive enhancer (HRE), Ets binding sequence (EBS).

element	positions	binding proteins	effect	methods	references
NF-κB	-85 to -76	p50, p65(RelA), c-Rel	+	site-directed mutation, EMSA, in vivo footprinting	(Kim et al. 1997, Diaz-Guerra et al. 1996b, Xie et al. 1994)
	-971 to -962				
ISRE	-926 to -909	ICSBP, IRF-1	+	site-directed mutation, EMSA, in vivo footprinting	(Martin et al. 1994, Xiong et al. 2003)
GAS	-942 to -934	STAT1α	+	site-directed mutation, EMSA, in vivo footprinting	(Gao et al. 1997)
Oct	-61 to -54	Oct-1, HMG-I(Y), Brn-3a, Brn-3b	+	site-directed mutation, EMSA, in vivo footprinting	(Xie 1997, Sawada et al. 1997, Kim et al. 1999) (Perrella et al. 1999, Gay et al. 1998)
EBS	-190 to -180	ESE-1	+	site-directed mutation, EMSA	(Rudders et al. 2001)
C/EBPβ	-153 to -142	C/EBPβ	+	site-directed mutation, EMSA, in vivo footprinting	(Dlaska and Weiss 1999)
HRE	-227 to -209	HIF-1	+	site-directed mutation, EMSA	(Melillo et al. 1997)
AP-1	-524 to -518		-	site-directed mutation	(Kizaki et al. 2001)
	-488 to -482		+	site-directed mutation	(Okada et al. 2003)
E-box	-893 to -888	USF-1, USF-2	-	site-directed mutation, EMSA, in vivo footprinting	(Gupta and Kone 2002)

Table 2. Regulatory elements in the mouse iNOS promoter. IFN-stimulated response element (ISRE), IFN consensus sequence binding protein (ICSBP), gamma activated site (GAS), octamer (Oct), high mobility group protein-I(Y) (HMG-I(Y)), hypoxia responsive enhancer (HRE), hypoxia-inducible factor-1(HIF-1), Ets binding sequence (EBS), epithelium specific Ets (ESE-1), upstream stimulatory factor 1 and 2 (USF-1, USF-2), electrophoretic mobility shift assay (EMSA).

The promoter of the human iNOS gene is different from the murine iNOS promoter. Deletional analysis of the human iNOS 5' flanking region indicates the presence of regulatory elements on the length of 16 kb (de Vera et al. 1996). In two reports, constructs containing the first 3.2 kb (Nunokawa et al. 1996) and 1 kb (Kolyada et al. 1996) were found to be cytokine-inducible in some cell types. However, other studies have revealed only basal activity but no cytokine inducibility with constructs containing the first 1-4.7 kb (Sakitani et al. 1998, de Vera et al. 1996, Laubach et al. 1997, Chu et al. 1998, Taylor et al. 1998). A proximal NF-κB site has been identified (Table 3) and this appears to be important in controlling inducible transcription (Kolyada and Madias 2001, Marks-Konczalik et al. 1998), though also no functionality for this site has been reported (Taylor et al. 1998). The reasons for the discrepancies between these reports are unclear, but differences between different cell types in regulating iNOS expression may exist. Several other functional NF-κB sites have been found further upstream from the promoter. The NF-κB site at -8.2 kb is essential for iNOS promoter activity, whereas the other sites enhance transcription (Taylor et al. 1998).

The binding of C/EPBβ to the A-activator-binding site is necessary but not sufficient for iNOS promoter activity (Guo et al. 2003). Krüppel-like factor 6 is a transcription factor, the expression of which is induced by tissue injury and which regulates the expression of genes mediating wound healing. The proximal region of the human iNOS promoter contains two Krüppel-like factor 6 responsive sites in close proximity. Whether Krüppel-like factor 6 regulates cytokine-induced iNOS transcription is not known, but overexpression of it can induce iNOS expression and NO production (Warke et al. 2003). Both positive and negative regulatory AP-1 sites have also been found in the human iNOS promoter (Table 3). A site at -5.3 kb has been shown shown to be essential for iNOS promoter activity (Marks-Konczalik et al. 1998). There are also data suggesting binding to this site also on an endogenous chromatin in situ (Mellott et al. 2001), which further supports its functionality. The proximal AP-1-like site regulates negatively both basal and inducible transcription. Cytokines can induce binding of nuclear proteins to this site, but these proteins have not been identified (Pance et al. 2002). Interestingly, activity of the human promoter can also be suppressed by overexpressing c-Jun and c-Fos (Kleinert et al. 1998).

IFN-γ is a cytokine essential for iNOS expression in human cells. However, the IFN-γ inducible factors and elements which regulate the activity of the human iNOS promoter are less well characterized compared with the mouse promoter. Two functional gamma-activated sites have been found (Table 3). Interestingly, the upstream site contains overlapping NF-κB and STAT1 binding sites and binding of both factors to this site is required for promoter activity (Ganster et al. 2001). STAT1 interacts with c-Fos at the -5.3 kb binding site and mutation of the distal AP-1 sites reduces the responsiveness of the promoter to IFN-γ. STAT1 could thus augment iNOS promoter activity by interacting with AP-1 (Xu et al. 2003). The role of STAT1 in human iNOS transcription seems to be cell type-dependent. In human epithelial cells overexpression of STAT1 increases iNOS promoter activity, whereas in human fibroblasts the effect of STAT1 seems to be the opposite (Ganster et al. 2001). In addition to the proximal AP-1 site only one negative regulatory element has been reported. NF-κB repressing factor is a constitutively expressed silencer which binds to a negative regulatory element in the iNOS promoter and acts as a suppressor of basal transcription (Feng et al. 2002).

The three-dimensional structure of chromatin affects the ability of transcription factors to bind DNA. Most of the data presented in Tables 2 and 3 are based on use of plasmid-based promoter constructs, which do not necessarily attain the natural chromatin structure. Data relying solely on promoter constructs and electrophoretic mobility shift assays (EMSA) may overestimate the significance of the regulatory elements studied (Mellott et al. 2001). In vivo footprinting data reflect the binding of proteins to endogenous chromatin in vivo and thus support the data obtained using promoter constructs.

The apparent differences between human and mouse iNOS promoters are thought mostly to explain the differences observed in the inducibility of iNOS expression (Chu et al. 1998). Interestingly, mouse promoter transfected to human colon epithelial cells is unresponsive to cytokines (Laubach et al. 1997) and human promoter transfected to mouse macrophages is inducible with LPS and cytokines (Kolyada et al. 1996, Zhang et al. 1996). In addition to interspecies differences, there are also marked cell type-dependent differences in the regulation of iNOS expression (Ganster et al. 2001, Guo et al. 2003, Nunokawa et al. 1996,

Mellott et al. 2001). These differences in inducibility of iNOS expression probably reflect differences between species and cell types in the range of signal transduction components and transcription factors expressed and activated. However, activation of NF-κB and STAT1 pathways seems to be important for both human and mouse iNOS transcription (Table 2 and 3). Activation of a single transcription factor is not sufficient for transcription of the iNOS gene; this requires coordinate activity of several factors.

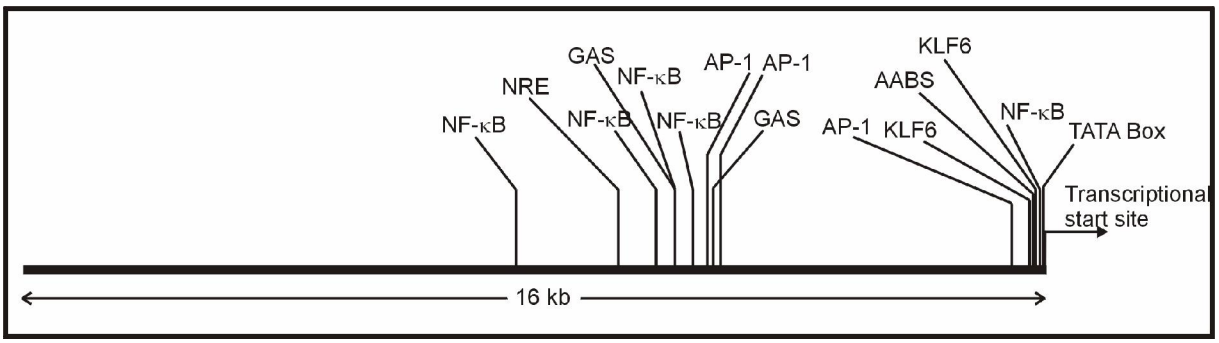


Figure 3. Schematic representation of the human iNOS promoter showing the relative positions of the regulatory elements. A-activator-binding site (AABS), Krüppel-like factor 6 (KLF-6), gamma-activated site (GAS), negative regulatory element (NRE).

element	positions	binding proteins	effect	methods	references
NF-κB	-115 to -106	p50, p65	+	site-directed mutation, EMSA	(Kolyada and Madias 2001, Sakitani et al. 1998, Marks-Konczalik et al. 1998, Taylor et al. 1998)
	-5486 to -5458				
	-5808 to -5798	p50, p65			
	-6080 to -6070				
	-8270 to -8287	p50, p65			
AABS	-192 to -184	C/EBPβ	+	site-directed mutation, EMSA	(Sakitani et al. 1998, Kolyada and Madias 2001, Guo et al. 2003)
KLF6	-168 to -164	KLF6	+	site-directed mutation, EMSA, ChIP	(Warke et al. 2003)
	-261 to -265				
AP-1	-531 to -523		-	site-directed mutation, EMSA	(Pance et al. 2002, Marks-Konczalik et al. 1998, Xu et al. 2003)
	-5113 to -5106	JunD, Fra2	+		
	-5300 to -5293	JunD, c-Fos, Fra2, c-Jun			
GAS	-5220 to -5205	STAT1	+	site-directed mutation, EMSA	(Ganster et al. 2001)
	-5808 to -5794				
NRE	-6749 to -6739	NRF	-	site-directed mutation, EMSA	(Feng et al. 2002)

Table 3. Regulatory elements in the human iNOS promoter. A-activator-binding site (AABS), Krüppel-like factor 6 (KLF-6), gamma-activated site (GAS), negative regulatory element (NRE), NF-κB repressing factor (NRF), chromatin immunoprecipitation assay (ChIP).

2.2 Post-transcriptional regulation of iNOS expression and NO production

There is evidence to support the conception that regulation of iNOS mRNA stability is an important means to regulate iNOS expression. In unstimulated cells, nuclear run-on assays show the basal rate of iNOS transcription and human iNOS promoter constructs to evince high basal activity. However, no iNOS mRNA or protein can be detected in these cells, suggesting that iNOS mRNA is highly unstable in unstimulated cells (Linn et al. 1997, de Vera et al. 1996, Laubach et al. 1997). High basal activity of the human iNOS promoter is suppressed and inducibility increased if the luciferace construct also contains the 3' untranslated region (UTR) of the human iNOS mRNA (Nunokawa et al. 1997), suggesting that both regions regulate iNOS expression. The observed reduction in basal activity of the reporter could result from reduced stability of the reporter mRNA. Both human and murine 3'-UTRs contain AU-rich elements (AREs), which are characterized by the presence of AUUUA pentamers or UUAUUUAUU nonamers. AREs have been shown to control mRNA stability and the translation of many transiently expressed cytokines and growth factors (Zhang et al. 2002).

Very little is known regarding the regulation of iNOS mRNA stability. Treatment with translation inhibitor cycloheximide stabilizes mouse iNOS mRNA, suggesting that the stability of this is regulated by factors which require *de novo* protein synthesis or that the stability is coupled to translation (Evans et al. 1994, Hattori and Gross 1995, Park and Murphy 1996). The role of human iNOS 3'-UTR in regulation of expression has been investigated in another study using the reporter gene approach (Rodriguez-Pascual et al. 2000). Based on these results, it would seem that the 3'-UTR ARE sequences of human iNOS mRNA alone are not sufficient for destabilization; additional elements in the 3'-UTR are required. Accelerated decay of mRNA mediated by 3'-UTR seems to be constitutive and treatment with cytokines does not alter the rate of mRNA degradation. However, in the study described the luciferace mRNA levels were measured at only one time point and the actual rate of mRNA degradation was not investigated. 3'-UTR of iNOS mRNA was shown to bind HuR, a protein belonging to the Hu family of RNA-binding proteins. Downregulation of HuR expression by anti-sense expression technique has resulted in reduced iNOS expression and

NO production, without reducing promoter activity, suggesting that HuR stabilizes iNOS mRNA (Rodriguez-Pascual et al. 2000). 3'-UTR of mouse iNOS mRNA has been shown to bind proteins extracted from livers of untreated mice. These proteins were characterized as heterogenous nuclear ribonucleoproteins I and L (hnRNP I and L), which have been implicated in the regulation of translation and mRNA stability. In addition, binding of these factors was reduced in protein extracts from livers of mice with septic shock (Soderberg et al. 2002). Protein kinase C-δ (PKC-δ) has also been though to be involved in the regulation of iNOS mRNA stability. In IL-1β stimulated rat insulinoma cells overexpresion of kinase-dead PKC-δ leads to reduced iNOS mRNA stability, iNOS expression and NO production (Carpenter et al. 2001). Reduced iNOS mRNA stability has also been observed after treatment with transforming growth factor-β (TGF-β) (Vodovotz et al. 1993), dexamethasone (Korhonen et al. 2002), 8-bromo-cGMP (Perez-Sala et al. 2001) and $[Ca^{2+}]_i$-elevating agents (Geng and Lotz 1995). Increased iNOS mRNA stability has been observed after treatment with the adenylate cyclase stimulator forskolin or dibutyryl cAMP (membrane permeable cAMP analogue) (Kunz et al. 1994, Oddis et al. 1995), BH_4 (Linscheid et al. 1998) and β-adrenergic agonist isoproterenol (Gustafsson and Brunton 2000).

The intracellular L-arginine concentration is an important regulator of NO synthesis. Cells which contain argininosuccinate synthetase and argininosuccinate lyase can generate L-arginine from citruline. However, in majority of cell types the most important regulators of the intracellular L-arginine concentration are a family of cationic amino acid transporters which transport L-arginine from extracellular space into the cell (Mori and Gotoh 2000). In mouse macrophages the same stimuli which induce iNOS expression also activate cationic amino acid transporter expression, resulting in increased L-arginine transport. This maintains intracellular L-arginine concentrations sufficient for NO synthesis (Caivano 1998, Shibazaki et al. 1996). Arginase is an enzyme which can convert L-arginine to ornithine. Proinflammatory stimuli can induce arginase expression or activity in some cell types, which can function as a negative regulator of NO production (Mori and Gotoh 2000). Interestingly, the intracellular L-arginine concentrations required for maximal NO production are much higher than would be expected from the K_m of L-arginine for iNOS. Recently it was shown that overexpression of arginase or depletion of extracellular arginine leads to reduced NO

production and, surprisingly, to inhibition in iNOS protein expression. L-arginine depletion was shown to reduce iNOS mRNA translation due to inactivation of eukaryotic initiation factor 2α, an important factor in the initiation of translation (Lee et al. 2003). IL-13 increases arginase activity in macrophages, which leads to L-arginine depletion and reduced iNOS mRNA translation (El-Gayar et al. 2003). The same mechanism may apply for inhibition of iNOS mRNA translation after TGF-β treatment (Vodovotz et al. 1993), as TGF-β has also been shown to enhance arginase activity (Boutard et al. 1995).

Postranslational mechanisms can affect the activity of expressed iNOS enzyme. The limited availability of cofactors such as BH_4 or haem can affect iNOS activity and NO production (Simmons et al. 1996, Park et al. 2002, Bertholet et al. 1999, Albakri and Stuehr 1996). A single article on tyrosine phosphorylation of iNOS has been published (Pan et al. 1996), but whether it has any effect on enzymatic activity is not known. Peroxynitrite mediated nitration of tyrosine residues in iNOS leads to decreased catalytic activity (Lanone et al. 2002). Recently it has been shown that iNOS protein is degraded by the proteasome pathway (Musial and Eissa 2001, Kolodziejski et al. 2002). The rate of iNOS protein degradation seems to be regulated, as it can be facilitated by TGF-β treatment (Vodovotz et al. 1993).

2.3 Signaling pathways regulating iNOS expression

The inducing stimuli determine the intracellular signaling pathways, which are involved in the regulation of iNOS expression. The most important here are the pathways regulating the activation of NF-κB and STAT1. In unstimulated cells NF-κB is bound to inhibitor κB (IκB) and is unable to translocate to the nucleus. When a cell is stimulated, the IκB kinase complex phosphorylates IκB, which leads to its ubiquination and subsequent degradation by proteasome. Released NF-κB then translocates to the nucleus and activates transcription (Li and Verma 2002). Although the events downstream of the IκB kinase complex are common for all stimuli, the signaling cascades activating IκB kinase complex are different depending on the stimulus used and are for the most part poorly defined. Phosphorylation and acetylation of the p65 subunit enhances the ability of NF-κB to activate transcription and thus provide

another means to regulate NF-κB activity (Li and Verma 2002). Binding of IFN-γ to its receptor activates the Janus-activated kinase (JAK) associated with the receptor. Activated JAK phosphorylates STAT1, which then dimerizes and translocates to the nucleus and activates transcription. The activity of STAT1 can be further enhanced by phosphorylation by other kinases (Kisseleva et al. 2002). JAKs are a family of tyrosine kinases comprising several members. Of these, JAK2 has been shown to regulate iNOS expression (Doi et al. 2002). Suppressors of cytokine signaling (SOCSs) are negative feedback regulators of the IFN-γ signaling pathway. Overexpression of SOCS-1 or SOCS-3 inhibits IL-β- or LPS+IFN-γ-induced iNOS expression (Karlsen et al. 2001, Crespo et al. 2002). In addition, pancreatic islet cells from SOCS-1 knockout mice have been observed to increase iNOS expression compared with cells from wild-type mice (Chong et al. 2002).

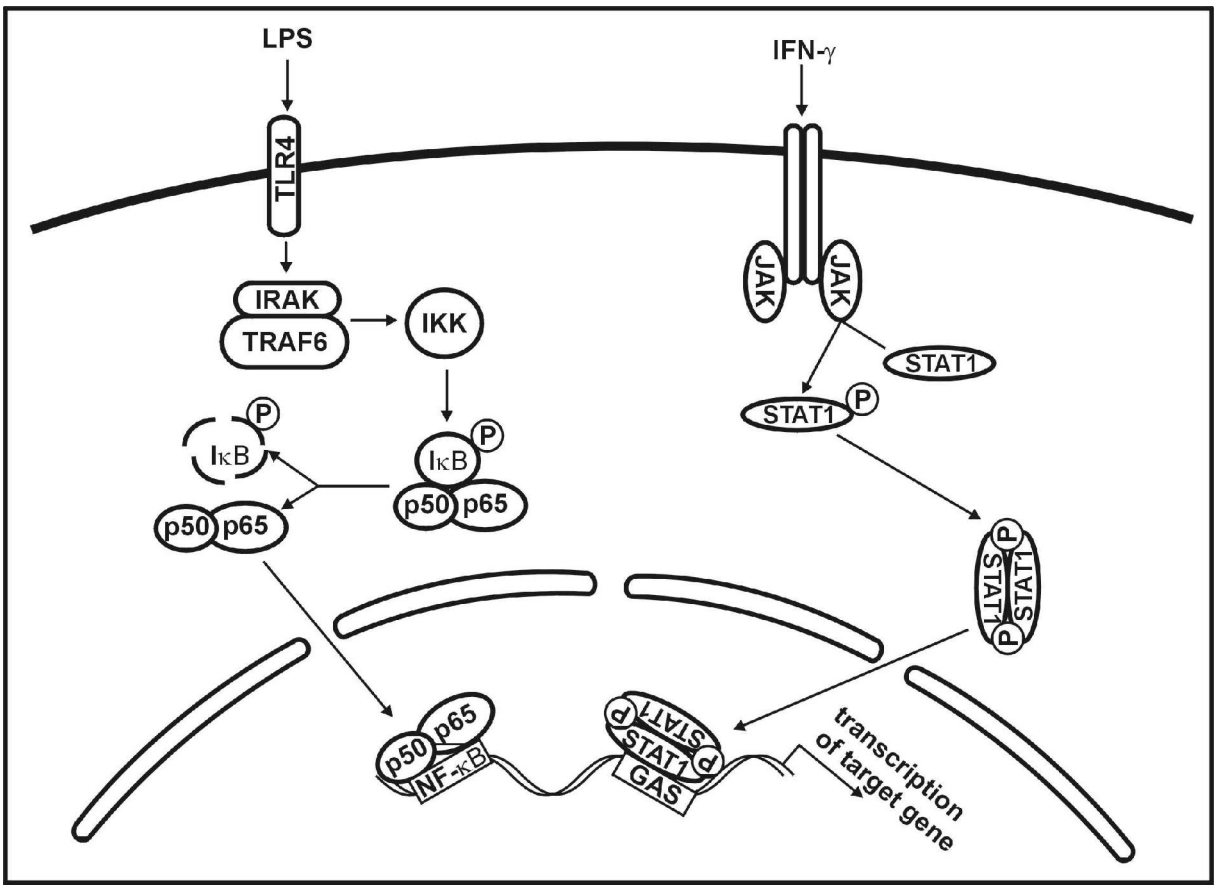


Figure 4. A simplified scheme of the NF-κB and STAT1 pathways. Toll-like receptor 4 (TLR4), interleukin-1-receptor-associated kinase (IRAK), TNF-receptor-associated factor 6 (TRAF6), IκB kinase complex (IKK).

Available reports on the role of cAMP and protein kinase A (PKA) in the regulation of iNOS expression are contradictory. Compounds which elevate the cAMP concentration can both enhance (Mullet et al. 1997, Kunz et al. 1994, Koide et al. 1993, Pahan et al. 1997) and inhibit (Mustafa and Olson 1998, Pahan et al. 1997) cytokine-induced iNOS expression. The molecular mechanisms whereby cAMP and PKA regulate iNOS transcription are unclear. PKA has been shown to phosphorylate the p65 subunit of NF-κB, which enhances its ability to activate transcription (Zhong et al. 1998), thus offering a possible mechanism for the stimulatory effect of cAMP-elevating compounds.

PKC has also been proposed to regulate iNOS expression. Use of PKC-activating phorbol esters or PKC inhibitors has mainly suggested a positive role for PKC in cytokine-induced iNOS expression (Chen et al. 1998a, Paul et al. 1997, Chen et al. 1998b), but a negative role has also been reported (Muhl and Pfeilschifter 1994, Banan et al. 2002). Different PKC isoforms seem to have different regulatory functions in iNOS expression. Activation of PKC-ζ inhibits oxidant-induced iNOS expression (Banan et al. 2002), whereas activation of PKC-δ stimulates it (Banan et al. 2003) in intestinal cells. Also there are cell type-dependent differences. In mouse macrophages down-regulation of PKC-α, -β1 and -δ, but not -η inhibits iNOS expression (Chen et al. 1998b), whereas in primary astrocytes down-regulation of PKC-η, but not - δ inhibits iNOS expression (Chen et al. 1998a). PKC-ε and -ζ have also been thought to have a regulatory role (Diaz-Guerra et al. 1996a, Miller et al. 1997).

Phospholipase C activation leads to an increase in $[Ca^{2+}]_i$. Although iNOS activity is independent of $[Ca^{2+}]_i$, changes in $[Ca^{2+}]_i$ regulate iNOS expression. Both stimulation (Park et al. 1995, Chen et al. 1998) and inhibition (Geng and Lotz 1995) (Bereta et al. 1994)of iNOS expression by $[Ca^{2+}]_i$-elevating agents have been reported. Interestingly, the effect of $[Ca^{2+}]_i$ seems to depend on the extent of the inducing stimulus. At low LPS concentrations an increase in $[Ca^{2+}]_i$ stimulates iNOS expression, whereas at high LPS concentrations it inhibits it (Korhonen et al. 2001). Furthermore, the effect of increased $[Ca^{2+}]_i$ appears to be PKC-independent (Korhonen et al. 2001, Chen et al. 1998).

3. Mitogen-activated protein kinases

3.1 Overview of MAPK signaling pathways

The MAPK signaling pathways are an important part of the signal transduction system of the cell conserved during evolution from yeast to human. The MAPKs are serine and threonine kinases, the substrates of which include many transcription factors, other protein kinases, phospholipases and cytoskeleton-associated proteins. Several MAPKs, which can be categorized into subfamilies, have been identified in mammalian cells. Better characterized subfamilies are extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) (Widmann et al. 1999, Pearson et al. 2001).

The core of the MAPK pathway is composed of three kinases which establish a sequential activation pathway. The first kinase of the three-component module is MAPK kinase kinase (MKKK), which phosphorylates and activates the next kinase module, a MAPK kinase (MKK). MKKs recognize and phosporylate a Thr-X-Tyr motif in the activation loop of MAPK. Phosphorylation of both threonine and tyrosine residues is required for MAPK to be activated.

Studies in yeast have shown that scaffolding proteins play an important part in MAPK signaling, and potential scaffolding proteins have also been discovered in mammalian cells. Scaffolding proteins help to organize MAPK cascades for efficient serial activation of the components and increase the specificity of signaling by interacting with a limited repertoire of MAPK cascade components and localizing the cascade to a selected site of action (Garrington and Johnson 1999).

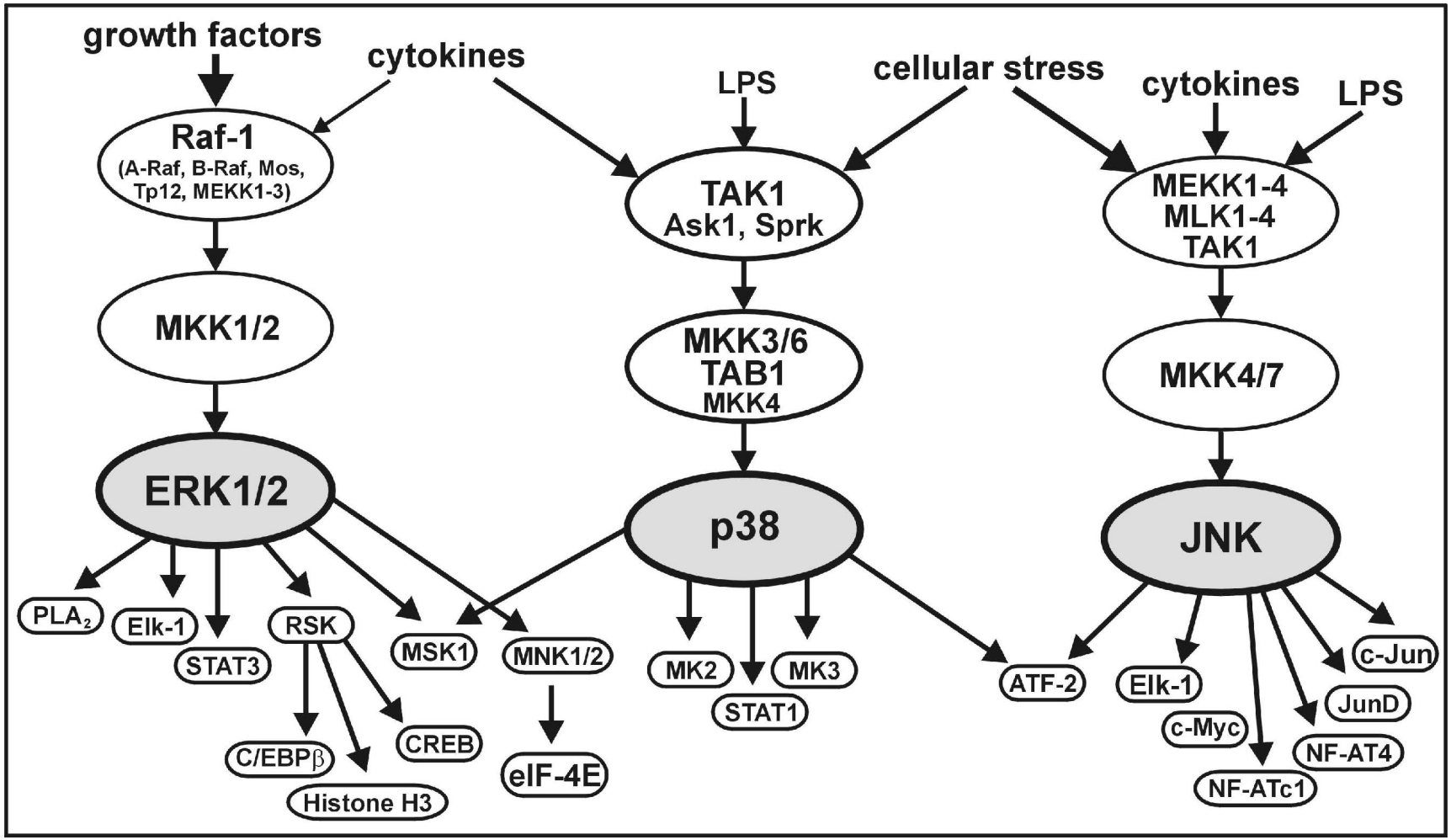


Figure 5. A simplified scheme of the MAPK pathways and their targets. Phospholipase A_2 (PLA_2), cAMP-response element binding protein (CREB), MAPK-interacting kinase 1 and 2 (MNK1/2), eukaryotic initiation factor 4E (eIF-4E), apoptosis-inducing kinase 1 (ASK1), nuclear factor of activated T-cells 4 and c1 (NF-AT4 and NF-ATc1).

3.2 ERK1/2 pathway

ERK1 and ERK2 share 85 % sequence homology. Both are ubiquitously expressed but evince some variability in their relative abundance in tissues; e.g. in many immune cells ERK2 is predominant (Pearson et al. 2001). Mitogens and growth factors are potent activators of the ERK1/2 pathway, but also other extracellular signals such as cytokines can activate it. MAPK/ERK kinase 1 and 2 (MKK1/2 or MEK1/2) are MKKs which specifically activate ERK1/2. Inactive ERK1/2 are localized in the cytoplasm. Upon phosphorylation by MKK1/2, ERK1/2 activity is increased >1000 fold and they are rapidly translocated to the nucleus (Widmann et al. 1999). Nuclear translocation is required for ERK1/2 to control gene expression and regulate the cell cycle (Pouyssegur et al. 2002).

Members of the Raf family (Raf-1, A-Raf, B-Raf) are the MKKKs of the ERK1/2 pathway, which are the most fully studied and best understood (Widmann et al. 1999). The ERK1/2 pathway can also be activated in a Raf-1-independent manner and MKKKs shown to activate MKK1/2 include Mos, Tp12, MAPK/ERK kinase kinase-1, -2 and -3 (MEKK1-3) (Widmann et al. 1999). A kinase suppressor of Ras and MKK partner 1 are thought to act as scaffolding proteins of the ERK1/2 pathway. MKK partner 1 associates with MKK1 and ERK1 and enhances phosphorylation of ERK1. In response to growth factors the kinase suppressor of Ras is translocated from the cytoplasm to the plasmamembrane, where it brings MKK and ERK into the close vicinity of the active Raf signaling complex (Pouyssegur et al. 2002)

The most prominent biological role of the ERK1/2 pathway is to regulate cell proliferation and differentiation in response to growth factors. ERK1 knockout mice are viable and fertile but have reduced thymocyte maturation and proliferation in response to activation, suggesting that ERK1 participates in the regulation of immune response but its loss can mostly be compensated by ERK2 (Pages et al. 1999). In contrast, ERK2 knockout results in embryonic lethality due to defective mesoderm differentiation and placental development (Hatano et al. 2003, Yao et al. 2003). Disruption of the other components of the ERK1/2 pathway, MKK1 and Raf-1, also results in embryonic lethality (Pearson et al. 2001).

ERK1/2 regulate cell proliferation by several mechanisms. They regulate the activity of carbomoyl phosphate synthetase, the rate-limiting enzyme in pyrimidine nucleotide synthesis (Graves et al. 2000). They activate RNA polymerase I-specific transcription initiation factor, which regulates growth factor-induced pre-rRNA synthesis (Zhao et al. 2003). In addition, ERK1/2 regulate cell cycle progression (Treinies et al. 1999). The ERK1/2 pathway is also an important signaling pathway for cell survival in cellular stresses or insults (Ballif and Blenis 2001). ERK1/2 participate in the development of long-term potentiation in neurons and are thus involved in long-term memory (Adams and Sweatt 2002).

ERK1/2 regulate gene expression by directly activating transcription factors or through activation of other regulatory protein kinases. Transcription factors directly activated by ERK1/2 include members of the ternary complex factor Elk-1, Sap-1 and Net. Ternary complex factors regulate the expression of a number of genes, e.g. c-Fos , TNF-α and junB (Shaw and Saxton 2003). ERK1/2 also phosphorylate members of the Fos family Fra-1 and -2 (Gruda et al. 1994). Downstream kinase effectors of ERK1/2 include p90 ribosomal S6 kinases (RSK) (Pearson et al. 2001) and mitogen- and stress-activated protein kinase 1 (MSK1) (Deak et al. 1998). One of the targets of RSK is C/EBPβ, a transcription factor which regulates cell proliferation and survival (Buck and Chojkier 2003). Additional targets of RSK include the transcription factors cAMP-response element binding protein (Xing et al. 1996) and c-Fos (Chen et al. 1993). Serine phosphorylation of STAT3 by ERK1/2 enhances its transcriptional activity (Decker and Kovarik 2000).

ERK1/2 can regulate transcription also through modulation of the chromatin structure. Histone H3 is phosphorylated by RSK and MSK1, which could directly influence the chromatin structure or help to recruit histone acetyltransferases (Clayton and Mahadevan 2003). ERK1/2 also target steroid receptor coactivator-1, which possesses intrinsic histone acetyltransferase activity (Pearson et al. 2001). ERK1/2 can regulate translation through phosphorylation and activation of MAPK-interacting kinase 1 and 2 (Fukunaga and Hunter 1997, Scheper et al. 2001). This activation in turn results in phosphorylation of translation initiation factor 4E (Waskiewicz et al. 1997). ERK1/2 phosphorylate and activate cytosolic phospholipase A_2, which leads to increased arachidonic acid release (Lin et al. 1993).

3.3 p38 pathway

There are four isoforms of the p38 MAPK family, p38α, p38β, p38γ and p38δ, which are all products of different genes. p38β is also expressed as a splice variant, which lacks an eight-amino acid insertion. Mxi2 is a splice variant of p38α in which 80 amino acid residues have been replaced with a 17-residue C terminus (Pearson et al. 2001). p38α , p38β, p38δ are expressed in several tissues, whereas p38γ is mainly expressed in skeletal muscle (Kumar et al. 2003). The p38 pathway is activated by cytokines (e.g. TNF-α, IL-1) and cellular stress such as hyperosmolarity.

MKKKs of the p38 pathway include TGF-β-activated protein kinase 1 (TAK1), apoptosis-inducing kinase 1 and Sprk, which phosphorylate MKK3 and MKK6 (Widmann et al. 1999). MKK3 and MKK6 specifically phosphorylate and activate p38. Also MKK4 can phosphorylate p38 in response to certain stimuli (Brancho et al. 2003). Recently, an MKK-independent activation of p38α has been described. Association of p38α with TAK1-binding protein 1 (TAB-1), leads to autophosphorylation and activation of p38α. LPS-induced p38α activation was shown to be mostly TAB-1-mediated (Ge et al. 2002). Furthermore, in mouse embryonic fibroblasts isolated from MKK3 knockout mice, p38 activation was impaired in response to TNF-α, but not IL-1 or osmotic shock. Thus, p38 activation in response to different stimuli seems to be mediated through different MKKs.

The p38 pathway has been implicated in the regulation of a variety of biological functions. Knockout of the p38α gene results in embryonic lethality due to a placental defect (Adams et al. 2000). However, embryos which survive this defect have normal morphology, but are anemic due to diminished erythropoietin expression (Tamura et al. 2000). The p38 pathway is an important regulator of the expression of inflammatory mediators and is therefore most often associated with regulation of inflammatory responses (Guo et al. 2003). Macrophages and dendritic cells from MKK3 knockout mice have a defect in IL-12 production (Lu et al. 1999) and p38α knockout mouse embryonic fibroblasts have a defect in IL-6 production (Allen et al. 2000). Inhibition of the p38 pathway results in reduced IFN-γ production by Th1

cells, which leads to impaired Th1 responses (Rincon et al. 1998). p38 activates MAPK-activated protein kinase 2 (MK2 or MAPKAPK-2). LPS-treated MK2 knockout mice have decreased levels of TNF-α protein in serum, and splenocytes isolated from the same mice show reduced TNF-α protein levels in response to LPS without reduction in mRNA levels (Kotlyarov et al. 1999). In addition to TNF-α, the p38-MK2 pathway has been shown to regulate post-transcriptionally, by regulating translation and mRNA stability, a number of other genes (e.g. cyclooxygenase-2 (COX-2) and IL-6) in an ARE-dependent manner (Clark et al. 2003a).

There is evidence of an association of p38 with the pathophysiology of cardiac hypertrophy. The p38α pathway controls the apoptosis of cardiac myocytes (Wang et al. 1998) and cardiac-specific transgenic mice expressing dominant-negative p38α showed exacerbation of stimulus-induced cardiac hypertrophy (Braz et al. 2003). p38 also regulates the cell cycle by inhibiting progression at G1/S- and G2/M-phase transition (Ambrosino and Nebreda 2001).

In addition to MK2, a number of other substrates for the p38 pathway have been discovered. Protein kinase substrates of p38 include MK 3 (Cuenda et al. 1997), MSK1 (Deak et al. 1998), MAPK-interacting kinase 1 and 2 (Fukunaga and Hunter 1997, Scheper et al. 2001). p38 also regulates the activity of several transcription factors, such as ATF-2 (Cuenda et al. 1997), Sap-1a (Janknecht and Hunter 1997) and STAT1 (Decker and Kovarik 2000).

Different p38 isoforms evince differences in selectivity of substrates. p38α activates MK2 and MK3 far more efficiently than p38γ (Cuenda et al. 1997). Mxi2 affinity for p38α substrates is diminished (Sanz et al. 2000). Furthermore, MKK3 and MKK6 selectively activate different p38 isoforms (Enslen et al. 2000). These differences suggest that p38 isoforms have different biological functions. Such a conception is further supported by findings that in cardiac myocytes, p38β enhances hyperthropic responses, whereas p38α increases apoptosis (Wang et al. 1998), and vitamin D receptor gene promoter activity is activated by p38β, but inhibited by p38γ or p38δ (Pramanik et al. 2003).

3.4 JNK pathway

There are three members in the JNK family, JNK1, JNK2 and JNK3. JNK1 and 2 are ubiquitously expressed, whereas JNK3 expression is restricted to brain and testes. Alternative splicing of the three genes results in 2 isoforms each, with and without COOH-terminal extension, yielding proteins of 46 and 55 kDa. The functional significance of these splice variants is unknown. In addition, JNK1 and JNK2 are expressed as splice variants which involve selection between two alternative exons encoding part of the kinase domain (Davis 2000). These isoforms bind transcription factors with different efficacy (Gupta et al. 1996). The JNK pathway is activated in response to cytokines and cellular stress such as heat shock, DNA damage, reactive oxygen species (H_2O_2), hyperosmolarity and ultraviolet radiation.

MKK4 and MKK7 are MKKs which specifically recognize and activate JNK. Different stimuli require different MKK to activate JNK. JNK activation in response to environmental stress (e.g. UV radiation) requires both MKK4 and MKK7, whereas MKK7 is essential for JNK activation by inflammatory cytokines (Tournier et al. 2001), this however possibly varying depending on cell type (Pearson et al. 2001). A number of MKKKs have been proposed for the JNK pathway. At least MEKK1-4, apoptosis-inducing kinase 1, TAK1, Mst, mixed lineage kinases 1-4 (MLK1-4), Sprk, Muk and Tp12 activate MKK4 or MKK7, as evidenced in transfection experiments and in vitro kinase assays (Barr and Bogoyevitch 2001, Davis 2000). However, it is unclear whether all of these are physiological regulators of the JNK pathway. Putative scaffolding proteins have also been found for the JNK pathway. MEKK1 binds both MKK4 and JNK, and may help to coordinate activation of the pathway (Garrington and Johnson 1999). The JNK interacting protein family binds JNK, MKK4, MKK7 and MLKs. Furthermore, JNK interacting proteins potentiate JNK activation (Morrison and Davis 2003). Recently it was shown that JNK interacting protein 3 associates with Toll-like receptor 4 and is involved in LPS-mediated JNK activation (Matsuguchi et al. 2003).

JNK has been associated with many biological functions, including regulation of immune responses and apoptosis. JNK1 or JNK2 knockout mice develop normally, are fertile and

appear morphologically normal but have defects in T cell function and are immunodeficient (Davis 2000). CD4+ T cells from JNK1 knockout mice hyperproliferate, exhibit decreased activation-induced apoptosis and preferentially differentiate into Th2 cells (Dong et al. 1998). In JNK2 knockout mice the differentiation of CD4+ T cell to Th1 cells is impaired due to defective IFN-γ production (Yang et al. 1998). In addition, immature CD4+ CD8+ double positive thymocytes exhibit reduced apoptosis induced by anti-CD3 antibody, suggesting that JNK contributes to negative selection of autoreactive T cells in the thymus (Sabapathy et al. 1999). In addition to IFN-γ, JNK has also been shown to regulate the expression of vascular endothelial growth factor, IL-2 and IL-3 at posttranscriptional level (Pages et al. 2000, Chen et al. 2000, Ming et al. 1998). JNK3 knockout mice were otherwise normal but show reduced seizure activity and hippocampal neuron apoptosis in response to exitotoxic glutamate-receptor agonist kainic acid (Yang et al. 1997). JNK1/JNK2 double knockouts have severe defects in the regulation of apoptosis during brain development and die during embryonal development (Pearson et al. 2001). Furthermore, knockout JNK1/JNK2 embryonic fibroblasts are protected against stress-induced apoptosis (Tournier et al. 2000). MKK4 and MKK7 knockout causes embryonic death, which is in MKK4 knockouts induced by increased apoptosis in the developing liver (Weston and Davis 2002). Both pro- and anti-apoptotic roles have been suggested for JNK and it seems that the role of JNK in apoptosis is very much cell type- and death-stimulus dependent (Lin 2003).

JNK is also thought to play a role in tumor development, a conception supported by observations that Ras-induced tumorigenecity requires c-Jun, an important target of JNK. JNK phosphorylates c-Jun on serine 63 and 73, which increases its transactivation potential (Derijard et al. 1994, Smeal et al. 1992). Mice bearing oncogenic transgenes and transgenic c-Jun in which the JNK phosphorylation sites have been mutated, develop less spontaneous tumors than mice expressing normal c-Jun (Behrens et al. 2000). In addition, JNK is constitutively activated in several tumor cell lines. The mechanism of JNK action in tumor development is however unclear (Davis 2000). Studies using knockout animals suggest that JNK is associated with the pathogenesis of non-insulin dependent diabetes mellitus. JNK activation is associated with insulin resistance and apoptosis of pancreatic β-cells (Bennett et al. 2003). JNK-regulated apoptosis is also held to be part of the pathogenetic mechanism of

neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Manning and Davis 2003).

The substrates of JNK discovered to date are mainly transcription factors. One of the most important regulatory targets of JNK pathway is the AP-1 family of transcription factors. In addition to c-Jun, also ATF-2 and JunD are phosphorylated by JNK (Gupta et al. 1995, Davis 2000). Additional substrates of JNK include Elk-1 (Zinck et al. 1995), c-Myc (Noguchi et al. 1999) and the nuclear factor of activated T cells 4 and c1 (Chow et al. 1997, Chow et al. 2000).

3.5 Other MAPK pathways

Other MAPKs have been described, but their regulation and function are poorly known. ERK5 is a protein of ~90 kDa, hence also called the big MAPK. It is ubiquitously expressed in mammalian cells and is activated by MKK5. Ras and MEKK3 are MKKKs of the ERK5 pathway in response to some stimuli. ERK5 is activated in response to some mitogens (e.g. epidermal growh factor (EGF) and phorbol ester) and cell-stressing agents (e.g. sorbitol and H_2O_2) (Pearson et al. 2001). The physiological role of the ERK5 pathway is largely unclear, but it has an important function in embryonic development. ERK5 knockout mice die during embryonic development due to defects in the development of blood vessels and heart (Regan et al. 2002, Yan et al. 2003). ERK5 is also required for the EGF-induced proliferative signal (Kato et al. 1998). Protein targets of ERK5 include the myocyte enhancer factor 2C and Sap-1a (Kato et al. 1997, Kamakura et al. 1999) and ERK5 indirectly regulates the phosphorylation and stability of c-Fos and Fra-1 (Terasawa et al. 2003).

Even less is known regarding ERK7 and the more recently discovered ERK8. ERK7 is constitutively active in serum-starved cells and no known activator of other MAPKs is able to increase ERK7 activity (Abe et al. 2001). The biological function of ERK7 is unknown, but it has been shown to regulate estrogen receptor degradation (Henrich et al. 2003). ERK8 is ubiquitously expressed and has low basal activity. Serum or constitutively active c-Src can increase ERK8 activity (Abe et al. 2002).

3.6 Regulation of MAPK activity

MAPK pathways do not function as simple on/off switch signal transducers, but the magnitude and duration of MAPK activity determines the physiological outcome of various extracellular stimuli (Hazzalin and Mahadevan 2002). Different stimuli activate different MAPK pathways, but also the temporal pattern of MAPK activation depends on the stimulus. The first evidence of the importance of the duration of MAPK activation in determining physiologic response came from studies on PC12 cells. Stimulation of PC12 cells with EGF caused only transient ERK1/2 activation and led to proliferation of cells, whereas stimulation with nerve growth factor caused sustained ERK1/2 activation and led to neurite outgrowth and cell differentiation. Data from a number of studies collectively show that sustained ERK1/2 activation is the key event in PC12 differentiation (Marshall 1995).

Similar observations have been made in different settings. In CCL39 cells thrombin-induced sustained ERK1/2 activation was followed by expression of Fra-1, JunB, c-Jun and cyclin D1 and cell cycle re-entry, whereas thrombin receptor peptides only induced transient ERK1/2 activation and expression of c-Jun (Balmanno and Cook 1999). In naive double-positive thymocytes, ligation of T cell receptor with a strong agonist leads to strong but transient ERK1/2 activation and negative selection, whereas ligation with a weak agonist leads to sustained low level ERK1/2 activation and positive selection (Mariathasan et al. 2001). Alterations in environmental conditions of erythroblasts causes either sustained low-level p38 activity, which is essential for optimal proliferation and CD36 expression or high-level activity, which leads to apoptosis (Somervaille et al. 2003). In an ovarian carcinoma cell line sensitive to cisplatin treatment with cisplatin leads to prolonged JNK and p38 activation, expression of Fas ligand and apoptosis. In contrast, in an ovarian carcinoma cell line resistant to apoptosis, treatment with cisplatin leads to transient JNK and p38 activation, but not to expression of Fas ligand or apoptosis (Mansouri et al. 2003).

In addition to activating stimuli, also inactivation by phosphatases is an important regulatory mechanism in defining the magnitude and duration of MAPK activity (Bhalla et al. 2002). Since phosphorylation of both threonine and tyrosine residues is required for MAPK

activation, dephosphorylation of either residue is sufficient for inactivation. Thus in theory inactivation of MAPKs can be achieved by tyrosine-specific, serine/threonine-specific or dual specificity phosphatases (Keyse 2000).

MAPK phosphatases (MKPs) are a family of dual-specificity phosphatases which dephosphorylate both threonine and tyrosine residues of target proteins. Ten members of the MPK family have been found and most are ubiquitously expressed, while others are expressed in only a limited subset of tissues. MKPs also differ in their subcellular localization. Some (e.g. MKP-1 and MKP-2) are inducible and are located in the nucleus and others are located predominantly in the cytosol (MKP-3, MKP-7) or in both (MKP-4, MKP-5). All MKPs contain an MAPK docking site, which specifies the MAPK which is the preferential substrate. MKP-3 and MKP-4 are more efficient in dephosphorylating ERK1/2 than JNK or p38, whereas MKP-1 and MKP-5 preferentially inactivate p38 and JNK (Theodosiou and Ashworth 2002). The regulation of MKP-1 is the most fully studied. Depending on cell type, expression of MKP-1 is induced by activation of any of the MAPKs or by only one or two of the MAPK members. Binding of MKP to MAPK causes a conformational change in MKP which leads to its catalytic activation. MKP-1 is also phosphorylated by ERK1/2, which reduces the degradation rate of the otherwise labile MKP-1 (Tamura et al. 2002).

Protein tyrosine phosphatases (PTPs) have also been implicated in the regulation of MAPK activity. These include striatal enriched phosphatase and PTP-striatal enriched phosphatase-like, which are expressed in neuronal cells and target ERK1/2 and p38 and lymphoid specific haemopoietic PTP and leukocyte PTP, which also target ERK1/2 and p38 (Tamura et al. 2002). Additional phosphatases shown to regulate MAPK activity include serine/threonine protein phosphatase 2Cα (PP2Cα), PP2Cβ, PP2A and Wip1 (Tamura et al. 2002). Although phosphatases have been shown to regulate MAPK activity in vitro, their importance in vivo and their physiological role is unclear. MKP-1 knockout mice have normal phenotype and cultured cells from these mice show no differences in MAPK activation or inactivation (Theodosiou and Ashworth 2002).

3.7 MAPK inhibitors

Considering the role of MAPK pathways in regulating cell physiology and their suggested involvement in different pathological states, e.g. inflammation and tumorigenesis, they have been seen to constitute an interesting target for drug development (English and Cobb 2002).

Screening of a group of pyridinyl imidazole compounds revealed molecules which inhibited the LPS-induced production of IL-6 and TNF-α at micromolar concentrations. The search for the target of these compounds led to identification and cloning of p38α (Lee et al. 1994). The first potent compound in this group, SB203580, inhibited LPS-induced TNF-α and IL-6 production at an IC_{50} value of ~0,2 μM, alleviated inflammation and reduced joint destruction in a murine arthritis model and reduced mortality in a mouse model of endotoxin-induced shock (Badger et al. 1996). This group of pyridinyl imidazoles inhibited activated p38 by binding to the ATP site (Young et al. 1997). p38α and p38β were sensitive to pyridinyl imidazoles, whereas p38γ and p38δ were insensitive (Kumar et al. 1997, Davies et al. 2000). An additional pyridinyl imidazole class of p38 inhibitors, e.g. SB220025 and SB239063, have since been developed. These compunds have been shown to inhibit inflammatory angiogenesis (Jackson et al. 1998), to inhibit ovalbumin-induced airway eosinophilia in mice (Underwood et al. 2000), to increase survival and reduce ventricular hypertrophy and dysfunction in spontaneously hypertensive stroke-prone rats (Behr et al. 2001) and to reduce infarct volume and neurological deficits in rat stroke (Barone et al. 2001).

Several pharmaceutical companies have p38 inhibitors under development and a few have already advanced to phase I and phase II clinical trials for treatment of rheumatoid arthritis, inflammatory bowel diseases and psoriasis (Kumar et al. 2003). Some data have been published from phase I trials. N,N'-diaryl urea-based BIRB 796 was tested in a human endotoxemia model, where healthy volunteers were given an i.v. dose of LPS. BIRB 796 given prior to LPS inhibited LPS-induced increases in TNF-α and IL-6 plasma concentrations (Branger et al. 2002). In the same study it was observed that BIRB 796 also inhibited LPS-induced coagulation, fibrinolysis and endothelial cell activation (Branger et al. 2003). The effect of pyridinyl imidazole RWJ-67657 was tested in a similar setting. RWJ-67657 dose-

dependently inhibited LPS-induced increases in TNF-α, IL-6 and IL-8 plasma concentrations (Fijen et al. 2001). VX-745 is a compound which has shown good effect in rheumatoid arthritis in a 12-week phase II trial. However, VX-745 is able to cross the blood brain barrier and has had neurological adverse effects in animals receiving it in high doses. Therfore the development of VX-745 was suspended (Kumar et al. 2003).

The first specific ERK1/2 pathway inhibitor, PD98059, was discovered by screening a compound library with a cascade assay measuring phosphorylation of myelin basic protein in the presence of MKK1 and ERK1 (Dudley et al. 1995). PD98059 has an interesting mechanism of action. It inhibits MKK1 by binding inactive MKK1 and preventing its activation by upstream kinases, but does not inhibit activated MKK1. Inhibition of MKK1 activation prevents activation of downstream kinases and PD98059 inhibits the phosphorylation of ERK2 at an IC_{50} value of 2 μM (Alessi et al. 1995). U0126 is another MKK1 inhibitor, with a mechanism of action similar to PD98059 (Favata et al. 1998, Davies et al. 2000). Both compounds have been widely used as tools to investigate the role of ERK1/2 in various physiological settings. A second generation MKK1 inhibitor PD184352, suppresses cell cycle progression and inhibits the growth of implanted colon tumors in mice (Sebolt-Leopold et al. 1999). In addition, the MKK1 inhibitor was shown to reduce the development of structural changes in an experimental osteoarthritis model in rabbits (Pelletier et al. 2003) and had an antihyperalgesic effect in an animal model of chronic pain (Ciruela et al. 2003). PD184352 is currently under clinical trials for the treatment of cancer (Allen et al. 2003). Raf inhibitors are also considered to be potential targets for drug intervention and Raf inhibitor BAY-43-9006 is likewise under clinical trials for treatment of cancer (Bollag et al. 2003).

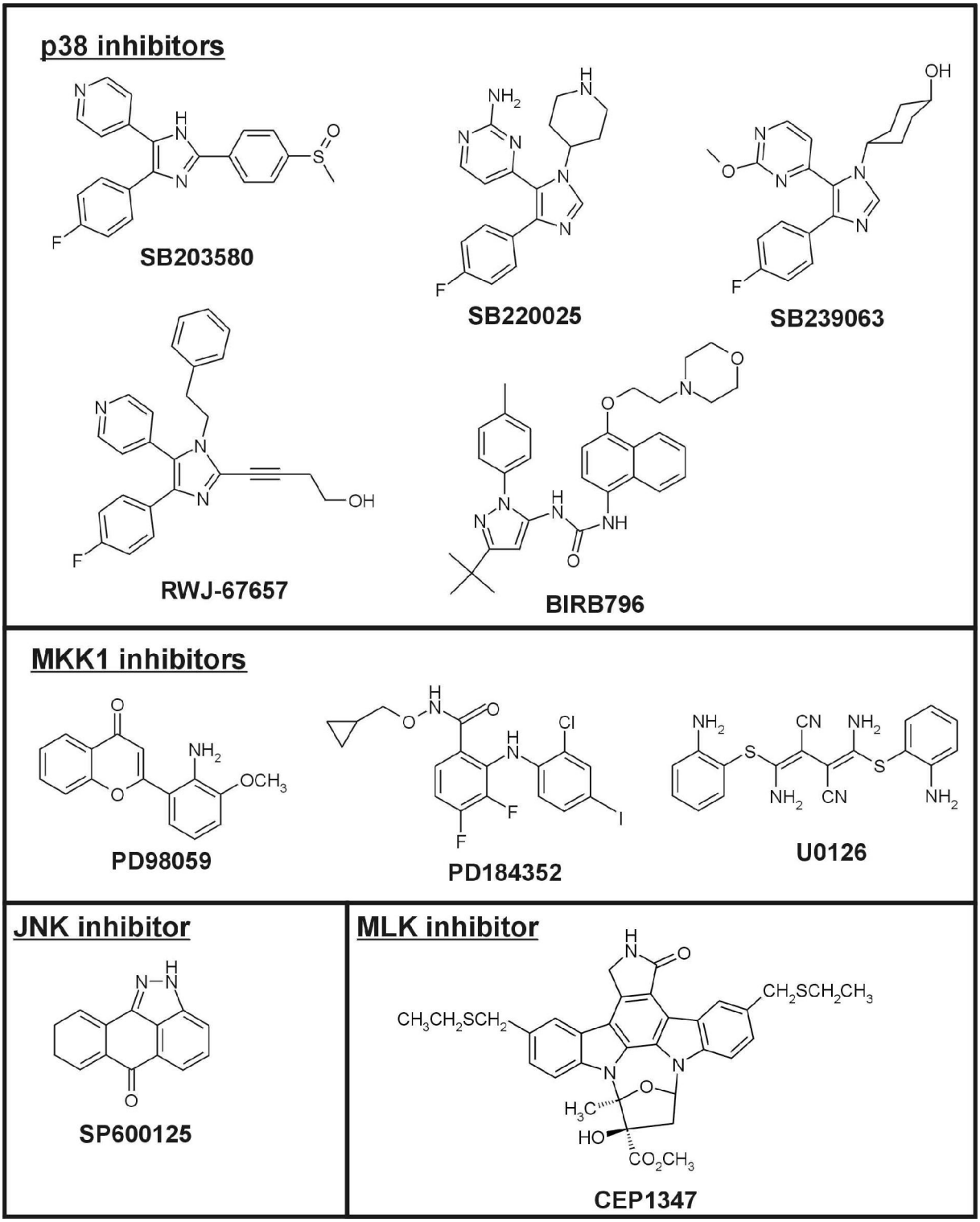


Figure 6. Inhibitors of MAPK pathways.

The first specific JNK inhibitor SP600125 was only recently described. SP600125 is a reversible ATP-competitor which inhibits c-Jun phosphorylation by JNK with an IC_{50} ~5 μM in intact cells and inhibits LPS-induced expression of COX-2, IL-2, IFN-γ and TNF-α (Bennett et al. 2001). In rat adjuvant-induced arthritis, SP600125 has inhibited inflammation only modestly but significantly inhibited the progression of joint damage in radiographic evaluation (Han et al. 2001). SP600125 also inhibited airway eosinophilia and infiltration of CD2+ positive T-cells in the bronchial submucosa in ovalbumin-sensitized rats (Eynott et al. 2003). CEP-1347 is an inhibitor of the MLK family of kinases. It has been shown to inhibit the MLK-mediated activation of JNK and protect neurons from cell death induced by UV irradiation or oxidative stress. CEP-1347 has also proved to be neuroprotective in vivo in a number of different animal models of neuronal damage (Wang et al. 2004). It is reported to be safe and well tolerated in patients with Parkinson's disease in a phase I trial (Parkinson Study Group 2004). Many pharmaceutical companies are developing inhibitors of the JNK pathway for the treatment of inflammatory conditions, cancer and neurodegenerative diseases (Manning and Davis 2003).

It has been shown that glucocorticoids inhibit the activity of MAPKs, which could be part of the anti-inflammatory mechanism of glucocorticoid action. This would seem not to be a direct effect on MAPKs; glucocorticoids induce the expression of MKP-1. This in turn leads to dephosphorylation and inactivation of MAPKs and inhibition of TNF-α and COX-2 expression (Clark and Lasa 2003b).

MAPKs regulate several factors which in turn are known to regulate iNOS expression and NO production. It is therefore likely that MAPKs form part of the signaling pathways regulating iNOS expression and NO production. The work for this thesis was started in 1997. At that time only four reports on the role of MAPKs in the regulation of iNOS expression had been published. Since then, the number of reports dealing wih this topic has rapidly increased. This literature will be reviewed thoroughly in the discussion section of this thesis.

AIM OF THE STUDY

NO is recognized as an important signaling molecule in immune responses. In inflammation, iNOS is mainly responsible for NO production by inflammatory and tissue cells. MAPK pathways are evolutionarily conserved, intracellular signal transduction systems which connect extracellular signals to intracellular regulatory proteins. MAPK pathways have been implicated in the regulation of the synthesis of various inflammatory mediators. MAPK inhibitors are under development for the treatment of inflammatory diseases. The purpose of the present study was to investigate the effects of MAPK inhibitors and the role of MAPKs in the regulation of iNOS expression and NO production. The results could provide new information on the regulation of iNOS expression, which might then reveal new targets for drug development.

The aim of the present study was to test the hypothesis that ERK1/2, p38 and JNK MAPKs regulate iNOS expression and NO production and to continue by studying the mechanisms whereby MAPKs regulate iNOS expression.

MATERIALS AND METHODS

1. Materials

Reagents were obtained as follows; rabbit polyclonal mouse and human iNOS, c-Jun, JNK1 and goat anti-rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc), PhosphoPlus® p44/42 MAP kinase (Thr202/Tyr204) antibody kit, PhosphoPlus® p38 MAP kinase (Thr180/Tyr182) antibody kit (New England BioLabs Inc), rabbit polyclonal phospho-SAPK/JNK (Thr183/Tyr185), phospho-c-Jun (Ser63) II and (Ser73) antibodies (Cell Signaling technology, Beverly, MA, USA), recombinant human TGF-β1 and anti-TGF-β1 neutralizing antibody (R&D Systems).

MAPK inhibitors and other small molecular inhibitors used in the present study are listed in Table 4 and were obtained as follows: SP600125, *N*1-methyl substituted pyrazolanthrone, PD98059, SB202474, SB220025, SC68376 (Calbiochem), sepiapterin, SB203580 and SB202190 (Alexis Corporation), PDTC (Tocris Cookson) All other reagents were from Sigma.

2. Cell culture

J774A.1 mouse macrophages (I-III) (American type culture collection), J774.2 mouse macrophages (IV) (The European Collection of Cell Cultures) and human T84 colon epithelial cells (I,III) (from Prof. Markku Mäki, University of Tampere) were cultured at 37°C, 5% CO_2 atmosphere, in Dulbecco's Modified Eagle's Medium with glutamax-I (Cambrex Bioproducts Europe) containing 5% (T84) or 10% (J774A.1 and J774.2) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (all from Invitrogen). Cells were seeded in 24 well plates for nitrite and L-arginine transport measurements and in 6 well plates for Western blot and RT-PCR, and grown for 48 h (IV) or 72 h (I-III) prior to experiments.

compound	chemical name	action
SP600125	anthra(1,9-cd)pyrazol-6(2H)-one	JNK inhibitor
*N*1-methyl substituted pyrazolanthrone	N^1-Methyl-1,9-pyrazoloanthrone	inactive control compound for JNK inhibitor
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one	MKK1 inhibitor
SB202474	4-ethyl-2-(4-methoxyphenyl)-5-(4-hydroxyphenyl)-5-(4pyridyl)-imidazole	inactive control compound for p38 inhibitors
SB220025	5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)-imidazole	p38 inhibitor
SC68376	2-methyl-4-phenyl-5-(4-pyridyl)oxazole	p38 inhibitor
sepiapterin	S(-)-2-amino-7,8-dihydro-6-(2-hydroxy-1-oxopropyl)-4(1H)-pteridinone	BH_4 precursor
SB203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4 pyridyl)-1H-imidazole	p38 inhibitor
SB202190	4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4 pyridyl)-1H-imidazole	p38 inhibitor
PDTC	ammonium pyrrolidinedithiocarbamate	NF-κB inhibitor
L-NMMA	N-monomethyl-L-arginine	NOS inhibitor
cycloheximide	3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide	translation inhibitor
actinomycin D		transcription inhibitor

Table 4. MAPK inhibitors and other small-molecular compounds used in this study.

3. Nitrite assays

At indicated time points the culture medium was collected for 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% napthalethylenediamine dihydrochloride, 1% sulphanilamine, 2.5% H_3PO_4) and the absorbance was measured at 540 nm. The concentration of nitrite was calculated using sodium nitrite as standard (Green et al. 1982).

4. Western blotting

At indicated time points cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1 % Triton-X-100, 5 mM PMSF, 2 mM sodiumorthovanadate, 10 µg/ml leupeptin, 25µg/ml aprotinin, 1.25 mM NaF, 1 mM sodiumpyrophosphate and 10 mM n-octyl-β-D-glucopyranoside. After incubation for 20 minutes on ice, lysates were centrifuged (14600 g, 15 min.) and supernatants mixed 1:4 with SDS loading buffer (62.5 mM Tris-HCl pH 6.8, 1%

glycerol, 2% SDS, 0.025% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. The protein content of the samples was measured by the Coomassie blue method (Bradford 1976). An equal amount of protein was loaded in each well of 10 % SDS-polyacrylamide electrophoresis gel and electrophoresed for 4 h at 100 V in buffer containing 95 mM Tris-HCl, 960 mM glycine and 0.5 % SDS. After electrophoresis the proteins were transferred to Hybond ECL™ nitrocellulose membrane (Amersham) with a semi-dry blotter at 2.5 mA/cm^2 for 60 min. After transfer the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1 % Tween-20) containing 5 % bovine serum albumin for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 °C overnight. Thereafter the membrane was washed 4x with TBS/T for 5 min, incubated with secondary antibody in the blocking solution for 0.5 h at room temperature and washed 4x with TBS/T for 5 min. Bound antibody was detected using SuperSignal® West Pico chemiluminescent substrate (Pierce) and the FluorChem™ 8800 imaging system (Alpha Innotech). Quantitation of the chemiluminescent signal was carried out with FluorChem™ software v. 3.1.

5. Arginine transport measurements

At indicated time points the medium was replaced with DMEM containing L-[^{14}C]arginine (0.2 nmol/ml, 296 mCi/mmol; Amersham) and incubated for 5 min after which the medium was removed and the cells rapidly washed 3 times with ice-cold PBS. The cells were solubilized (0.2 M NaOH, 0.2% SDS) and radioactivity quantified by liquid scintillation counting.

6. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were seeded on 10 cm dishes and grown for 72 h prior to experiments. Cells were incubated in the presence of the tested compounds for 30 min. Thereafter they were rapidly washed with ice cold PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1mM EGTA, 1 mM Na_2VO_4, 1mM NaF). After incubation for 10 min on

ice, the cells were vortexed for 30 s and the nuclei separated by centrifugation at 4 °C, 21000 g for 10 s. Nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1mM EGTA, 1 mM Na_3VO_4, 1mM NaF) and incubated for 20 min on ice. They were then vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4 °C, 21000 g for 2 min. The protein content of the nuclear extracts was measured by the Coomassie blue method (Bradford 1976). Transcription factor consensus oligonucleotides (5'-AGTTGAGGGGACTTTCCCAGGC-3') for NF-κB (Promega) were 5'[^{32}P]-end labelled with the DNA 5'-End Labelling Kit (Boehringer Mannheim). For binding reactions 5 µg of nuclear extract was incubated in 20 µl of a total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM dithiotreitol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl, 10 % glycerol for 20 min at room temperature. A ^{32}P-labeled oligonucleotide probe (0.2 ng) was added and the reaction mixture was incubated for 10 min. Protein/DNA complexes were separated from the DNA probe by electrophoresis on a native 4 % polyacrylamide gel. This was dried and autoradiographed using an intensifying screen at – 70 °C. The quantitation of densities of specific bands was carried out with FluorChem™ software v. 3.1.

7. RNA extraction

At indicated time points cell monolayers were rapidly washed with ice-cold PBS and cells were homogenized using QIAshredder™ (QIAGEN Inc.). RNA extraction was carried out with an RNeasy® kit for isolation of total RNA (QIAGEN Inc.).

8. RT-PCR (III)

Synthesis of cDNA and subsequent amplification of cDNA was performed with the RobusT RT-PCR kit (Finnzymes). The primers for RT-PCR are listed in Table 5. RT-PCR reaction conditions were as follows: 250 ng of total RNA in reaction mixture containing 1.5 mM Mg^{2+} was subjected to RT reaction at 48 °C for 30 min, denaturation at 94 °C for 30 s, annealing at

60 °C for 30 s, extension at 72 °C for 1 min and 22 cycles for mouse iNOS and mouse GAPDH; 1 mM Mg^{2+}, RT reaction at 45 °C for 30 min, amplification 94 °C for 35 s, 60 °C for 2 min, 72 °C for 2 min, 42 and 30 cycles for human iNOS and β-actin, respectively. Products were analysed on 1.5 % agarose gel containing ethidium bromide and visualized with UV light.

9. Real time RT-PCR (II, IV)

Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems). Reverse transcriptase (RT) reaction parameters were as follows: incubation at 25 °C for 10 min, RT at 48 °C for 30 min and RT inactivation at 95 °C for 5 min. cDNA obtained from the RT reaction (amount corresponding approximately to 1 ng of total RNA) was subjected to PCR using the TaqMan® Universal PCR Master Mix and ABI PRISM® 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimized according to manufacturer's guidelines in TaqMan® Universal PCR Master Mix Protocol Part Number 4304449 Rev. C. The primers and probes used in this study are listed in Table 5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probes were obtained from the TaqMan® Rodent GAPDH Control Reagents kit (Applied Biosystems) and were used in the following concentrations: forward and reverse GAPDH primers, both 300 nM and GAPDH probe 50 nM, containing VIC as 5'-reporter dye and TAMRA as 3'-quencher. PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min and thereafter 40 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min. Each sample was determined in duplicate.

A standard curve method was used to determine the relative mRNA levels as described in Applied Biosystems User Bulletin #2. In short, a standard curve for each gene was created using mRNA isolated from LPS-stimulated J774 macrophages. Isolated RNA was reverse-transcribed as described. Dilution series were made from the cDNA obtained ranging from 1 pg to 10 ng and were subjected to real time PCR as described. Threshold cycle values

obtained were plotted against dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated from the standard curve.

10. Statistics

Results are expressed as mean ± standard error of mean (SEM). When indicated, statistical significance was calculated by analysis of variance supported by Dunnett adjusted significance levels (I, III) or by Bonferroni multiple comparisons test (II, IV). Differences were considered significant when $P < 0.05$.

RT-PCR primers	
5'-TCACTGGGACAGCACAGAAT-3'	forward mouse iNOS primer
5'-TGTGTCTGCAGATGTGCTGA-3'	reverse mouse iNOS primer
5'-CGGAGTCAACGGATTTGGTCGTAT-3'	forward mouse GAPDH primer
5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	reverse mouse GAPDH primer
5'-CCATGGAACATCCCAAATAC-3'	forward human iNOS primer
5'-TCTGCATGTACTTCATGAAGG-3'	reverse human iNOS primer
5'-TGACTGACTACCTCATGAAGATCCTCACCG-3'	forward human β-actin primer
5'-CCACGTCACACTTCATGATGGAGTTG-3'	reverse human β-actin primer
Real time RT-PCR primers and probes	
5'-CCTGGTACGGGCATTGCT-3'	forward mouse iNOS primer
5'-GCTCATGCGGCCTCCTT-3'	reverse mouse iNOS primer
5'-CAGCAGCGGCTCCATGACTCCC-3'	mouse iNOS probe
5'-GGTTTTGGTGAGGTTGAATCCATA-3'	forward mouse AUF1 primer
5'-TCTTCACTGGCTCCTCTTCCTTAA-3'	reverse mouse AUF1 primer
5'-CAGAACCCACGCCTCTTATTGGTCTTCTTG-3'	mouse AUF1 probe
5'-TGTCCCCGGCAATGCT-3'	forward mouse HuR primer
5'-TCACGAATCACTTTCACATTGGT-3'	reverse mouse HuR primer
5'-CCTCATCGGCGTCTTGCCCAA-3'	mouse HuR probe

Table 5. Primers and probes used in RT-PCR and real time RT-PCR.

RESULTS

1. LPS activates MAPKs and induces iNOS expression and NO production in J774 macrophages

ERK1/2, p38 and JNK were inactive in resting J774 macrophages. Stimulation with LPS induced rapid activation of the three MAPKs (I-IV). ERK1/2 activation peaked at 15 min, p38 at 30 min and JNK at 30 min after LPS. The activity of all MAPKs decreased rapidly after the initial peak of high activity, but this was followed by sustained low-level activity for hours.

No iNOS mRNA or protein and no NO production was detected in unstimulated J774 macrophages (Figure 7). iNOS mRNA levels increased after 2 h of LPS stimulation and iNOS protein was detectable 4 h after LPS. As a marker of NO production, nitrite started to accumulate in the growth medium after 6 h. The peak in iNOS mRNA levels was reached between 6 and 8 h after addition of LPS and mRNA levels rapidly decreased after 8 h. iNOS protein levels reached maximum at 12 h and remained high up to 24 h. NOS inhibitor L-NMMA, NF-κB inhibitor PDTC and protein synthesis inhibitor cycloheximide completely inhibited the accumulation of nitrite in growth medium. These results confirm that nitrite was derived from NO generated by iNOS (I).

2. MKK1 inhibitor PD98059 reduces LPS-induced ERK1/2 activation and iNOS expression, NO production and L-arginine transport (I)

PD98059 is a pharmacological tool widely used to inhibit the ERK1/2 pathway because it inhibits MKK1, an upstream activator of ERK1/2. In the present study, PD98059 completely inhibited LPS-induced ERK1/2 activation at a 10 µM concentration in J774 macrophages. It also inhibited iNOS expression, which resulted in reduced NO production. The reduction was 50 % at 10 µM PD98059. PD98059 had no effect on LPS-induced NO production if added 6 h after LPS stimulation, suggesting that PD98059 has no direct effect on iNOS activity. Similarly, PD98059 inhibited LPS-induced iNOS expression and NO production by 40 % in T-84 human colon epithelial cells.

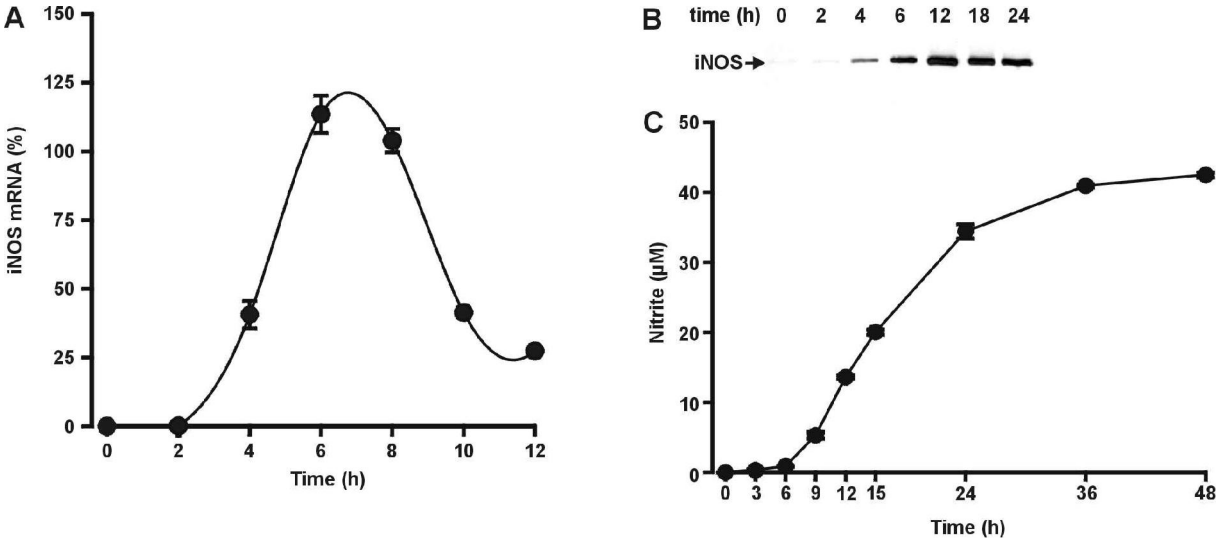


Figure 7. The effect of LPS on iNOS mRNA and protein expression and NO production in J774 macrophages. (A) Cells were stimulated with LPS (10 ng/ml). Incubations were terminated at time points indicated and extracted total RNA was subjected to real time RT-PCR. iNOS mRNA levels were normalized against GAPDH. Values are mean±SEM (n=3). (B) Cells were stimulated with LPS (10 ng/ml). Incubations were terminated at times indicated and iNOS detected by immunoblot. (C) Cells were stimulated with LPS (1 µg/ml). Growth medium was collected at time points indicated and the nitrite concentrations in growth medium were measured. Values are mean±SEM (n=6).

As PD98059 reduced iNOS expression, we investigated whether PD98059 had an effect on the activity of NF-κB, which is a transcription factor critical for iNOS. Stimulation of J774 macrophages by LPS increased the NF-κB nuclear translocation and DNA binding activity as measured by EMSA. PD98059 had no effect on LPS-induced NF-κB binding activity. To test whether the effect of PD98059 on LPS-induced NO production was due to reduced BH_4 availability, sepiapterin was added to the growth medium. Sepiapterin increases the cellular BH_4 levels by the pterin salvage pathway (Werner et al. 1989). Addition of sepiapterin to growth medium did not alter the inhibitory effect of PD98059 on iNOS expression, suggesting that PD98059 has no effect on BH_4 availability. The rate of L-arginine transport can be a rate limiting factor in NO synthesis (Bogle et al. 1992). We therefore tested the effect of PD98059 on L-arginine transport into the cells. LPS induced a 50 % increase in L-arginine transport to cells. PD98059 inhibited L-arginine transport by 18 % in LPS-treated cells.

3. JNK inhibitor SP600125 inhibits iNOS expression and NO production by destabilizing iNOS mRNA (II)

JNK phosphorylates c-Jun at residues Ser 63 and 73 (Derijard et al. 1994) and we therefore used phosphorylation of c-Jun as a measure of JNK activity. SP600125 inhibited LPS-induced c-Jun phosphorylation at an IC_{50} value of 5-10μM (Figure 8A), but had no effect on activation of p38 or ERK1/2. At a 20 μM concentration, which totally inhibited c-Jun phosphorylation, SP600125 inhibited NO production by 80 %. However, the N^1-methyl-substituted form of SP600125, which is a >100 fold less potent inhibitor of JNK than SP600125, inhibited NO production by only 17 %.

SP600125 inhibited LPS-induced NO production with comparable efficacy when added 30 min before or 1 h after LPS. SP600125 also inhibited iNOS expression at both protein and mRNA levels. Interestingly, SP600125 had no significant effect on iNOS mRNA levels when measured 4 h after LPS stimulation, but iNOS mRNA levels were dramatically reduced when measured 8 h after LPS (Figure 8C). This result suggested that JNK does not participate in the regulation of iNOS gene transcription, but may rather regulate iNOS mRNA stability. This hypothesis was confirmed in the iNOS mRNA degradation assay, in which iNOS mRNA levels decreased faster in cells treated with SP600125 than in cells treated with LPS only in experimental conditions where transcription was inhibited by actinomycin D. The half-life of iNOS mRNA was ~5 h in cells treated with LPS only, but was reduced to ~2 h in cells treated with a combination of LPS and SP600125 (Figure 8D).

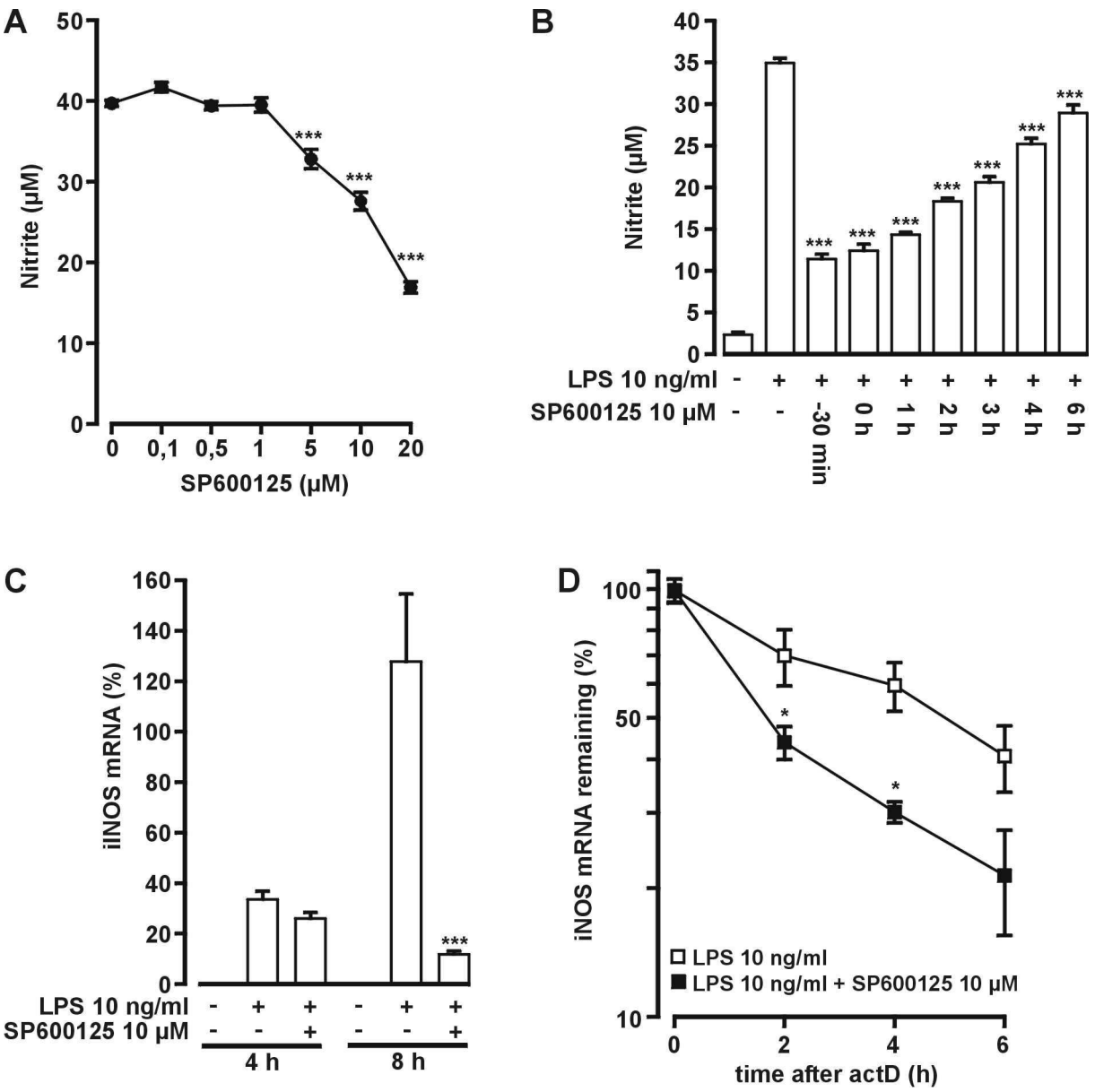
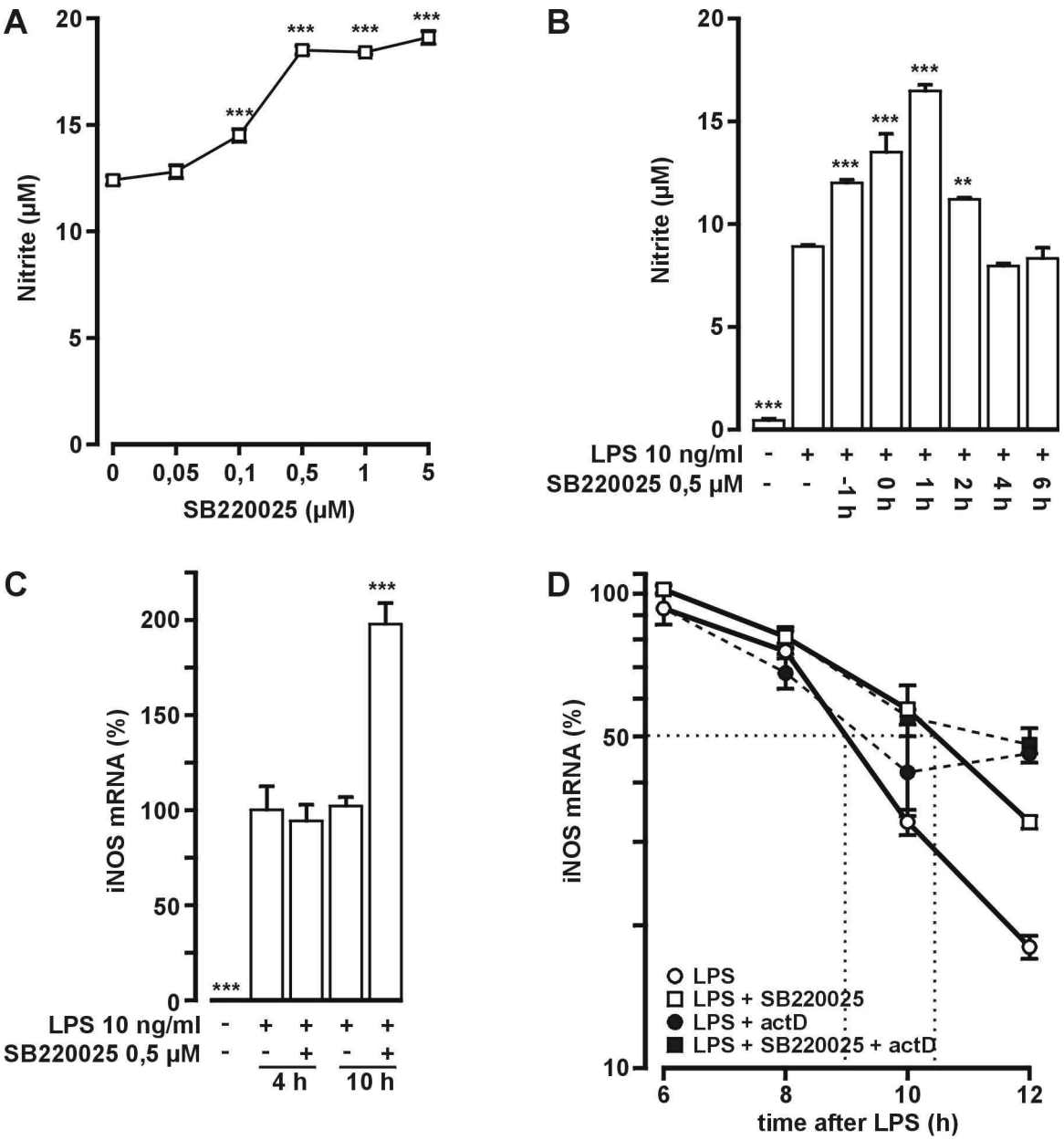


*Figure 8. The effect of JNK inhibitor SP600125 on LPS-induced NO production and iNOS mRNA expression in J774 macrophages. (A) Cells were stimulated with LPS (10 ng/ml) and treated with increasing concentrations of SP600125. (B) SP600125 was added to cells 30 min before or at the time points indicated after LPS. (A,B) 24 h after the addition of LPS, the nitrite concentrations in the culture medium were measured as a marker of NO production. (C) Cells were incubated with SP600125 30 min before stimulation with LPS. (D) Cells were incubated with SP600125 30 min before stimulation with LPS. Actinomycin D (actD) (0,5 µg/ml) was added to cells 6 h after LPS. (C,D) Incubations were terminated and total RNA isolated at time points indicated. iNOS and GAPDH mRNA was measured by real time RT-PCR. iNOS mRNA levels were normalized against GAPDH. Values are mean±SEM (A,B,D n=6)(C n=3).*** ,P<0.001 and * ,P<0.05 compared with the respective control treated with LPS only. (Reprinted with permission from: Lahti et al. 2003, Mol Pharmacol 64: 308-315. © American Society for Pharmacology and Experimental Therapeutics)*

4. p38 inhibition increases LPS-induced iNOS expression and NO production by stabilizing iNOS mRNA (III, IV)

The role of the p38 pathway in the regulation of iNOS expression and NO production was investigated using SB203580, SB202190, SB220025 and SC68376 as pharmacological tools to inhibit p38. SB203580 and SB202190 stimulated LPS-induced NO production at an EC_{50} value of ~0,5 µM. Similarly, SB220025 stimulated NO production at an EC_{50} value of ~0,1 µM (Figre 9A). SC68376 is a structurally different and less potent p38 inhibitor than the three SB-compounds. SC68376 was also found to stimulate NO production at a 30 µM concentration. SB202474 is a compound structurally related to SB203580, but does not inhibit p38 (Lee et al. 1994). SB202474 did not stimulate NO production. In addition, both SB203580 and SB220025 stimulated LPS-induced iNOS protein and mRNA expression, whereas SB202474 did not.

The effects of SB203580 and SB220025 on iNOS expression and NO production were strongest when the compounds were added to cells 1 h after LPS stimulation, and both compounds were effective even when added up to 2 h after LPS (Figure 9B). Interestingly, SB220025 had no significant effect on LPS-induced iNOS mRNA levels when measured 4 h after LPS stimulation, but had a marked stimulatory effect when measured 10 h after LPS (Figure 9C). This result suggested that p38 does not regulate iNOS transcription, but may rather regulate iNOS mRNA stability. This hypothesis was supported by the finding that iNOS mRNA levels were reducing more slowly in cells treated with a combination of LPS and SB220025 compared with cells treated with LPS only. However, there was no significant difference in the degradation rate of iNOS mRNA between cells treated with LPS+SB220025 and LPS only in the presence of actinomycin D, an inhibitor of transcription. Interestingly, the level of mRNA was falling at a slower rate in cells exposed to actinomycin D 6 h after LPS compared with cells treated with LPS only, suggesting that no significant transcription of iNOS gene occurs in cells 6 h after LPS stimulation and that actinomycin D itself inhibits the degradation of iNOS mRNA (Figure 9D). Thus, the slowed disappearance of iNOS mRNA in cells treated with SB220025 was most likely due to reduced degradation of mRNA.



<u>Figure 9. The effect of p38 inhibitor SB220025 on LPS-induced NO production and iNOS mRNA expression in J774 macrophages.</u> (A) Cells were incubated for 1 h with LPS (10 ng/ml) before addition of increasing concentrations of SB220025. (B) SB220025 was added to cells 1 h before or at the time points indicated after LPS. (A,B) After 24 h incubation, the nitrite concentrations in the culture medium were measured as a marker of NO production. (C) Cells were incubated with LPS for 1 h before addition of SB220025. (D) Cells were incubated with LPS for 1 h before addition of SB220025. Actinomycin D (actD) (0,5 µg/ml) was added to cells 6 h after LPS. (C,D) Incubations were terminated and total RNA isolated at indicated time points. iNOS and GAPDH mRNA were measured by real time RT-PCR. iNOS mRNA levels were normalized against GAPDH. Values are mean±SEM (A,B, n=6, C,D, n=3). ****,P<0.001 and* ***,P<0.01 compared with respective control treated with LPS only.*

At concentrations higher than 5 µM, SB202190 and SB203580 inhibited LPS-induced NO production, the inhibition being >90 % at the highest concentration used (30 µM). Similar inhibition was observed in iNOS protein and mRNA expression after treatment with 30 µM SB203580. In addition, SB203580 had a bi-directional effect on LPS-induced iNOS expression and NO production also in a T-84 human colon epithelial cell line. However, at the same concentration the negative control compound SB202474 also inhibited NO production to the same extent. This would indicate that the inhibitory effect of SB203580 on iNOS expression is not related to inhibition of p38.

5. p38 inhibition increases JNK activity: Mechanism of increased iNOS expression (IV)

The JNK and p38 inhibitors had opposite effects on LPS-induced iNOS expression and both regulated mRNA stability and were active when added 1-4 h after LPS. On the basis of these results we hypothetized that the effects of p38 and JNK inhibitors may be somehow coupled. SB220025 markedly increased the levels of phosphorylated JNK in LPS-treated cells, while SP600125 had no effect on p38 activation. The JNK was maximally activated within 30 min after the addition of LPS and the activity was thereafter reduced. SB220025 added to cells 1 h after LPS caused a prolonged increase in the levels of phosphorylated JNK (Figure 10A). The increase in JNK phosphorylation resulted in increased JNK activity, as SB220025 induced a similar increase in c-Jun phosphorylation, which could be inhibited by SP600125.

Because inhibition of p38 clearly increased JNK activity, we hypothetized that stimulation of LPS-induced NO production by p38 inhibitors was due to increased JNK activity. To test this hypothesis, we investigated the effect of SP600125 on SB220025-augmented NO production and iNOS mRNA expression. SP600125 clearly reversed the SB220025-induced increase in iNOS mRNA expression and NO production (Figure 10B).

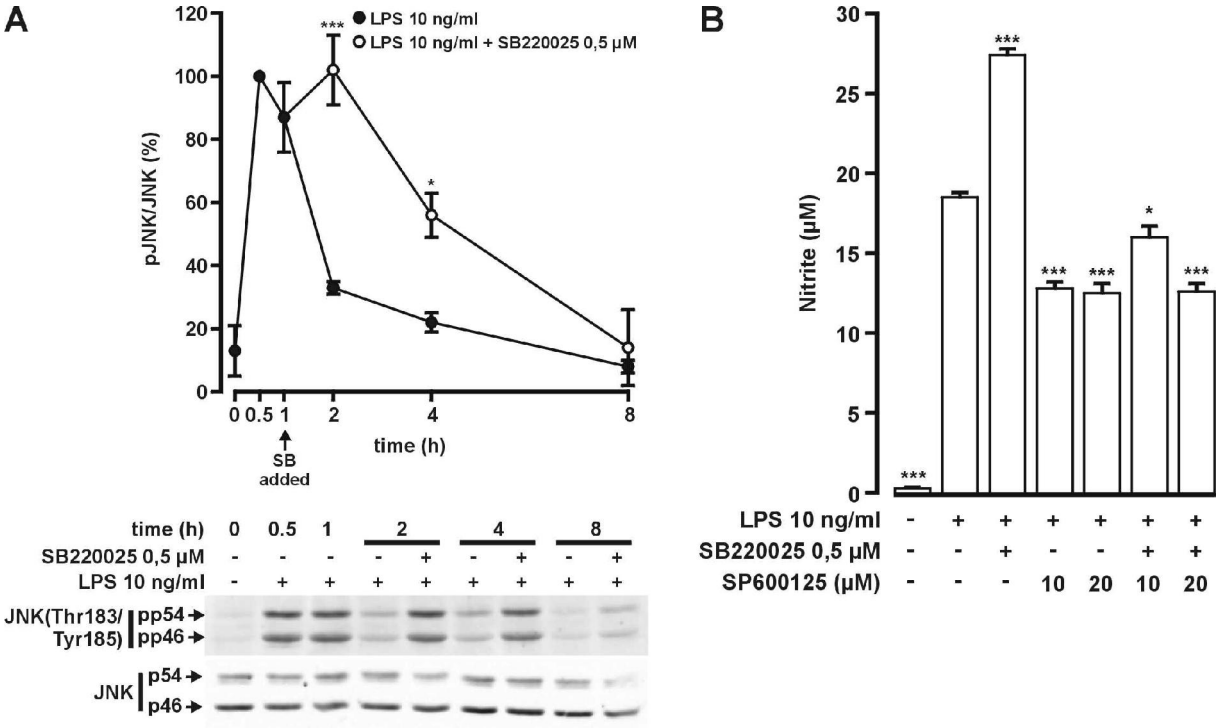


Figure 10. Effect of SB220025 on JNK activation and effect of SP600125 on LPS- and SB220025-stimulated NO production. (A) J774.2 macrophages were stimulated with LPS. SB220025 was added to cells 1 h after LPS stimulation. Incubations were terminated at the time points indicated and parallel immunoblots were run from the same cell lysates using antibodies against Thr183/Tyr185 phosphorylated JNK (pp54 and pp46) and total JNK (p54 and p46). The chemiluminescent signal was quantitated as described in materials and methods. Phosphorylated JNK and c-Jun values were normalized to total JNK and c-Jun values, respectively. (B) Cells were stimulated with LPS 1 h before addition of tested compounds. 24 h after addition of LPS the nitrite concentrations in the culture medium were measured as a marker of NO production. Results are expressed as mean±SEM (A n=3, B n=6). ***,P<0.001 and *,P<0.05 compared with respective control treated with LPS only.

DISCUSSION

1. Methodology

As nitrite is a stable metabolite of NO in cell culture conditions, nitrite measurements can be used to determine NO production by cells (Laurent et al. 1996). LPS-induced nitrite accumulation could be inhibited by L-NMMA, which showed that the increased nitrite concentrations observed were due to NO produced by NOS. Furthermore, this nitrite accumulation required de novo protein synthesis and activation of NF-κB, as it could be inhibited by cycloheximide and PDTC. These are characteristics of NO production by iNOS. The observed effects on NO production were confirmed to occur at the level of iNOS protein expression by Western blot and/or iNOS mRNA expression with conventional RT-PCR or real time RT-PCR. These are standard methods in molecular and cellular biology.

We measured NF-κB activity in nuclear protein extracts by EMSA, which gives information regading the nuclear translocation and DNA binding activity of NF-κB. However, the ability of NF-κB to activate transcription can also be modulated by phosphorylating the p65 subunit (Li and Verma 2002). This modification does not affect the DNA binding ability of NF-κB and thus cannot be measured by EMSA.

The “actinomycin D assay” has been widely used to investigate the half-life of different mRNAs. By measuring the levels of a given mRNA at different intervals after inhibition of transcription by actinomycin D, one can estimate the half-life of RNA. This approach was used in the present study, but it has its pitfalls. Actinomycin D has been shown to inhibit degradation of some mRNAs, e.g. c-Fos, transferrin receptor and neurofilament (Shyu et al. 1989, Seiser et al. 1995, Schwartz et al. 1992). We also observed that iNOS mRNA levels were decreasing faster in cells treated with LPS only compared with cells treated with actinomycin D + LPS. This would suggest that no significant transcription occurs in cells 8 h

after LPS stimulation and that actinomycin D has a stabilizing effect on iNOS mRNA. Stabilization of iNOS mRNA by actinomycin D might result from inhibition of transcription of putative mRNA stability-regulating genes or be due to some mechanism unrelated to inhibition of transcription. The latter conception is supported by findings indicating that actinomycin D can inhibit ARE-dependent degradation of mRNAs already 10 min after treatment (Chen et al. 1995). Thus, the ability of actinomycin D to stabilize mRNAs complicates interpretation of the results of mRNA degradation assays.

Macrophages activated by LPS produce cytokines which then have an autocrine and/or paracrine effect on the macrophage. At least IFN-β expression can be induced by LPS in macrophages and this endogenous IFN-β significantly increases NO production (Fujihara et al. 1994, Jacobs and Ignarro 2001). Thus, the observed effects of MAPK inhibitors in this study might result from modulation of responses activated directly by LPS or through modulation of responses induced by cytokines expressed in response to LPS.

In this study, specific inhibitors of MAPKs were used to assess the role of MAPK pathways in regulation of iNOS expression and NO production. The conclusions are based on the assumption that the observed effects are a result of specific action of these inhibitors. These issues are discussed separately for each MAPK in the following text.

2. The role of ERK1/2 in the regulation of iNOS expression and NO production

In the present study, the MKK1 inhibitor PD98059 was used as a pharmacological tool to study the role of the ERK1/2 pathway in iNOS expression and NO production. PD98059 inhibited LPS-induced ERK1/2 activation completely at a 10 μM concentration. PD98059 has been reported to inhibit EGF-induced MKK1 activation with an IC_{50} value of 2-7 μM. The inhibitory effect of PD98059 is dependent on the potency of the activating stimuli and if the concentration of EGF is increased the IC_{50} for MKK1 inhibition is also increased (Alessi et al.

1995) . Our results also suggest that MKK2 is not an important mediator of LPS-induced ERK1/2 activation, since considerably higher concentrations of PD98059 (>50 μM) are required to inhibit MKK2 activation (Alessi et al. 1995).

PD98059 inhibited LPS-induced iNOS expression by 30 % and NO production by 50 %, suggesting that the ERK1/2 pathway regulates iNOS expression. Similar results have been reported by others. In J774 macrophages treated with IFN-γ alone, 10 μM PD98059 inhibited IFN-γ-induced iNOS mRNA and protein expression and NO production by ~90 % (Blanchette et al. 2003). In other mouse macrophage cell lines, PD98059 has been reported to inhibit iNOS expression and NO production induced by LPS (Jeon et al. 2000), LPS+IFN-γ (Ajizian et al. 1999, Chan and Riches 2001b), lipoarabinomannan+IFNγ (Chan et al. 2001a) and hemozoin+IFN-γ (Jaramillo et al. 2003). However, PD98059 has also been reported to have no effect on LPS-, LPS+IFN-γ- or thrombin-induced iNOS expression and NO production in mouse macrophages (Chen and Wang 1999, Caivano 1998, Kang et al. 2003). In addition, no role for the ERK1/2 pathway in the regulation of iNOS expression has been reported in some other cell types activated by different stimuli (Table 6). The reasons for these discrepancies are unclear. It is evident, however, that the role of this pathway in iNOS expression is dependent on the stimulus (Kan et al. 1999) and the cell type (Bhat et al. 1998). Further evidence for a role of the ERK1/2 pathway in the regulation of iNOS expression is provided by blocking ERK1/2 activation by overexpressing dominant negative MKK1. this overexpression inhibited IL-1β and TNF-α induced iNOS expression in rat vascular smooth muscle cells (Doi et al. 2000, Jiang et al. 2004) and LPS+IFN-γ-induced human iNOS promoter activity in human alveolar epithelial cells (Kristof et al. 2001).

PD98059 has been tested against a number of different kinases without effect (Alessi et al. 1995, Davies et al. 2000). PD98059 has been reported to inhibit COX-1 and -2 and aryl hydrocarbon receptor at concentrations similar to those at which it inhibits MKK1 (Börsch-Haubold et al. 1998, Reiners et al. 1998). Aryl hydrocarbon receptor is a transcription factor which regulates the expression of number of genes (Reiners et al. 1998). However, it is

unlikely that the effect of PD98059 on iNOS expression seen in the present study is mediated through the aryl hydrocarbon receptor, since 2,3,7,8-tetrachlorodibenzo-p-dioxin, an activator of this transcription factor, had no effect on iNOS expression (Lahti et al., unpublished data). PD98059 has also been shown to inhibit MKK5, an activator of ERK5, at more or less the same potency which it inhibits MKK1 (Kamakura et al. 1999, Mody et al. 2001). ERK5 is activated in mouse macrophages in response to LPS and participates in the regulation of TNF-α expression (Zhu et al. 2000), whereas the role of the MKK5-ERK5 pathway in the regulation of iNOS expression is not known.

Whether ERK1/2 regulates iNOS expression at the transcriptional or posttranscriptional level was not directly investigated in this study. Results by others suggest, however that ERK1/2 regulates iNOS gene transcription, as PD98059 and overexpression of dominant negative MKK1 inhibits LPS- and/or cytokine-induced activity of iNOS promoter constructs (Table 6). ERK1/2 can regulate iNOS gene transcription through several different mechanisms. They have been reported to regulate many of the transcription factors involved in the regulation of iNOS transcription. We and others have observed no change in LPS-induced NF-κB activity by PD98059 treatment as measured by EMSA (Chen and Wang 1999, Jeon et al. 2000). In contrast, PD98059 suppresses hemozoin+IFN-γ-induced NF-κB activity (Jaramillo et al. 2003). The effect of PD98059 on NF-κB activity seems to be partly dependent on the activating stimuli. In rat ventricular myocytes PD98059 has had no effect on IL-1β -induced NF-κB activity, but reduces TNF-α+IL-1β-induced activity (Kan et al. 1999). Interestingly, it was recently reported that ERK1/2 is not involved in the early activation of NF-κB but is required for persistent activation of NF-κB. PD98059 had no effect on NF-κB activity when measured 30 min after IL-1β stimulation but reduced NF-κB activity when measured 6 h after stimulation. This was shown to be due to inhibition of IκBβ degradation by PD98059. Thus, ERK1/2 is required for degradation of IκBβ, an important factor in the regulation of persistent NF-κB activity (Jiang et al. 2004). In the present study, the effect of PD98059 on NF-κB was measured 30 min after stimulation with LPS but not hours after stimulation. Therefore the possible effect of PD98059 on later phases of NF-κB activity was not detected.

Table 6. Summary of studies of the role of the ERK1/2 pathway in the regulation of iNOS expression and NO production. dn=dominant negative, hiNOS=human iNOS, miNOS=mouse iNOS.

Cells	Inhibitor	Stimulus	Response	Result	Reference
rat cardiac microvascular endothelial cells	PD98059 (100 µM)	IL-1β	iNOS mRNA	⇓	(Singh et al. 1996)
primary mouse astrocytes	PD98059 (20 µM)	TNF-α+IL-1α	nitrite/iNOS protein	⇓ ~15 %	(Da Silva et al. 1997)
C6 rat glioma cells	PD98059	LPS+IFN-γ	nitrite	-	(Nishiya et al. 1997)
rat primary microglia	PD98059 (25 µM)	LPS	nitrite	⇓ ~50 %	(Bhat et al. 1998)
			iNOS protein/mRNA	⇓	
rat primary astroglia	PD98059 (25 µM)	IFN-γ+LPS	nitrite/iNOS mRNA	-	
RAW 264.7 mouse macrophages	PD98059 (50 µM)	LPS+IFN-γ	nitrite/iNOS protein	-	(Caivano 1998)
DLD-1 human colon epithelial-like cells	PD98059 (50 µM)	IFN-γ+IL-1β+TNF-α	iNOS mRNA	-	(Kleinert et al. 1998)
rat pancreatic β-cells	PD98059 (100 µM)	IL-1β	nitrite/iNOS mRNA	⇓ ~70 %	(Larsen et al. 1998)
RAW 264.7 mouse macrophages	PD98059 (50 µM)	LPS+IFN-γ	iNOS protein	⇓ ~90 %	(Ajizian et al. 1999)
rat aortic smooth muscle cells	PD98059 (100 µM)	LPS+IFN-γ	nitrite	-	(Baydoun et al. 1999)
mouse bone marrow-derived macrophages	PD98059 (30 µM)	TNF-α+IFN-γ	nitrite	-	(Chan et al. 1999)
NIH3T3 mouse fibroblasts				-	
RAW 264.7 mouse macrophages	PD98059 (50 µM)	LPS	nitrite/iNOS protein	-	(Chen and Wang 1999)
fetal mouse skin dendritic cells	PD98059 (30 µM)	LPS	nitrite	-	(Cruz et al. 1999)
bovine retinal pigmented epithelial cells	PD98059 (10 µM)	LPS+IFN-γ	nitrite/ miNOS promoter	⇓ ~60 %	(Faure et al. 1999)
rat ventricular myocytes	PD98059 (20 µM)	IL-1β	nitrite/iNOS mRNA	-	(Kan et al. 1999)
		IL-1β+TNF-α	nitrite	⇓ ~50 %	
			iNOS protein/mRNA	⇓	
bovine cartilage explants	PD98059 (100 µM)	IL-1α	nitrite	-	(Badger et al. 2000)
rat vascular smooth muscle cells	PD98059 (100 µM)	IL-1β	nitrite	⇓ ~50 %	(Doi et al. 2000)
			iNOS protein/mRNA	⇓	
			miNOS promoter	⇓ ~100 %	
	dnMKK1	IL-1β	iNOS protein	⇓	
			nitrite	⇓ ~100 %	
			miNOS promoter	⇓ ~90 %	
		TNF-α	nitrite	⇓ ~100 %	
RAW 264.7 mouse macrophages	PD98059 (50 µM)	LPS	iNOS mRNA	⇓ ~90 %	(Jeon et al. 2000)
J774 mouse macrophages	PD98059 (10 µM)	LPS	nitrite/iNOS protein	⇓ 30-50 %	(Lahti et al. 2000)

RAW 264.7 mouse macrophages	PD98059 (30 µM)	LPS+IFN-γ	nitrite	⇓ ~30 %	(Chan and Riches 2001b)
			iNOS protein	⇓	
RAW 264.7 mouse macrophages	PD98059 (30 µM)	lipoarabinomannan +IFN-γ	nitrite	⇓ ~100 %	(Chan et al. 2001a)
			iNOS protein	⇓	
A549 human alveolar type II epithelial cells	PD98059 (10 µM)	IFN-γ+LPS	hiNOS promoter	⇓ ~40 %	(Kristof et al. 2001)
		IFN-γ+IL-1β+TNF-α		⇓ ~10 %	
	dnMKK1	IFN-γ+IL-1β+TNF/ IFN+LPS		⇓ 40-60 %	
RAW 264.7 mouse macrophages	PD98059 (50 µM)	bovine type I collagen	nitrite	⇓ ~40 %	(Cho et al. 2002)
			iNOS protein	⇓	
	dnMKK1		nitrite	⇓ ~30 %	
			iNOS protein	⇓	
human fetal astrocytes	PD98059 (10 µM)	IL-1/ IL-1+IFN-γ	nitrite	-	(Hua et al. 2002)
		IL-1+TNF-α		-	
J774 mouse macrophages	PD98059 (10 µM)	IFN-γ	nitrite	⇓ ~90 %	(Blanchette et al. 2003)
			iNOS protein/mRNA	⇓	
	Apigenin (50 µM)		nitrite	⇓ ~100 %	
			iNOS protein/mRNA	⇓	
BV2 mouse microglia	U0126	IFN-γ+PMA	nitrite	⇓ ~100 %	(Han et al. 2003)
B10R mouse macrophages	PD98059 (40 µM)	hemozoin+IFN-γ	nitrite	⇓ ~60 %	(Jaramillo et al. 2003)
			iNOS protein/mRNA	⇓	
	Apigenin (40 µM)		nitrite	⇓ ~80 %	
			iNOS protein/mRNA	⇓	
RAW 264.7 mouse macrophages	PD98059 (50 µM)	thrombin	iNOS protein	-	(Kang et al. 2003)
C6 rat glioma cells	PD98059 (10 µM)	LPS+ TNF-α	iNOS mRNA	⇓ ~70 %	(Lee et al. 2003)
rat primary astroglia	PD98059 (100 µM)	IL-1β+TNF-α	nitrite	⇓ ~40 %	(Marcus et al. 2003)
			protein	⇓	
	U0125 (10 µM)		nitrite	⇓ ~50 %	
A498 human kidney epithelial cells	U0125 (20 µM)	IFN-γ+IL-1β+TNF-α	nitrite	-	(Poljakovic et al. 2003)
rat vascular smooth muscle cells	PD98059 (20 µM)	IL-1β	iNOS protein/mRNA	⇓	(Jiang et al. 2004)
	U0125 (20 µM)		iNOS protein/mRNA	⇓	
	dnMKK1		iNOS protein	⇓	
BV2 mouse microglia	PD98059 (50 µM)	hyaluronan fragments	nitrite	-	(Wang et al. 2004)

ERK1/2 also regulates the transactivation potential of NF-κB (Vanden Berghe et al. 1998). The mechanism of this regulatory effect was recently reported. MSK1, a target of ERK1/2, phosphorylates the p65 subunit of NF-κB, which leads to an increase in its transactivation potential (Vermeulen et al. 2003). The ERK1/2 pathway is also involved in the regulation of AP-1 activity, which is an additional mechanism whereby ERK1/2 might regulate iNOS expression (Cho et al. 2002, Kristof et al. 2001). ERK1/2 also regulates IFN-γ-activated transcription. PD98059 and U0126 have been shown to inhibit IFN-γ-stimulated IRF-1 expression (Faure et al. 1999, Han et al. 2003), STAT1α phosphorylation (Blanchette et al. 2003) and C/EBPβ-dependent transcriptional activity (Hu et al. 2001). All these transcription factors have been shown to regulate iNOS expression.

Our results on the effect of PD98059 on LPS-induced L-arginine transport are in line with those reported by Caivano (1998), who also observed a similar small reduction in LPS+IFN-γ-induced L-arginine uptake by PD98059 in mouse macrophages. In contrast, PD98059 had no effect on LPS+IFN-γ-induced L-arginine transport in rat vascular smooth muscle cells (Baydoun et al. 1999). How much the reduction in the rate of L-arginine transport influences the level of intracellular L-arginine concentration was not studied. However, the reduction in L-arginine transport by PD98059 may have been a factor contributing to the inhibition of NO production.

PD98059 inhibited LPS-induced iNOS expression and NO production only partially at concentrations which completely inhibited ERK1/2 activation. This suggests that the ERK1/2 pathway augments but is not essential for iNOS expression.

3. The role of JNK in iNOS expression and NO production

In the present study, the JNK inhibitor SP600125 was used to evaluate the role of the JNK pathway in the regulation of iNOS expression and NO production. In kinase assays, at 0,1 mM ATP, SP600125 inhibits JNK1 and JNK2 with IC_{50} values of 5,8 µM and 6,1 µM, respectively (Bain et al. 2003). In the present study SP600125 inhibited the LPS-induced Ser63 phosphorylation of c-Jun in J774 macrophges at an IC_{50} value of 5-10 µM, which is similar to the IC_{50} value previously reported in Jurkat T cells (Bennett et al. 2001). Total inhibition of c-Jun phosphorylation in J774 cells was achieved at a 20 µM concentration. In the concentration range of 5-20 µM SP600125 inhibited iNOS expression and NO production. N^1-methyl-1,9-pyrazoloanthrone, a N^1-methylated form of SP600125 and >100-fold less potent inhibitor of JNK, had only a minimal inhibitory effect on NO production, which further supports the conclusion that the effect of SP600125 on LPS-induced iNOS expression is related to JNK inhibition.

SP600125 has been tested against a number of other kinases such as other MAPKs, without effect (Bennett et al. 2001, Bain et al. 2003). Similarly, we observed no effect of SP600125 on LPS-induced ERK1/2 or p38 activation. However, SP600125 has been shown to inhibit 13 other kinases at similar or greater potency than JNK (Bain et al. 2003). Of these 13 kinases, only AMP-activated protein kinase is directly associated with the regulation of iNOS expression, but AMP-activated protein kinase activators inhibit iNOS expression (Pilon et al. 2004). Although MSK1, another kinase inhibited by SP600125, has not been directly associated with iNOS, it is possible that the effect of SP600125 is partly mediated through inhibition of MSK1, as this kinase has been show to regulate NF-κB activity (Vermeulen et al. 2003). However, such a mechanism is not evidenced by our results, which suggest that SP600125 inhibits iNOS expression mainly at the posttranscriptional level.

SP600125 has also been shown to inhibit LPS- and hyaluronan fragment-induced iNOS expression in mouse and rat microglia (Wang et al. 2004), as well as iNOS expression

induced by overexpressing TAK1 and TAB1 (Bhat et al. 2003). Further support for the role of JNK in iNOS expression is provided by studies in which blockade of the JNK pathway by overexpressing dominant negative forms of MKKK1, MKK4/7 and JNK1/2 inhibited iNOS expression (Table 7). SP600125 had only little effect on LPS-induced iNOS mRNA expression when measured 4 h after LPS stimulation, but significantly reduced mRNA levels when measured 8 h after LPS. Similar results are reported in mouse and rat microglia, where SP600125 had no or only small effect on iNOS mRNA levels when measured 5 h after stimulation with LPS or hyaluronan fragments, in contrast to the >90 % inhibition seen in iNOS protein expression (Wang et al. 2004). One would expect a significant decrease in mRNA levels at 4 h after stimulation if the regulation occurs at the level of transcription. These results suggest that JNK has no or only a minor effect on iNOS transcription and regulates iNOS expression mostly at posttranscriptional level. This is in contrast with reports of 50-100 % inhibition in mouse iNOS promoter activity by overexpression of dominant negative MKK4 or MKK7 (Table 7). Since the actual rate of transcription or the activity of iNOS promoter was not investigated in the present study, the effect of SP600125 on iNOS gene transcription cannot be totally ruled out. In this study, SP600125 considerably reduced the half-life of iNOS mRNA, which would imply that JNK regulates iNOS mRNA stability.

Cycloheximide reversed the effect of SP600125 at a 1 µg/ml concentration. One possible interpretation of these results is that the effect of SP600125 requires *de novo* protein synthesis. However, at this concentration cycloheximide also activated JNK and in these conditions the activity of JNK was only partially inhibited by SP600125. In further studies we have observed that lower concentrations of cycloheximide (0,1 µg/ml), which do not inhibit protein synthesis, also augmented LPS-induced JNK activation and iNOS mRNA expression. JNK activity induced by low concentrations of cycloheximide was totally inhibited by SP600125, but the cycloheximide-induced increase in iNOS mRNA expression was only partially reversed by SP600125. Thus, the effect of cycloheximide on iNOS mRNA expression seems to be independent of protein synthesis and only partially mediated through increased JNK activity. The question whether JNK-mediated stabilization of iNOS mRNA requires protein synthesis or is mediated by other mechanisms remains unanswered.

Table 7. Summary of studies of the role of the JNK pathway in the regulation of iNOS expression and NO production. dn=dominant negative, miNOS=mouse iNOS.

Cells	Inhibitors	Stimulus	Response	Result	Reference
NIH3T3 mouse fibroblasts	dnMKKK1	TNF-α+IFN-γ	miNOS promoter	⇓ 100 %	(Chan et al. 1999)
	dnMKK4			⇓ 100 %	
rat primary mesangial cells	dnJNK1	IL-1β	nitrite	⇓ ~70 %	(Guan et al. 1999)
			iNOS protein	⇓	
	dnJNK2		nitrite	⇓ ~70 %	
			iNOS protein	⇓	
	dnMKK4		nitrite	⇓ ~40 %	
			iNOS protein	⇓	
RAW 264.7 mouse macrophages	dnMKK7	lipoarabinomannan+IFN-γ	miNOS promoter	⇓ ~90 %	(Chan and Riches 2001b)
	dnMKK4			-	
RAW 264.7 mouse macrophages	dnMKKK1	LPS+IFN-γ	miNOS promoter	-	(Chan et al. 2001a)
	dnMKK4		miNOS promoter	⇓ ~50 %	
RAW 264.7 mouse macrophages	dnJNK1	bovine type I collagen	nitrite/iNOS protein	-	(Cho et al. 2002)
human fetal astrocytes	dnJNK	IL-1+IFN-γ	nitrite	⇓ 30-60 %	(Hua et al. 2002)
HAPI rat microglia	SP600125 (25 μM)	overexpression of wtTAK1+wtTAB1	nitrite	⇓ ~70 %	(Bhat et al. 2003)
			iNOS mRNA	⇓ ~20 %	
RAW 264.7 mouse macrophages	dnJNK1	thrombin	iNOS protein	⇓ ~90 %	(Kang et al. 2003)
J774 mouse macrophages	SP600125 (20 μM)	LPS	nitrite	⇓ ~80 %	(Lahti et al. 2003)
			iNOS protein/mRNA	⇓ ~80 %	
BV2 mouse microglia	SP600125 (20 μM)	hyaluronan fragments	nitrite	⇓ ~90 %	(Wang et al. 2004)
			iNOS protein	⇓ ~90 %	
			iNOS mRNA	⇓ ~40 %	
		hyaluronan fragments+IFN-γ	nitrite	⇓ ~10 %	
		LPS	nitrite	⇓ ~90 %	
			iNOS mRNA	-	
rat primary microglia			nitrite	⇓ ~70 %	
			iNOS mRNA	-	
		hyaluronan fragments	nitrite	⇓ ~80 %	
			iNOS mRNA	-	

4. The role of the p38 pathway in iNOS expression and NO production

In the present study, SB203580 and SB220025 augmented LPS-induced NO production at EC_{50} values of ~0,5 µM and ~0,1 µM, respectively. These are close to their reported IC_{50} values for p38 inhibition (~0,6 µM and ~60 nM respectively) (Alessi et al. 1995, Jiang et al. 1996, Jackson et al. 1998). SB202474, a structurally related compound which does not inhibit p38, had no effect on NO production at a 1 µM concentration. This would suggest that the increase in NO production induced by these compounds was due to inhibition of p38 and not to some unspecific effect. However, at concentrations >5 µM, SB202190 and SB203580 inhibited NO production and iNOS expression. The inhibition was >90 % at 30 µM. The negative control compound, SB202474, also inhibited NO production to the same extent at a 30 µM concentration. These findings suggest that the inhibition of NO production and iNOS expression brought about by these compounds is unrelated to p38 inhibition at concentrations >10 µM.

SB203580 has been tested against a number of other kinases without effect (Alessi et al. 1995, Davies et al. 2000). It inhibits p38α and p38β with similar potency, but has no effect on p38γ or p38δ (Kumar et al. 1997). However, SB203580 has some unspecific effects. It inhibits COX-1 and -2 (Börsch-Haubold et al. 1998), some isoforms of JNK (Clerk and Sugden 1998), type I TGF-β receptor (Eyers et al. 1998) and c-Raf (Hall-Jackson et al. 1999), but at higher concentrations than required for inhibition of p38α. Also SB220025 has been tested against other kinases without effect (Jackson et al. 1998).

The role of the p38 pathway in the regulation of iNOS expression is controversial. Inhibition of p38 has been reported to stimulate, to have no effect or to inhibit iNOS expression induced by various cytokines in vitro (Table 8). In most studies, high concentrations (>10 µM) of SB203580 have been used to block the p38 pathway. The inhibition in iNOS expression and NO production by SB203580 at concentrations >10 µM may result from unspecific action of the compound. However, inhibition of iNOS expression has also been observed with lower

concentrations of SB203580 (≤10 μM) (Table 8). The reason why inhibition of this pathway inhibits iNOS expression in some experimental settings and augments it in others is unclear. Partially these discrepancies may be related to cell type- and species-dependent differences in regulation of iNOS expression as well as to the use of different stimuli in inducing iNOS expression. In one in vivo study, SB203580 was reported to inhibit iNOS expression in the lung in mice with endotoxin shock (Kan et al. 2004).

A recent report suggests that the p38α and p38β2 isoforms may have opposite effects on iNOS expression (Lui et al. 2004). Overexpression of dominant negative p38α and wild-type p38β2 both inhibited iNOS expression. Thus, it is possible that the increase in LPS-induced iNOS expression induced by p38 inhibitors results from inhibition of p38β2. Human monocytes or macrophages express p38α abundantly but no or only low-level p38β (Hale et al. 1999, Fearns et al. 2000). We have not investigated which p38 isoforms are expressed in J774 macrophages, and to our knowledge no such information has been reported for mouse macrophages.

Interestingly, we observed that the stimulatory effect of p38 inhibitors increased when they were added 1 h after LPS stimulation compared with addition of 30 min before LPS. This suggests two explanations. First, p38 may have overlapping inhibitory and stimulatory effects on iNOS expression. Second, the early peak of high activity is important for the positive regulatory effect, whereas the later low but sustained phase of p38 activity seems to be important for the negative regulatory effect. The conclusion that the p38 pathway may also have positive regulatory effect on iNOS expression is supported by the observation that iNOS expression is increased by overexpressing constitutively active MKK3 and MKK6 (Bhat et al. 2002) and by reports of iNOS expression being inhibited by overexpressing dominant negative MKK3, MKK6 and p38α (Guan et al. 1999, Kristof et al. 2001). Reporter gene assays using rat and human iNOS promoter constructs suggest that p38 regulates iNOS transcription positively (Kristof et al. 2001, Bhat et al. 2002). This might result from a MSK1-mediated increase in NF-κB transactivational activity (Vermeulen et al. 2003).

Table 8. Summary of studies of the role of the p38 MAPK pathway in the regulation of iNOS expression and NO production. dn=dominant negative, wt=wild type, ca=constitutively active, hiNOS=human iNOS, riNOS=rat iNOS.

Cells	Inhibitor	Stimulus	Response	Result	Reference
rat primary mesangial cells	SC68376 (10 μM)	IL-1β	nitrite	⇑ ~150 %	(Guan et al. 1997)
			iNOS protein/mRNA	⇑	
rat primary microglia	SB203580 (20 μM)	LPS	nitrite	⇓ ~50 %	(Bhat et al. 1998)
			iNOS protein/mRNA	⇓	
RAW 264.7 mouse macrophages	SB230580 (10 μM)	LPS+IFN-γ	nitrite/ iNOS protein	-	(Caivano 1998)
DLD-1 human colon epithelial-like cells	SB203580 (5 μM)	IFN-γ+IL-1β+TNF-α	iNOS mRNA	-	(Kleinert et al. 1998)
rat pancreatic β-cells	SB203580 (10 μM)	IL-1β	nitrite/iNOS mRNA	⇓ ~35 %	(Larsen et al. 1998)
Primary human articular chondrocytes	SB230580 (10 μM)	IL-17	nitrite	⇓ ~30 %	(Shalom-Barak et al. 1998)
			iNOS protein	⇓	
RAW 264.7 mouse macrophages	SB203580 (20 μM)	LPS+IFN-γ	iNOS protein	⇓ ~90 %	(Ajizian et al. 1999)
rat aortic smooth muscle cells	SB203580 (0,1 μM)	LPS+IFN-γ	nitrite/iNOS protein	⇑ ~40 %	(Baydoun et al. 1999)
	SB203580 (10 μM)	LPS+IFN-γ	nitrite/iNOS protein	⇓ ~70 %	
CG4 rat oligodendrocyte cells	SB203580 (5 μM)	TNF-α+IFN-γ	nitrite	⇓ ~80 %	(Bhat et al. 1999)
			iNOS protein/mRNA	⇓	
mouse bone marrow-derived macrophages	SKF86002 (30μM)	TNF-α+IFN-γ	nitrite	-	(Chan et al. 1999)
NIH3T3 mouse fibroblasts	SKF86002 (10μM)	TNF-α+IFN-γ	nitrite	-	
RAW 264.7 mouse macrophages	SB203580 (10 μM)	LPS	nitrite	⇓ ~50%	(Chen and Wang 1999)
			iNOS protein	⇓	
J774 mouse macrophages	SB203580 (1 μM)	LPS	nitrite	⇓ ~50 %	(Chen et al. 1999)
			iNOS protein/mRNA	⇓	
fetal mouse skin dendritic cells	SB203580 (20 μM)	LPS	nitrite	⇓ ~21 %	(Cruz et al. 1999)
rat primary mesangial cells	dnp38α	IL-1β	nitrite	⇓ ~60 %	(Guan et al. 1999)
			iNOS protein	⇓	
	dnMKK3	IL-1β	nitrite	⇓ ~40 %	
			iNOS protein	⇓	
	dnMKK6	IL-1β	nitrite	⇓ ~60 %	
			iNOS protein	⇓	
RAW 264.7 mouse macrophages	SB203580 (1 μM)	LPS	nitrite	⇑ ~20 %	(Patel et al. 1999)
human articular chondrocytes	SB242235 (20 μM)	IL-1β	nitrite/iNOS mRNA	-	(Badger et al. 2000)

RAW 264.7 mouse macrophages	SB203580 (30 μM)	LPS	iNOS mRNA	⇓ ~90 %	(Jeon et al. 2000)
RAW 264.7 mouse macrophages	SB203580 (30 μM)	LPS+IFN-γ	nitrite	⇑ ~30 %	(Chan and Riches 2001b)
			iNOS protein	⇑	
RAW 264.7 mouse macrophages	SB203580 (30 μM)	lipoarabinomannan+IFN-γ	nitrite/iNOS protein	⇑ ~30 %	(Chan et al. 2001a)
A549 human alveolar type II epithelial cells	SB203850 (5μM)	IFN-γ+LPS	hiNOS promoter	⇓ ~45 %	(Kristof et al. 2001)
		IFN-γ+IL-1β+TNF-α		⇓ ~40 %	
	dnMKK3	IFN+IL-1+TNF/IFN+LPS		⇓ ~30 %	
	dnMKK6	IFN+IL-1+TNF/IFN+LPS		-	
C6 rat glioma cells	SB230580 (15 μM)	LPS+IFN-γ+TNF-α	nitrite	⇓ ~50%	(Bhat et al. 2002)
			riNOS promoter	⇓ ~50%	
	caMKK6	-	riNOS promoter	⇑	
	caMKK3	-	riNOS promoter	⇑	
		LPS+IFN-γ	nitrite	⇑ ~3x	
			protein	⇑	
RAW 264.7 mouse macrophages	SB203580 (10 μM)	bovine type I collagen	nitrite/iNOS protein	-	(Cho et al. 2002)
human fetal astrocytes	SB203580 (10 μM)	IL-1/ IL-1+TNF-α IL-1+IFN-γ	nitrite	⇓ ~100 %	(Hua et al. 2002)
			nitrite	⇓ ~30 %	
J774 mouse macrophages	SB203580 (1 μM)	LPS	nitrite/iNOS protein	⇑ 30-90 %	(Lahti et al. 2002)
	SB203580 (30 μM)		nitrite/iNOS protein	⇓ 50-80 %	
BV2 mouse microglia	SB203580	IFN-γ+PMA	nitrite	-	(Han et al. 2003)
RAW 264.7 mouse macrophages	SB230580 (10 μM)	thrombin	iNOS protein	⇑ ~100 %	(Kang et al. 2003)
C6 rat glioma cells	SB203580 (10 μM)	LPS+ TNF-α	iNOS mRNA	⇓ ~100 %	(Lee et al. 2003)
rat primary astroglia	SB203580 (1 μM)	IL-1β+TNF-α	nitrite	⇑ ~40 %	(Marcus et al. 2003)
A498 human kidney epithelial cells	SB203580 (1 μM)	IFN-γ+IL-1β+TNF-α	nitrite	⇓ ~45 %	(Poljakovic et al. 2003)
	SB203580 (10 μM)		iNOS protein	⇓	
rat primary mesangial cells	SC68376 (10 μM)	IL-1β	iNOS protein	⇑	(Lui et al. 2004)
	dnp38α	IL-1β	iNOS protein	⇓	
	wtp38α	IL-1β	iNOS protein	-	
	dnp38β2	IL-1β	iNOS protein	-	
	wtp38β2	IL-1β	iNOS protein	⇓	
RAW 264.7 mouse macrophages	SB203580 (10 μM)	LPS	iNOS protein	⇑ ~100 %	
BV2 mouse microglia	SB203580 (40 μM)	hyaluronan fragments	nitrite	⇓ ~60 %	(Wang et al. 2004)
			iNOS protein/mRNA	⇓ ~50 %	

In addition to increased iNOS expression, SB203580 has been shown also to increase LPS- or heat-killed Streptococcus Pneumoniae-induced IL-6 and TNF-α production in mouse macrophages in vitro and TNF-α production in mouse models of streptococcal pneumonia and tuberculosis in vivo (van den Blink et al. 2001). No mechanism for increased iNOS expression has previously been reported. In this study, SB220025 had no effect on iNOS mRNA levels when measured 4 h after LPS stimulation, whereas the levels were increased ~2 fold when measured 10 h after LPS. This would suggest that p38 regulates iNOS expression at the post-transcriptional level. iNOS mRNA levels fell faster in cells treated with LPS only compared with cells treated with LPS+SB220025, whereas SB220025 had no effect on the iNOS mRNA degradation rate when the same experiment was done in the presence of actinomycin D. The stabilizing effect of actinomycin D on iNOS mRNA might mask the effect of SB220025 and is one possible reason why no effect was seen in the actinomycin D assay. This suggests that inhibition of p38 inhibits iNOS mRNA degradation. Blocking of the p38 pathway has been mostly reported to facilitate the mRNA degradation rate of various cytokines (Clark et al. 2003a). Only MKK6 mRNA stability has been reported to be negatively regulated by this pathway (Ambrosino et al. 2003).

5. Regulation of JNK activity by the p38 pathway and its role in iNOS expression

SP600125 inhibited NO production even when added 1-3 h after LPS stimulation, suggesting that the later sustained phase of low JNK activity is important for the regulation of NO production, whereas the early high-activity peak is not. SB220025 added at the same time as LPS had no effect on the early high-activity peak of JNK activation, but considerably increased the late-phase levels of phosphorylated JNK. SB220025 increased JNK phosphorylation significantly also when added 1 h after LPS and the increased levels of phosphorylated JNK remained for hours. Similarly, SB220025 also increased phosphorylation of c-Jun. This increase was inhibited by SP600125, which confirms that the increase in c-Jun phosphorylation was due to increased JNK activity and not e.g. to reduced dephosphorylation.

Previously, inhibition of p38 has been shown to augment JNK activity induced by TNF-α in human neutrophils (Avdi et al. 2002), by TNF-α, IL-1 or sorbitol in human epithelial KB cells, by LPS in mouse macrophages (Cheung et al. 2003) and by IL-6 in mouse peritoneal macrophages (Hall and Davis 2002). In addition, ERK1/2 activity is increased when p38 is inhibited by SB203580 or dominant negative p38α (Westermarck et al. 2001, Liu and Hofmann 2004, Numazawa et al. 2003, Hall and Davis 2002). Furthermore, increased ERK1/2 activation has been observed in rats with remnant kidneys treated with p38 inhibitor (Ohashi et al. 2004). Thus the p38 pathway seems to regulate the activity of other MAPKs also in vivo. The mechanism whereby blocking of the p38 pathway regulates the activity of JNK and ERK1/2 is unclear. The increase in ERK1/2 and JNK activity has been suggested to be due to reduced dephosphorylation by PPA2. Inhibition of p38 reduces PP2A activity and the phosphatase inhibitors calyculin A and okadaic acid can induce a similar increase in MAPK activity (Westermarck et al. 2001, Avdi et al. 2002, Liu and Hofmann 2004). Another proposed mechanism is one mediated through increased TAK1 activity. TAB1, a subunit of TAK1, is phosphorylated by p38α, which leads to reduced activity of TAK1. Inhibition of p38 leads to increased activity of TAK1, which is followed by increased activity of downstream signalling pathways, e.g. JNK (Cheung et al. 2003).

The SB220025-induced increase in iNOS expression and NO production was reversible by SP600125. This suggests that the stimulatory effect of SB220025 on iNOS expression is mediated through increased JNK activity. Increased ERK1/2 activity by p38 inhibition results in increased matrix metalloproteinase 1 expression in human skin fibroblasts (Westermarck et al. 2001) and increased IL-6-induced IL-10 expression in mouse peritoneal macrophages (Hall and Davis 2002). Thus, the regulation of other MAPKs by the p38 pathway may be of functional significance. Furthermore, when assessing the role of the p38 pathway in various settings, one must be careful in interpreting results obtained by p38 inhibition. The observed effects might result from increased activity of other MAPKs as well as being due to inhibition of direct effects of the p38 pathway.

6. The role of mRNA stability in regulation of iNOS expression

iNOS mRNA levels reached maximum 6-8 h after LPS stimulation, whereafter the mRNA levels began to decline rapidly. Treatment with an inhibitor of transcription, actinomycin D, 6 h after LPS slightly increased the levels of mRNA when compared with cells treated with LPS only. This suggests that no or only little transcription occurs 6-8 h after LPS stimulation. Thus, at that point the rate of iNOS mRNA degradation mostly determines the level of iNOS mRNA expression. Neither p38 inhibitor or JNK inhibitor had any effect on the iNOS mRNA levels when measured 4 h after LPS, at a time point when iNOS mRNA levels were rapidly increasing. In contrast, both compounds had a significant effect on mRNA levels when measured 8 h after LPS, suggesting that these compounds modify the rate of iNOS mRNA degradation. Such a conclusion was supported by results of experiments in which the rate of iNOS mRNA disappearance was investigated. In addition, both compounds significantly modified iNOS protein expression and NO production. These findings together suggest that the rate of iNOS mRNA degradation has a significant effect on iNOS expression and NO production. However, the compounds tested may also have an effect on the rate of translation and iNOS protein stability (which were not investigated in the present study) and the observed effect on NO production being then a sum of these effects.

Only scattered information has been published regarding the regulation of iNOS mRNA stability. Involvement of the hnRNP I and L, HuR and ARE/poly(U)-binding/degradation factor-1 (AUF1) in the regulation of iNOS expression has been suggested (Soderberg et al. 2002, Rodriguez-Pascual et al. 2000, Kleinert et al. 2002). Results in the present study suggest that JNK is part of the signalling pathways regulating iNOS mRNA stability. The effect of JNK on iNOS mRNA stability has not previously been reported, but JNK has been shown to regulate the stability of vascular endothelial growth factor, IL-2, IL-3 and beta-adrenergic receptor mRNAs (Pages et al. 2000, Chen et al. 1998, Ming et al. 1998, Headley et al. 2004). The regulation of IL-3 mRNA stability by JNK is dependent on the AREs in the 3'-UTR (Ming et al. 1998). IL-2 mRNA stability is dependent on elements on the 5'-UTR, a region called the JNK-responsive element and an unidentified element in the 3'-UTR and interaction between these elements (Chen et al. 1998). Furthermore, JNK-dependent IL-2 mRNA

stabilization requires binding of nucleolin and Y-box binding protein to the JNK-responsive element (Chen et al. 2000). Y-box binding protein or nucleolin are not direct targets of JNK and it is unclear how these proteins regulate mRNA stability (Chen et al. 2000). The elements in iNOS mRNA which regulate its stability are unknown and no JNK-responsive element has been reported in iNOS mRNA, but it is possible that the regulation of iNOS mRNA by JNK is also dependent on the 3'-UTR AREs. No association has been reported between JNK and hnRNP I and L, HuR or AUF1.

Further characterization of the elements in the iNOS mRNA and the factors associated with these elements, would also provide information as to the regulation of other transiently expressed inflammatory genes. However, a more reliable method to measure the rate of iNOS mRNA degradation is required. Replacement of actinomycin D with a more specific inhibitor of transcription would provide more precise information of the rate of mRNA degradation. Other methods may also be adopted. Reporter gene constructs, transcription of which is controlled by tetracycline sensitive promoter, have been combined with the 3' UTR of the mRNA of interest. This method has been successfully employed to map more accurately the mRNA stability regulating elements in the 3'-UTR of COX-2 gene and to identify factors participating in the regulation (Lasa et al. 2000). An in vitro mRNA decay system which measures the decay of labelled mRNA in soluble cytoplasmic extracts has been used successfully to study IL-2 mRNA stability (Chen et al. 2000).

It is likely that the stability of the mRNAs of proinflammatory mediators is regulated by common factors which may significantly modulate immune responses. Tristetraprolin is an mRNA binding protein which participates in the regulation of at least TNF-α and granulocyte/macrophage colony-stimulating factor mRNA stability. In addition, tristetrapolin knockout mice develop a severe inflammatory syndrome (Blackshear 2002). Thus, characterization of the factors involved in the regulation of mRNA stability could provide new targets for the development of anti-inflammatory drugs.

SUMMARY AND CONCLUSIONS

The purpose of the present study was to investigate the role of three MAP-kinase pathways, i.e. ERK1/2, p38 and JNK, in the regulation of iNOS expression and NO production in activated macrophages. The findings and conclusions are the following:

1. The MKK1 inhibitor PD98059, which inhibits ERK1/2 activation, partially reduced LPS-induced iNOS expression and NO production. This suggests that activation of the ERK1/2 pathway is not essential for iNOS expression but is required for maximal NO production.

2. The JNK inhibitor SP600125 reduced LPS-induced iNOS expression by increasing the rate of iNOS mRNA degradation. This suggests that activation of JNK results in stabilization of iNOS mRNA, and subsequently increases iNOS expression and NO production.

3. p38 inhibitors increased LPS-induced iNOS expression at low drug concentrations, which are specific for p38 inhibition, whereas they inhibited iNOS expression at high, unspecific concentrations. p38 inhibition augmented iNOS expression by reducing the rate of iNOS mRNA degradation.

4. p38 inhibition resulted in a sustained increase in LPS-induced JNK activity and enhanced iNOS expression, which was reversed by a JNK inhibitor. This suggests that the increase in iNOS expression by p38 inhibitors is mediated through increased JNK activity.

MAPK inhibitors have exhibited anti-inflammatory properties in various animal models of inflammatory diseases and have therefore been intensively developed for clinical use in the treatment of inflammatory diseases. Inhibition of iNOS expression could partially explain the anti-inflammatory effect of the JNK inhibitor SP600125. The activity of the MAPK pathways

seems to be coordinately regulated. Thus, blocking of one pathway may alter the activity of the others. This might result in unexpected effects of MAPK inhibitors and warrants caution in interpretation of results when used for studying physiological roles of MAPK pathways in experimental settings.

It is increasingly evident that post-transcriptional mechanisms are important in the regulation of iNOS expression. The present study shows that JNK is one of the signalling pathways which regulate the rate of iNOS mRNA degradation. The targets of JNK which mediate these effects remain unknown. mRNA stability is a common mechanism to regulate the expression of transiently expressed inflammatory genes. Hence characterization of the JNK-regulated and other factors which regulate iNOS mRNA degradation could reveal potential targets for drug development.

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