TRANSMEMBRANE SIGNALLING MECHANISMS REGULATING EXPRESSION OF CATIONIC AMINO ACID TRANSPORTERS AND INDUCIBLE NITRIC OXIDE SYNTHASE IN RAT VASCULAR SMOOTH MUSCLE CELLS

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Running Title: Regulation of CAT and iNOS expression in RASMCs

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SYNOPSIS

The signalling mechanisms involved in the induction of nitric oxide synthase (iNOS) and L-arginine transport were investigated in bacterial lypopolysaccharide (LPS) and interferon-γ (IFN-γ)- stimulated rat cultured aortic smooth muscle cells (RASMC). The expression profile of transcripts for cationic amino acid transporters (CATs) and their regulation by LPS and IFN-γ were also examined. Control RASMC expressed mRNA for CAT-1, CAT-2A and CAT-2B. Levels of all three transcripts were significantly elevated in activated cells. Stimulated CAT mRNA expression and L-arginine transport occurred independently of protein kinase C (PKC), protein tyrosine kinase (PTK) and p44/42 mitogen activated kinases (MAPK), but were inhibited by the p38 MAPK inhibitor SB203580, which at 3 μM caused maximum inhibition of both responses. Induction of NO synthesis was independent of p44/42 MAPK activation and only marginally dependent on PKC, but was attenuated markedly by the PTK inhibitors, genistein and herbimycin A. SB203580 differentially regulated iNOS expression and NO production, potentiating both processes at low μM concentrations and inhibiting at concentrations of $\geq 1\mu M$. In conclusion, our data suggest that RASMC constitutively express transcripts for CAT-1, CAT-2A and CAT-2B, and that expression of these transcripts is significantly enhanced by LPS and IFN-γ. Moreover, stimulation of L-arginine transport and induction of NO synthesis by LPS and IFN-γ appears to be under critical regulation by the p38 MAPK, since both processes were significantly modified by SB203580 at concentrations so far shown to have no effect on other signalling pathways. Thus, in RASMCs, the p38 MAPK cascade represents an important signalling mechanism, regulating both enhanced L-arginine transport and induced NO synthesis.

INTRODUCTION

We have previously demonstrated that activation of rat cultured aortic smooth muscle cells (RASMC) with pro-inflammatory mediators results in expression of inducible nitric oxide synthase (iNOS) and upregulation in transport of L-arginine [1]. Induction of both processes is blocked by cycloheximide and therefore dependent on *de novo* protein synthesis. Expression of iNOS is attenuated selectively by dexamethasone, suggesting that the signalling events associated with regulation of iNOS expression may, at least in part, be distinct from those associated with upregulation of L-arginine transport [1,2].

Although considerable effort has been put into unraveling the signalling mechanisms associated with induction of iNOS and L-arginine transport, most of the reported studies have focused mainly on tyrosine kinase and/or protein kinase C signalling with conflicting conclusions. In this regard data in the literature present quite contradictory interpretations of the role of protein kinase C (PKC) and protein tyrosine kinases (PTKs) in bacterial lipopolysaccharide (LPS) and/or cytokine-induced iNOS expression. Initial studies suggested that activation of PKC may be the key signal transduction pathway in several cell types including rat hepatocytes [3], smooth muscle cells [4] and murine RAW 264.7 macrophages [5]. However, other studies have found no indication for PKC involvement in iNOS induction in RASMC [6], mouse astrocytes [7] or human chrondocytes [8]. Increasing evidence now suggests that activation of PTK rather than PKC may be obligatory for induction of iNOS not only in rat smooth muscle cells [5, 6, 9] but also in murine macrophages [5, 10], astrocytes [7] and mesangial cells [11].

The signal transduction mechanisms associated with induction of L-arginine transport are also poorly defined. L-arginine is predominantly transported across cell membranes via a

specific sodium-independent transport system selective for cationic amino acids and sensitive to *trans*-stimulation, thus referred to as system y^+ [12]. The specificity and characteristics of L-arginine transport in rat cultured aortic smooth muscle cells used in our study strongly support the existence of carrier system(s) resembling system y^+ [1]. At present, three related carriers that mediate the transport of cationic amino acids similar to system y^+ (K_m: 0.1-0.25mM) have been identified and referred to as cationic amino acid transporter-1 (CAT-1), CAT-2B and CAT-3 [13-19]. A fourth related carrier, CAT-2A, exhibits the same Na^+-independent transport activity specific for cationic amino acids but has at least 10-fold lower substrate affinity (K_m: 2.1-5.2 mM) and is much less sensitive to *trans*-stimulation [17, 20, 21]. CAT-2A and CAT-2B are most likely the product of differentially spliced mRNAs.

CAT-1 is ubiquitously expressed with the exception of the liver [for review see 22]. A more restricted expression pattern has been found for the other three isoforms: CAT-2A [20, 23] and CAT-3 [18, 19] are constitutively expressed in liver and brain, respectively, whereas CAT-2B expression can be induced in many cell types [for review see 22]. A variety of external stimuli, including mitogens[24], LPS [15, 25], interleukin-1β (IL-1β) , insulin [26], tumor necrosis factor-α (TNF-α) [27] and angiotensin II [27, 28], alter the expression of CAT transcripts.

Although CAT-expression has been studied in vascular smooth muscle cells, relatively little is known about the signalling pathways that regulate CAT expression and L-arginine transport in these cells. As with iNOS, the role of either PTK or PKC in enhanced L-arginine transport induced by pro-inflammatory mediators remains inconclusive. Limited reports have suggested that exposure to phorbol esters enhances L-arginine entry into rat peritoneal macrophages [29], human umbilical vein endothelial cells [30] and the human intestinal

epithelial cell line Caco-2 [31]. However, Schmidlin & Wiesinger [7] failed to modulate arginine transport in activated astrocytes using the PKC inhibitor staurosporine.

In this study we have examined the signalling mechanisms associated with induction of L-arginine transport and NO synthesis in cultured smooth muscle cells, the key cell type implicated as the major source of NO in endotoxin and/or cytokine induced shock [32, 33]. Changes in LPS and interferon-γ (IFN-γ) -induced expression of CATs and iNOS have been examined in the presence of various inhibitors of either PTK or PKC. We have extended these studies by examining the role of downstream kinases thought to be points of convergence for various signals originating from cell surface receptors known to be activated by LPS and cytokines. Two of these correspond to the 42 and 44 kDa forms of mitogen-activated protein kinase (MAPK) which are phosphorylated on tyrosine/threonine residues via an upstream dual-specific MAPK kinase [34]. An alternative pathway is the signalling cascade involving the stress-activated 38 kDa MAPK [35]. Involvement of either of these two pathways in our system has been investigated using PD98059 which inhibits activation of p42/44 MAPKs by blockade of their upstream activator, MAPK kinase (MEK) [36-38], and SB203580 which directly inhibits p38 MAPK [39]. Although SB203580 is routinely used to implicate p38 MAPK in cellular responses, it is worth noting that this compound also stimulate Raf-1 [40], inhibits c-Jun N-terminal kinase (JNK) [41] and the cyclooxygenase (COX) enzymes [42]. Some of these effects are, however, observed at concentrations of around 10 μM and above, with the IC_{50} for inhibition of p38 MAPK being around 0.1 μM. Thus in our study we have examined the effects of this compound over a wide concentration range and have ascribed responses observed at concentrations well below those shown to inhibit the above pathways to inhibition of p38 MAPK.

EXPERIMENTAL

Materials

Tissue culture reagents were from Gibco (Paisley, U.K.). Recombinant murine IFN-γ was from Genzyme (Cambridge, U.K.). LPS from *Escherichia coli* (serotype 0111:B4) was obtained from Sigma (Poole, U.K.). SB203580 was a generous gift from SmithKline Beecham Pharmaceuticals, U.K. PD98059 was purchased from Calbiochem (Nottingham, U.K.). Monoclonal antibody for inducible nitric oxide synthase was obtained from Affinity Research Laboratories and phospho-specific polyclonal antibodies for MAPK and p38 were purchased from New England BioLabs (Hertfordshire, U.K.). Other chemicals were from Sigma or BDH and of the highest analytical grade obtainable. L-[2,3-^{3}H]arginine (36.1 Ci/mmol) was obtained from New England Nuclear (Dreieich, Germany). [α-^{32}P]UTP (3000 Ci/mmol) was from ICN (Eschwege, Germany). Restriction enzymes, oligonucleotides (random hexamer, oligo (dT)$_{12\text{-}18}$, and CAT-specific), Taq DNA polymerase, Taq polymerase reaction buffer, ^{T7}Sequencing Kit, were from Pharmacia Biotech (Freiburg, Germany). Dnase I, Rnase A, RNase T1, proteinase K and T7 RNA polymerases were from Boehringer Mannheim (Mannheim, Germany). Vectors pCR-Script AmpSK$^+$, pbluescript KS$^+$, and pSG5 were from Stratagene (Heidelberg, Germany). The SuperScript reverse transcriptase (RT) Kit was from Life Technologies (Eggenstein, Germany).

Isolation and culture of aortic smooth muscle cells

Vascular smooth muscle cells were cultured from rat aortic explants as described previously [1]. Briefly, male Sprague-Dawley rats (250-300 g) were stunned, exsanguinated and the thoracic aorta dissected into Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.4% NaHCO$_3$, penicillin (100 units ml^{-1}) and streptomycin (100 mg ml^{-1}). Explants of

aorta were left in culture for upto 14 days to allow migration and proliferation of smooth muscle cells. Clusters of cells were subsequently harvested with trypsin/EDTA (0.01/0.02 % in phosphate buffered saline) and cultured to confluence in a T-75 flask. All isolates were identified as smooth muscle cells by phase contrast microscopy and immunostaining of smooth muscle α-actin [43].

Cell activation

Cells were plated at a seeding density of either 5×10^3 cells per well into 96 well plates for measurements of transport of L-arginine and nitrite production, or at 5×10^4 cells per well in 24 well plates for Western blot analysis. Cells cultured in T-75 flasks were used for RNA extraction and analysis. Confluent monolayers were incubated with DMEM supplemented with 2 mM glutamine and 10 % foetal bovine serum either alone or in combination with various compounds for a period of 30 min. Cells were subsequently activated with LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}). Incubations were terminated after 5-60 min for analysis of MAPK activation, after 24 h for detection of iNOS protein or 18 h for RNA extraction.

Assessment of cell viability

Cell viability was determined by monitoring mitochondrial-dependent reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] (MTT) to formazan as described [44]. Briefly, after each experimental protocol, cells in 96-well plates were incubated with MTT (0.5 mg ml^{-1}) for 4 h at 37°C. An equal volume of 10 % SDS in 0.01 M HCl was then added to all wells and incubated for a further 3 h to dissolve the accumulated crystals of formazan. Absorbance was measured at 560 nm using a Multiskan II plate reader (Titertek). Readings obtained from treated cells were compared to controls.

Determination of nitrite production

The culture medium from cell monolayers used either for transport studies or iNOS Western blotting was removed from each well for nitrite analysis. Total nitrite accumulated was determined colorimetrically using the standard Griess reagent [45] as described [1].

Measurement of L-arginine transport

Unidirectional transport of L-arginine was measured in confluent cell monolayers as described previously [1]. Briefly, cells were rinsed twice with a Hepes-buffered Krebs solution (mM: NaCl: 131; KCl: 5.5; $MgCl_2$: 1; $CaCl_2$: 2.5; $NaHCO_3$: 25; NaH_2PO_4: 1; D-glucose: 5.5; Hepes: 20; pH 7.4) maintained at 37^oC. Uptake was initiated by adding 50 μl of Krebs containing 100 mM L-[^{3}H]arginine (2 μCi ml^{-1}) to each well and influx was measured over 30 s. Uptake of L-[^{3}H]arginine was terminated by placing plates on ice and rinsing cells twice with 200 μl ice-cold Krebs containing 10 mM unlabelled L-arginine. Cells were lysed with 0.5 M NaOH, and protein concentrations determined using Brilliant Blue G [46]. Radioactivity (dpm) in cell lysates was measured by liquid scintillation counting. Transport was expressed in pmoles μg protein^{-1} min^{-1}.

Western Blot analysis

Western blotting was carried out as described previously [44]. Briefly, cell lysates (20 μg protein per lane) were separated by SDS-PAGE electrophoresis, transferred onto 0.2 μm nitrocellulose membrane (Anderman and Co., Kingston-upon-Thames, Surrey, U.K.) and blocked for 2 h in 100 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween-20, pH 7.4 (STT) containing 3 % (w/v) BSA. Membranes were then incubated overnight with either mouse

monoclonal anti-iNOS antibody (1:2500 dilution in STT containing 0.2 % (w/v) BSA) or polyclonal antibodies specific for phosphorylated p44/42 or p38 (1:1000 dilution in STT containing 0.2% (w/v) BSA). Blots were washed with STT (6 x 10 min) and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse (for iNOS) or anti-rabbit (for p44/42 or p38 MAPK) IgG for 1 h. Following further washing (8 x 10 min) in STT, immunoreactive bands were visualised using ECL detection System (Amersham).

Cloning of cDNA fragments of rat CAT-1, -2A and -2B

Total RNA was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction from rat ileum, rat liver and rat smooth muscle cells for the cloning of cDNA fragments of rat CAT-1, -2A and -2B, respectively. Single stranded cDNA was obtained by reverse transcription (RT) of 2 µg total RNA using the SuperScript RT kit and 80 ng hexamer oligonucleotides with random sequence as well as 0.5 µg oligo(dT)$_{12-18}$ as primers in a 20 µl reaction. The RT-generated cDNA served as templates in polymerase chain reactions (PCR). The sequence of the oligonucleotides used as primers were derived from a region of the mouse CAT-1 cDNA (GenBank accession number: M 26687) with high degree of identity with mCAT-2A/2B. The sense oligonucleotide for CAT-1 and CAT-2B was CCGGAATTCTTCGTGGGCTTTGACTGCAT (nucleotide (nt) 774 to 794 of mCAT-1 cDNA, additional Eco RI site underlined) and GCGAGATCTGCCTTATCAACAAGTCTTCT for CAT-2A (nt 1051 to 1071 of mCAT-2A cDNA, additional BglII site underlined, GenBank accession number L11600). The same antisense oligonucleotide was used for all three CATs: GCGAGATCTAAAAAGAAAGGCCATCACAGC (nt 1168 to 1188 of mCAT-1 cDNA, additional BglII site underlined) PCR was performed in 100 µl Taq polymerase buffer,

containing 0.2 mM dNTPs, 1.5 mM MgCl$_2$, 2 U Taq polymerase, 50 pmol oligonucleotides

and 2 µl cDNA. Thirty cycles of PCR were performed as follows: 1 min at 95°C, 2 min at

55°C and 3 min at 72°C.

The amplified cDNA fragments were cloned into the *Eco* RI/ *Bam* HI sites of pbluescript KS$^+$

(rCAT-1, 415 bp), the *Bgl*II site of pSG5 (rCAT-2A, 171 bp) or the *Eco* RV site of pCR-

Script AmpSK$^+$ (rCAT-2B, 415 bp) generating the plasmids prCAT-1/18, prCAT-2A/25 and

prCAT-2B/57, respectively. The DNA sequence of the cloned PCR product was determined

from plasmid templates using the dideoxy chain termination method with a ^{T7} Sequencing

Kit. The 230 bp *Nco*I/ *Bst* XI fragment from prCAT-1/18 was inserted into the *Eco* RV site

of pCR-Script AmpSK$^+$ resulting in the plasmid prCAT-1/41. A plasmid containing a cDNA

fragment of rat γ-actin (pCR_γ-actin_rat, 540 bp) was generously provided by Dr. Hartmut

Kleinert (Department of Pharmacology, Johannes Gutenberg University, Mainz). The

pCR_γ-actin_rat was restricted with *Asp*I and *Eco* RI, blunted and religated resulting in

pCR_γ-actin_rat_ ΔAspI_*Eco* RI that contains only 110 nt of the rat γ-actin cDNA.

Preparation of antisense RNA probes and RNase protection analysis of rCATs and rγ-actin mRNAs

RNAs were prepared from rat aortic smooth muscle cells using acid guanidinium

isothiocyanate-phenol-chloroform extraction and ribonuclease protection analyses performed

as described previously [47]. To generate radiolabeled antisense RNA probes, prCAT-1/41

was linearized with *Eco* RI, prCAT-2A/25 with *Xba* I, prCAT-2B/57 with *Bam* HI, and

pCR_γ-actin_rat_ΔAspI_Eco RI with *Bam* HI. *In vitro* transcription using T7 RNA

polymerase was performed to generate [α-^{32}P]-UTP-labeled riboprobes of 285 nt for rCAT-1,

339 nt for rCAT-2A, 203 nt for rCAT-2B, and 187 nt for rγ-actin.

Statistics

All values are means ± S.E. of measurements in at least three different cell cultures with 5-6 replicates per experiment. Statistical analyses were performed as described [44], using either an unpaired Student's *t*-test or a multiple means comparison test [48] validated by comparison with the Newman-Keuls multiple range test in the statistical package [49] with the overall confidence levels set at 95% (0.05).

RESULTS

Regulation of L-arginine transport and CAT mRNA expression by LPS and IFN-γ

In agreement with our previous findings [1], incubation of cultured smooth muscle cells with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) resulted in a marked increase in transport of L-[^{3}H]arginine which was elevated from a basal value of 3.2 ± 0.27 to 5.1 ± 0.18 pmoles μg protein^{-1} min^{-1} after 24 h exposure to LPS and IFN-γ.

Transcripts for rCAT-1, rCAT-2A and rCAT-2B were detected in total RNA isolated from control non-stimulated cells (Fig. 1). Moreover, levels of all three transcripts were significantly elevated following activation of cells with LPS and IFN-γ. The increases in mRNAs were about 3-fold for rCAT-1, 6-fold for rCAT-2A and 7-fold for rCAT-2B.

Effects of protein tyrosine kinase inhibition on L-arginine transport, CAT-expression and NO synthesis

Activated L-arginine transport was slightly reduced by genistein and herbimycin A, when applied at non-cytotoxic concentrations (≤ 30 μM and ≤ 1 μM for genistein and herbimycin A respectively) (Fig. 2A and 2C). L-arginine transport was reduced by 19 ± 3 % and 22 ± 7 % in the presence of 30 μM genistein or 1 μM herbimycin A respectively. At higher concentrations, both compounds caused a significant reduction in total cell protein and in the viability of the remaining adherent cells, and presumably reflect additional non-selective cytotoxic actions. In agreement with these transport data, RNase protection assays revealed that the LPS and IFN-γ-induced increases in expression of rCAT-1, -2A or -2B transcripts were unaffected by genistein (30 μM) or herbimycin A (1 μM) (data not shown).

In contrast to their marginal effects on L-arginine transport, genistein and herbimycin A caused a concentration-dependent inhibition of LPS and IFN-γ-induced nitrite accumulation (Fig.2B and 2D). At 30 μM genistein reduced stimulated nitrite levels by $77 \pm 5\%$ (Fig 2B). Similarly, nitrite production was inhibited by $73 \pm 6\%$ in the presence of 1 μM herbimycin A (Fig 2D). Daidzein, the inactive analogue of genistein, was without effect at concentrations of up to 30 μM (data not shown).

Effects of protein kinase C inhibition on L-arginine transport, CAT-expression and NO synthesis

The potent PKC inhibitor, RO318220, failed to cause any significant changes in either control or induced L-arginine transport at concentrations of ≤ 1 μM. Furthermore, RO318220 (1μM) did not alter mRNA levels of either rCAT-1, -2A or -2B (data not shown). Concentrations above 1 μM could not be investigated due to the marked cytotoxicity caused by this compound over the 24 h incubation period required in our studies. In addition to its marginal effects on transport, RO318220 was much less effective, compared to genistein or herbimycin A, in inhibiting NO synthesis, reducing the latter by ~22% at non-cytotoxic concentrations (≤ 1 μM).

Role of p44/42 mitogen activated protein kinase in induction of L-arginine transport, CAT-expression and NO synthesis

Neither L-arginine transport nor induced CAT-expression (data not shown) was affected by PD98059 (1-100 μM), which selectively blocks the upstream activator of p44/42 MAPK, MAPK kinase. In parallel experiments PD98059 (1-100 μM) also failed to modify LPS and IFN-γ-induced nitrite production. Western blot analysis using a phosphospecific polyclonal

antibody for p44/42 MAPK confirmed that both proteins are activated in rat cultured aortic smooth muscle cells, with maximum phosphorylation occurring 30 min after exposure to LPS and IFN-γ. More importantly, phosphorylation of both p44 and p42 MAP kinase was virtually abolished in the presence of 30 μM PD98059 (data not shown).

Role of p38 mitogen activated protein kinase in induction of L-arginine transport, CAT-expression and NO synthesis

SB203580 caused a concentration-dependent inhibition of enhanced L-arginine transport, completely abolishing the latter at 3 μM (Fig 3A). The basal rate of transport in control cells was not significantly altered over the same concentration range (0.01-10 μM). Similarly, SB203580 markedly attenuated the induced but not basal expression of CAT-1, -2A and -2B, (Fig. 4).

In addition to its effects on LPS and IFN-γ-enhanced L-arginine transport, SB203580 caused a concentration-dependent inhibition of nitrite production, reducing levels by 68 $\pm$ 11% at 10 μM (Fig 3B). Interestingly, inhibitions were only observed at concentrations of 1-10 μM. At 0.01 to 0.1 μM, SB203580 potentiated LPS and IFN-γ-induced nitrite production, increasing the latter by 40 $\pm$ 2 % at 0.1 μM. These findings suggest that expression of iNOS may be differentially regulated by different concentrations of SB203580. This was confirmed by Western blot analysis of iNOS expression using a selective monoclonal anti-iNOS antibody. As shown in Fig 5 the antibody recognised a single 130 kDa protein band in lysates from LPS and IFN-γ-activated cells but not from control cells. Expression of iNOS was increased 2.5 fold in the presence of 0.1μM SB203580 and virtually abolished at 3 μM.

DISCUSSION

One of the fundamental actions of pro-inflammatory mediators, including LPS and cytokines is the induction of the inducible NO synthase. This can be observed both *in vivo* and in a variety of cell types *in vitro*. Moreover, we have previously established that the sustained production of NO under these conditions is critically dependent on extracellular L-arginine and have further demonstrated that the rate of L-arginine transport is significantly upregulated by pro-inflammatory mediators [1, 50], thus providing a mechanism for sustained substrate supply during enhanced utilization of L-arginine for the generation of NO. Taken together these initial findings suggested that the transport system(s) for L-arginine play a critical role in the production of NO and that biosynthesis of NO under pathophysiological conditions can be regulated at the transporter level.

Our current findings provide the first evidence that rat smooth muscle cells constitutively express transcripts for CAT-1, 2A and -2B, and support a previous study in which we found evidence for high and low affinity transport activity for L-arginine in RASMCs [1]. The current study also demonstrate that mRNA for all three CAT isoforms may be significantly elevated (3-7 fold) by LPS and IFN-γ. Whether expression of CAT proteins reflect the increases in mRNA levels now remain to be established. Our results however contrast with findings in mouse RAW 264.7 macrophages [15] and rat astroglial cells [25], where only CAT-1 is expressed constitutively and CAT-2B is induced following activation with LPS and IFN-γ. In these latter cell types LPS and IFN-γ treatment did not alter CAT-1 mRNA levels. Transcripts of the low affinity CAT-2A have not been detected in RAW 264.7 macrophages (E. I. Closs unpublished observation). Whether CAT-2A is expressed in astroglial cells is unclear, since there are no published reports on this.

The induction pattern of CAT-1, -2A and -2B identified in our experiments is consistent with a previous study in rat cardiac myocytes, where increases in transcripts of CAT-1, 2A and -2B were observed following treatment with IL-1β and IFN-γ [26]. Thus, basal expression and induction of CATs seems to be cell/tissue specific. Moreover, the fact that all three CATs may be expressed in the same cell type raise the question of the relative contribution of each carrier to total arginine transport under physiological and pathophysiological conditions. It is also difficult to distinguish the role individual CATs play in supplying substrate for NO synthesis in activated cells.

Although other reports have examined changes in CAT expression in cultured smooth muscle cells, these studies did not distinguish between CAT-2A and -2B, since the probe used corresponded to a section of the CAT-2 mRNA that is identical in both CAT-2A and -2B [27, 28, 51, 52]. For our studies we generated isoform-specific probes and employed these probes in RNase protection analyses which enabled clear identification of individual rCAT-transcripts. We found that LPS and IFN-γ consistently caused induction of all three CATs and increased L-arginine transport. This contrasts with observations by Gill *et al*. [27], who reported that LPS and IFN-γ were without effect on L-arginine transport in rat smooth muscle cells. Changes in CAT expression in response to LPS and IFN-γ were not investigated.

Induction of L-arginine transport and iNOS expression requires *de novo* protein synthesis. However, our previous studies suggested that these two processes may, at least in part, be regulated differentially [1, 2]. This hypothesis is substantiated by our current findings which demonstrate that, unlike LPS and IFN-γ-induction of NO synthesis, induction of L-arginine transport is largely independent of protein tyrosine kinases. This process is also independent of PKC activation and contrasts with observations in rat peritoneal macrophages [29], human

umbilical vein endothelial cells [30] and the human intestinal epithelial cell line Caco-2 [31] where PKC has been implicated as the key signalling mechanism mediating enhanced L-arginine transport. Although there are cell and maybe species differences, it is worth noting that in some of the studies mentioned above, PKC was implicated simply on data obtained with PKC activators including PMA, or on the use of relatively high concentrations of PKC inhibitors which may not only be non selective but also cytotoxic.

In agreement with published data [5, 7, 9-11] our findings with the protein tyrosine kinase inhibitors confirm that one or more PTKs are critical for induction of NO synthesis in smooth muscle cells. Unlike effects with RO318220, both genistein and herbimycin A caused significant concentration-dependent inhibition of accumulated nitrite at concentrations well below those found to be cytotoxic.

In addition to the above findings, we are also reporting the first evidence that LPS and IFN-γ-induced stimulation of L-arginine transport in rat smooth muscle cells does not involve the p44/42 MAPK cascade but rather the p38 MAPK pathway. Inhibition of transporter activity is accompanied by parallel decreases in the levels of mRNA for rCAT-1, rCAT-2A and rCAT2B. These findings contrast with those reported by Caivano [53] suggesting that LPS and IFN-γ-stimulated L-arginine transport in RAW264 macrophages is partially inhibited both by PD98059 and SB203580. Moreover, as neither compound altered LPS-stimulated CAT-2B mRNA levels in RAW 264 cells (the primary CAT thought to mediate enhanced L-arginine transport in these cells), this suggest that these compounds may regulate arginine transport at a post-transcriptional level. In our studies PD98059 was without effect while SB203580 caused concentration-dependent inhibition of L-arginine transport in RASMCs. In

addition, at 3 μM, SB203580 blocked increases in mRNA levels for all three CATs suggesting a transcriptional regulation of the CAT proteins.

In parallel with its effects on L-arginine transport SB203580 also caused a concentration-dependent inhibition of NO production. Interestingly, and unlike its effects on transport, concentrations of SB203580 in the lower μM range potentiated NO synthesis. Both these effects were reflected by a concomitant decrease in iNOS protein expression at 3 μM and potentiation at 0.1 μM SB203580. Thus, depending on the concentration of SB203580 used NO production induced by LPS and IFN-γ in smooth muscle cells can be potentiated or inhibited.

In addition to our study there are at least four other reports describing the role of p38 MAPK in the induction of iNOS and NO synthesis in various cell lines. As with the controversy surrounding PKC and PTK the resulting effects of p38 inhibition appear to vary in different cell types. Inhibition of p38 in rat glomerular mesangial cells has been reported to cause up-regulation of IL-1β-induced NO synthesis [54] while in rat pancreatic islet [55] and mouse astrocytes [56] the same process is down-regulated following p38 inhibition. Furthermore, p38 MAPK was found to have no effect on induced NO synthesis in mouse RAW264 cells [53] and human colon carcinoma cells [57]. These diverse effects may reflect differences in p38 isoforms that may be differentially expressed and/or activated, and mediate divergent effects in different cells. In this regard, at least five different p38 isoforms have been described and of these the p38δ and p38γ isoforms are thought to be insensitive to inhibition by SB203580 and other pyridinyl imidazoles. [58, 59]. Thus, it is likely that in our system p38α and/or p38β may be the key isoforms associated with enhanced arginine transport and induced iNOS expression.

In addition to the selective expression and/or activation of different p38 isoforms, marked variations in the promoter region of the iNOS gene in different species may, in part, account for the disparity in some of the studies discussed above. Significant differences have been found between the rodent and human promoter of iNOS [60]. In rodents, only 1 kb of the proximal 5' flanking region of the iNOS gene is necessary to confer LPS and cytokine inducibility. In contrast, 3.8 kb of the 5' flanking region of the human iNOS gene exhibit basal but not cytokine-inducible promoter activity. The elements that mediate cytokine induction of the human iNOS promoter are located between -16 kb and -3.8 kb, demonstrating that the gene regulation of iNOS differs markedly in the two species. For the human and rodent CAT genes the promoters have yet to be identified.

In conclusion, we have demonstrated that in RASMC transcripts for the three non-neuronal CATs, CAT-1, CAT-2A and CAT-2B, are constitutively expressed, and that expression of these transcripts is significantly enhanced by LPS and IFN-γ. Moreover, our data also suggest that the upstream signalling mechanisms associated with enhanced L-arginine transport are at least in part distinct from those leading to the induction of iNOS, but converge on the p38 MAPK. Although we cannot be certain without further experiments that our conclusions are definitive, our data provide strong support that the actions of SB203580 result from the inhibition of p38. Other effects of this compound, including the activation of Raf-1 [40] and inhibition of either JNK [41] or COX [42] can be eliminated in our study on the following grounds: firstly, the concentrations over which SB203580 is reported to activate Raf-1 or inhibit JNK are well above those that caused changes in CATs and iNOS activity/expression. Secondly, although SB203580 can inhibit COX, we have shown that PD98059 (a more potent inhibitor of COX than SB203580 [42]) has no effect on either iNOS activity or L-arginine transport in LPS and IFN-γ-stimulated RASMCs. On these basis we conclude that effects observed with SB203580 are specifically related to its ability to inhibit p38 activation. Whether different isoforms of p38 mediate different functions in smooth muscle cells now

remains to be established. There is, however, emerging evidence for divergent functions for different p38 isoforms [61]. Thus a select role for distinct isoforms of p38 could account for the differential regulation of NO synthesis by SB203580 in our study and also explain why p38 is the point of convergence of upstream signalling events that mediate iNOS expression and enhanced L-arginine transport.

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ABBREVIATIONS

CAT: cationic amino acid transporter, IFN-γ: interferon-γ, IL-1β:interleukin-1β, iNOS: inducible nitric oxide synthase, LPS: bacterial lypopolysaccharide, MAPK: mitogen-activated protein kinase, NOS: NO synthase, nt: nucleotides, PCR: polymerase chain reaction, PKC: protein kinase C, PTK: protein tyrosine kinase, RASMC: rat aortic smooth muscle cell, rCAT: rat cationic amino acid transporter, TNF-α: tumor necrosis factor-α.

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FIGURE LEGENDS

Figure 1. Ribonuclease protection analyses of the mRNAs of rCAT-1 (panel i), rCAT-2A (panel ii) and rCAT-2B (panel iii). Total mRNA was prepared either from untreated rat aortic smooth muscle cells (RASMC) (C) or cells treated with 100 µg ml^{-1} LPS and 50 U ml^{-1} IFN-γ (A). The RNAs were hybridized with cRNA probes specific for either rCAT-1, -2A or -2B and for γ-actin. After RNase treatment, the protected RNA fragments (rCAT-1: 230 nt, rCAT-2A: 171 nt, rCAT-2B 153 nt, and γ-actin: 110 nt) were separated on 6 % denaturing polyacrylamid gels. T: t-RNA used as a negative control; P1: undigested probe for rCAT-1; P2A: undigested probe for rCAT-2A; P2B: undigested probe for rCAT-2B; PA: undigested probe for γ-actin (internal control); M: molecular size markers given as nt. This figure is representative of at least three independent blots.
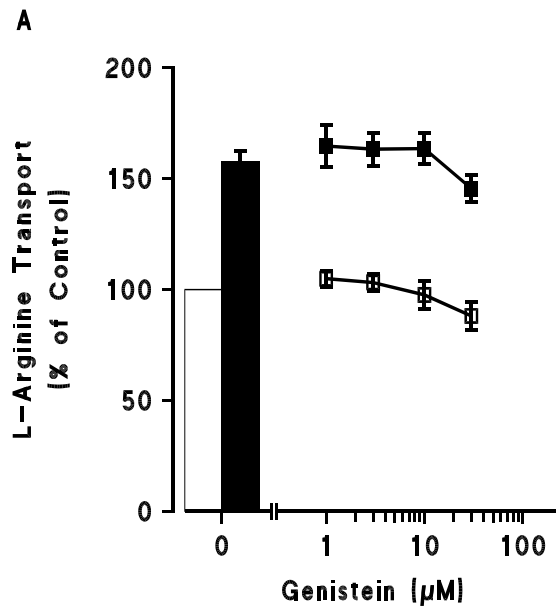
Figure 2: **Concentration dependent effects of genistein and herbimycin A on induced L-arginine transport and nitric oxide synthesis in cultured RASMC.** Cells were pre-incubated with increasing concentrations of genistein (figures 2A and 2B) or herbimycin A (figures 2C and 2D) for 30 min prior to activation with LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}). L-arginine transport into control (□-□) or activated (■-■) cells (Panels A and C) was monitored as described in methods. Results are expressed as percent of values in untreated cells: 100% corresponds to 2.9 ± 0.08 pmol µg protein^{-1} min^{-1}. Accumulated nitrite (Panels B and D) was determined by the Griess reaction. Results are expressed as percent of values from cells treated with LPS and IFN-γ: 100% corresponds to 0.12 ± 0.02 pmol µg protein^{-1} 24 h^{-1}. All values are the mean ± sem of 5 independent experiments with 5 replicates in each. ** denotes statistical significance at p<0.01 and * at p<0.05 when compared to LPS and IFN-γ controls.
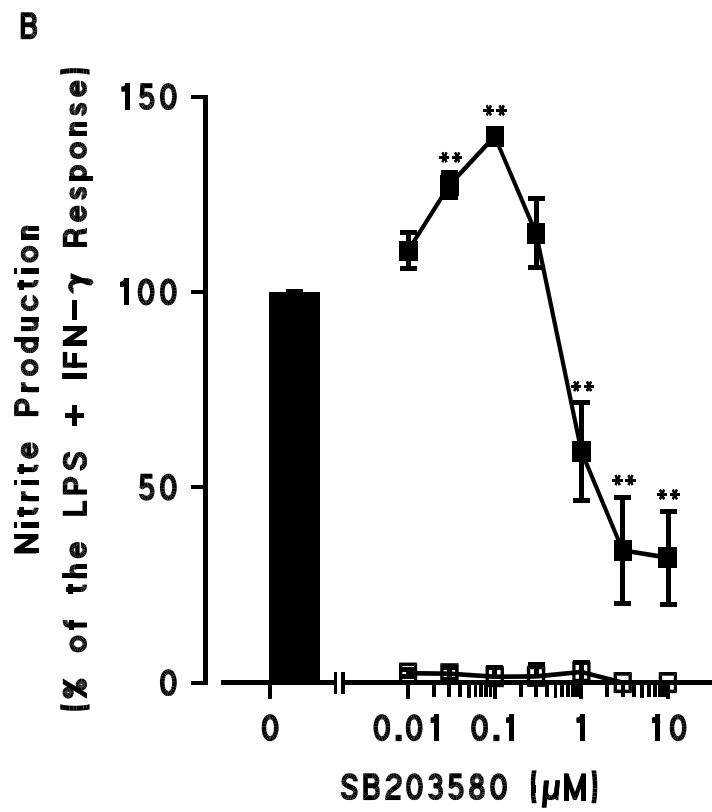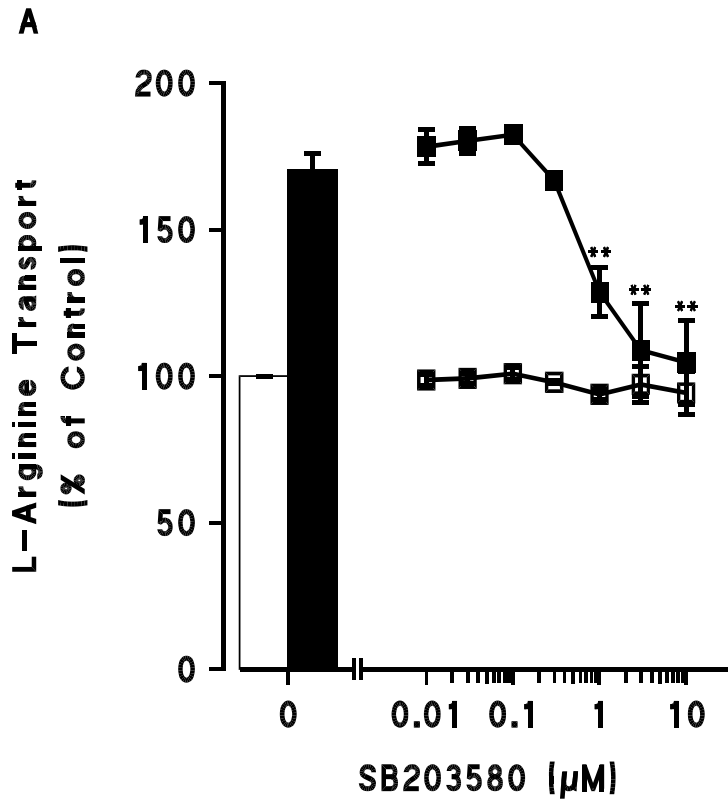
Figure 3: **Concentration dependent effects of SB203580 on induced L-arginine transport and nitric oxide synthesis in cultured RASMC.** Cells were pre-incubated with increasing concentrations of SB203580 for 30 min prior to activation with LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}). L-arginine transport into control (□-□) or activated (■-■) cells (Panel A) was monitored as described in methods. Results are expressed as percent of values in untreated cells: 100% corresponds to 3.4 ± 0.26 pmol µg protein^{-1} min^{-1}. Accumulated nitrite (Panel B) was determined by the Griess reaction. Results are expressed as percent of values from cells treated with LPS and IFN-γ: 100% corresponds to 0.12 ± 0.01 pmol µg protein^{-1} 24 h^{-1}. All values are the mean $\pm$ sem of 5 independent experiments with 5 replicates in each. ** denotes statistical significance at $p<0.01$ when compared to LPS and IFN-γ controls.
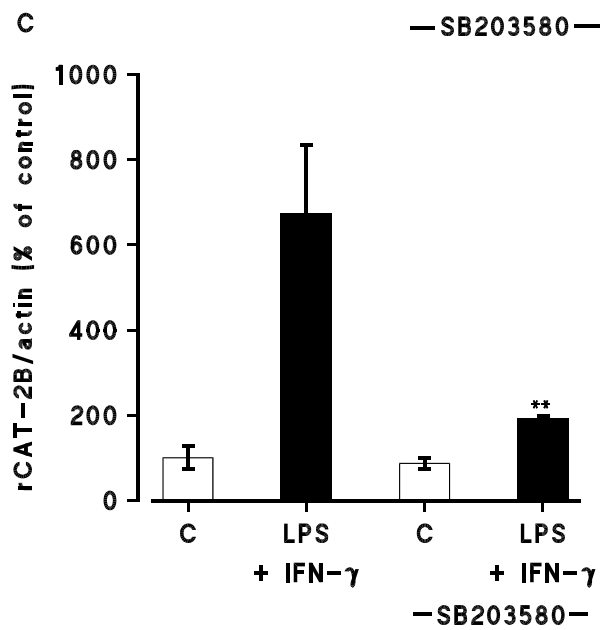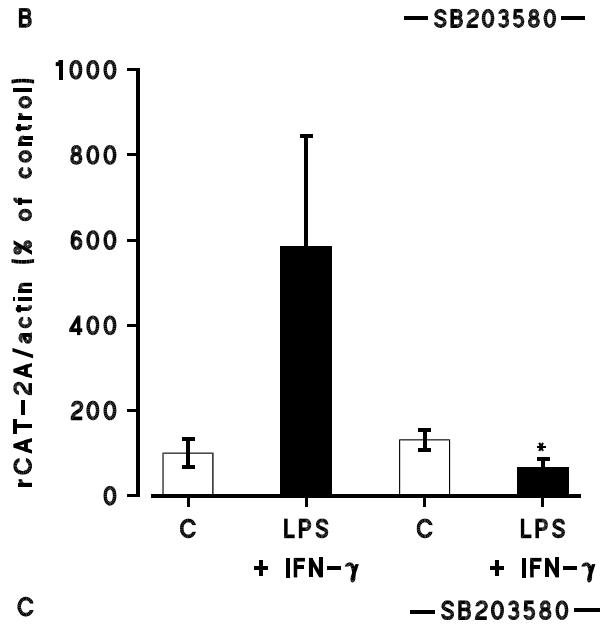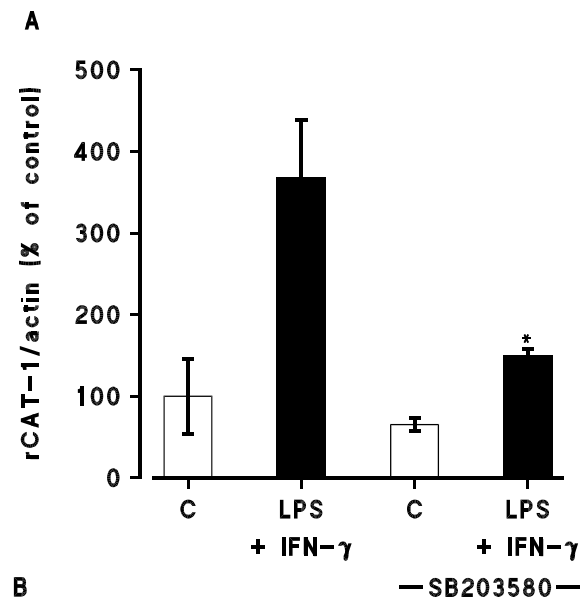
Figure 4: **Effect of SB203580 on basal and LPS and IFN-γ-induced rCAT mRNA expression in cultured RASMC**. Cells were pre-incubated for 30 min with DMEM alone or DMEM containing 3 µM SB203580 prior to a further 18 h incubation with either DMEM alone (C) or with LPS (100 µg ml^{-1}) and IFN-γ (50 U ml^{-1}). RNase protection analyses were performed for rCAT-1 (panel A), rCAT-2A (panel B) and rCAT-2B (panel C) as described in Fig. 1. The density of each band was determined by Phospho-Imager system (BioRad, Munich). The values obtained for each rCAT band were normalized to the corresponding γ-actin band and expressed as percent of the values in untreated cells. Columns represent densitometric analyses from three to four experiments (mean $\pm$ sem). ** denotes statistical significance at $p<0.01$ and * at $p<0.05$ compared to LPS and IFN-γ controls.
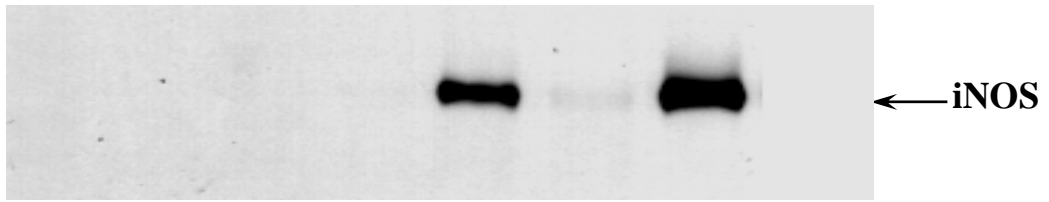
Figure 5: **Effects of SB203580 on LPS and IFN-γ-induced nitric oxide synthase expression in cultured RASMC**. Cells were pre-incubated for 30 min with DMEM alone or DMEM containing either 0.1 μM or 3 μM SB203580 prior to activation with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) for a further 24 h. Lysates obtained were seperated by SDS-PAGE electrophoresis and probed with a selective monoclonal anti-iNOS antibody. Lanes represent1: control, 2**:** 3 μM SB203580 in control cells, 3: 0.1 μM SB203580 in control cells, 4: LPS and IFN-γ activated cells, 5: 3 μM SB203580 in activated cells and 6: 0.1 μM SB203580 in activated cells. This figure is representative of three independent blots.

A

L−Arginine Transport (% of Control) vs Genistein [μM]

B

Nitrite Production (% of the LPS + IFN−γ Response) vs Genistein [μM]

C

L−Arginine Transport (% of Control) vs Herbimycin A [μM]

D

Nitrite Production (% of the LPS + IFN−γ Response) vs Herbimycin A [μM]

A

B

Lanes: **1**   **2**   **3**   **4**   **5**   **6**

← **iNOS**