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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The signalling mechanisms involved in the induction of nitric oxide synthase and L-arginine transport were investigated in bacterial lipopolysaccharide (LPS)- and interferon- γ (IFN- γ)-stimulated rat cultured aortic smooth muscle cells (RASMCs). The expression profile of transcripts for cationic amino acid transporters (CATs) and their regulation by LPS and IFN- γ were also examined. Control RASMCs 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 (MAPKs), but were inhibited by the p38 MAPK inhibitor SB203580, which at 3 μ M 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 inducible NO synthase expression and NO production, potentiating both processes at low micromolar concentrations and inhibiting at concentrations of ≥ 1 μ M. In conclusion, our results suggest that RASMCs 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 appear 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.

Key words: L-arginine, mitogen-activated kinases, nitric oxide, protein kinase C.

INTRODUCTION

We have previously demonstrated that activation of rat cultured aortic smooth muscle cells (RASMCs) 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 unravelling the signalling mechanisms associated with induction of iNOS and L-arginine transport, most of the reported studies have focused mainly on protein tyrosine kinase (PTK) and/or protein kinase C (PKC) signalling, with conflicting conclusions. In this regard, data in the literature present quite contradictory interpretations of the role of PKC and 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 RASMCs [6], mouse astrocytes [7] or human chondrocytes [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 RASMCs 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 : 0.1–0.25 mM) 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

Abbreviations used: CAT, cationic amino acid transporter; COX, cyclo-oxygenase; DMEM, Dulbecco's modified Eagle's medium; IFN- γ , interferon- γ ; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, bacterial lipopolysaccharide; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NOS, NO synthase; PKC, protein kinase C; PTK, protein tyrosine kinase; RASMC, rat aortic smooth muscle cell; mCAT, mouse CAT; rCAT, rat CAT; RT, reverse transcriptase.

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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-1 β (IL-1 β), insulin [26], tumour necrosis factor- 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 and 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 [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 stimulates Raf-1 [40] and inhibits c-Jun N-terminal kinase (JNK) [41] and the cyclo-oxygenase (COX) enzymes [42]. Some of these effects are, however, observed at concentrations of around 10 μ M and above, with the IC_{50} for inhibition of p38 MAPK being around 0.1 μ M. 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, Dorset, U.K.). SB203580 was a gift from Smith-Kline Beecham Pharmaceuticals, U.K. PD98059 was purchased from Calbiochem (Nottingham, U.K.). Monoclonal antibody for iNOS was obtained from Affinity Research Laboratories

(Nottingham, U.K.), 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)_{12–18}, and CAT-specific], *Taq* DNA polymerase, *Taq* polymerase reaction buffer and the 77 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 Super-Script 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% NaHCO₃, penicillin (100 units·ml^{−1}) and streptomycin (100 mg·ml^{−1}). Explants of aorta were left in culture for up to 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 PBS) 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% (v/v) foetal bovine serum either alone or in combination with various compounds for a period of 30 min. Cells were subsequently activated with LPS (100 μ g·ml^{−1}) and IFN- γ (50 units·ml^{−1}). Incubations were terminated after 5–60 min for analysis of MAPK activation, after 24 h for detection of iNOS protein or after 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-diphenyl-2H-tetrazolium 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, Huntsville, AL, U.S.A.). Readings obtained from treated cells were compared with controls.

Determination of nitrite production

The culture medium from cell monolayers used either for transport studies or iNOS Western blotting was removed from

Figure 1 Ribonuclease protection analyses of the mRNAs of rCAT-1 (i), rCAT-2A (ii) and rCAT-2B (iii)

Total mRNA was prepared either from untreated RASMCs (C) or cells treated with $100 \mu\text{g} \cdot \text{ml}^{-1}$ LPS and 50 units $\cdot \text{ml}^{-1}$ IFN- γ (A). The RNAs were hybridized with cRNA probes specific for either rCAT-1 (i), rCAT-2A (ii) or rCAT-2B (iii) 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 polyacrylamide 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 on the left of the gel. This Figure is representative of at least three independent blots.

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°C . Uptake was initiated by adding $50 \mu\text{l}$ of Krebs solution containing $100 \mu\text{M}$ L-[^3H]arginine ($2 \mu\text{Ci} \cdot \text{ml}^{-1}$) to each well and influx was measured over 30 s. Uptake of L-[^3H]arginine was terminated by placing plates on ice and rinsing cells twice with $200 \mu\text{l}$ of ice-cold Krebs solution containing 10 mM unlabelled L-arginine. Cells were lysed with 0.5 M NaOH and protein concentrations were determined using Brilliant Blue G [46]. Radioactivity (d.p.m.) in cell lysates was measured by liquid-scintillation counting. Transport was expressed in $\text{pmol} \cdot \mu\text{g}$ of protein $^{-1} \cdot \text{min}^{-1}$.

Western blot analysis

Western blotting was carried out as described previously [44]. Briefly, cell lysates ($20 \mu\text{g}$ of protein per lane) were separated by SDS/PAGE, transferred on to $0.2 \mu\text{m}$ 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 (6×10 min) and incubated with a 1:10000 dilution of horseradish peroxidase-conjugated goat anti-mouse (for iNOS) or anti-rabbit (for p44/42 or p38 MAPK) IgG for 1 h. After further washing (8×10 min) in STT, immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL; Amersham).

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

Total RNA was isolated by acid guanidinium isothiocyanate/phenol/chloroform (4 M; 25:24:1) 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 of $2 \mu\text{g}$ of total RNA using the SuperScript RT kit and 80 ng of hexamer oligonucleotides with random sequence, as well as $0.5 \mu\text{g}$ of oligo(dT) $_{12-18}$ as primers in a $20 \mu\text{l}$ reaction mixture. The RT-generated cDNAs served as templates in PCR. The sequence of the oligonucleotides used as primers were derived from a region of the mouse CAT-1 (mCAT-1) cDNA (GenBank accession number M 26687) with a high degree of identity with mCAT-2A/2B. The sense oligonucleotide for CAT-1 and CAT-2B was CCGGAATTCTTCGTGGGCTTTGACTGCAT (nts 774–794 of mCAT-1 cDNA, additional *Eco*RI site underlined) and GCGAGATCTGCCTTATCAACAAGTCTTCT for CAT-2A (nt 1051–1071 of mCAT-2A cDNA, additional *Bgl*II site underlined, GenBank accession number L11600). The same antisense oligonucleotide was used for all three CATs: GCGAGATCTAAAAGAAAGGCCATCACAGC (nt 1168–1188 of mCAT-1 cDNA, additional *Bgl*II site underlined). PCR was performed in $100 \mu\text{l}$ of *Taq* polymerase buffer, containing 0.2 mM dNTPs, 1.5 mM MgCl_2 , 2 units of *Taq* polymerase, 50 pmol oligonucleotides and $2 \mu\text{l}$ of 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 $^+$ [rat CAT-1 (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 ^{32}P 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 provided by Dr. Hartmut Kleinert

Figure 2 Concentration-dependent effects of genistein and herbimycin A on induced L-arginine transport and NO synthesis in cultured RSMCs

Cells were pre-incubated with increasing concentrations of genistein (**A** and **B**) or herbimycin A (**C** and **D**) for 30 min before activation with LPS ($100 \mu\text{g} \cdot \text{ml}^{-1}$) and IFN- γ ($50 \text{ units} \cdot \text{ml}^{-1}$). L-Arginine transport into control (□) or activated (■) cells (**A** and **C**) was monitored as described in the Experimental section. Results are expressed as a percentage of values in untreated cells: 100% corresponds to $2.9 \pm 0.08 \text{ pmol} \cdot \mu\text{g protein}^{-1} \cdot \text{min}^{-1}$. Accumulated nitrite (**B** and **D**) was determined by the Griess reaction. Results are expressed as a percentage of values from cells treated with LPS and IFN- γ : 100% corresponds to $0.12 \pm 0.02 \text{ pmol} \cdot \mu\text{g protein}^{-1} \cdot 24 \text{ h}^{-1}$. All values are means $\pm$ S.E.M. of five independent experiments with five replicates in each. Results were statistically significant at $**P < 0.01$ and $*P < 0.05$ when compared with LPS and IFN- γ controls.

(Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany). The pCR- γ -actin-rat was restricted with *AspI* and *EcoRI*, blunted and religated, resulting in pCR- γ -actin-rat- Δ AspI-*EcoRI* that contains only 110 nt of the rat γ -actin cDNA.

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

RNAs were prepared from RSMCs using acid guanidinium isothiocyanate/phenol/chloroform extraction and ribonuclease protection analyses performed as described previously [47]. To generate radiolabelled antisense RNA probes, prCAT-1/41 was linearized with *EcoRI*, prCAT-2A/25 with *XbaI*, prCAT-2B/57 with *BamHI*, and pCR- γ -actin-rat- Δ AspI-*EcoRI* with *BamHI*. *In vitro* transcription using T7 RNA polymerase was performed to generate [α - ^{32}P]UTP-labelled riboprobes of 285 nt for rCAT-1, 339 nt for rCAT-2A, 203 nt for rCAT-2B and 187 nt for rat γ -actin.

Statistics

All values are means $\pm$ S.E.M. 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 ($100 \mu\text{g} \cdot \text{ml}^{-1}$) and IFN- γ ($50 \text{ units} \cdot \text{ml}^{-1}$) resulted in a marked increase in transport of L-[^3H]arginine, which was elevated from a basal value of

Figure 3 Concentration-dependent effects of SB203580 on induced L-arginine transport and NO synthesis in cultured RASMCs

Cells were pre-incubated with increasing concentrations of SB203580 for 30 min before activation with LPS ($100 \mu\text{g} \cdot \text{ml}^{-1}$) and IFN- γ ($50 \text{ units} \cdot \text{ml}^{-1}$). L-Arginine transport into control (□) or activated (■) cells (A) was monitored as described in the Experimental section. Results are expressed as a percentage of values in untreated cells: 100% corresponds to $3.4 \pm 0.26 \text{ pmol} \cdot \mu\text{g protein}^{-1} \cdot \text{min}^{-1}$. Accumulated nitrite (B) was determined by the Griess reaction. Results are expressed as a percentage of values from cells treated with LPS and IFN- γ : 100% corresponds to $0.12 \pm 0.01 \text{ pmol} \cdot \mu\text{g protein}^{-1} \cdot 24 \text{ h}^{-1}$. All values are the means $\pm$ S.E.M. of five independent experiments with five replicates in each. **Statistical significance at $P < 0.01$ when compared with LPS and IFN- γ controls.

3.2 ± 0.27 to $5.1 \pm 0.18 \text{ pmol} \cdot \mu\text{g of protein}^{-1} \cdot \text{min}^{-1}$ 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 (Figure 1). Moreover, levels of all three transcripts were significantly elevated after 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 ($\leq 30 \mu\text{M}$ and $\leq 1 \mu\text{M}$ for genistein and herbimycin A respectively) (Figures 2A and 2C). L-Arginine transport was reduced by $19 \pm 3\%$ and $22 \pm 7\%$ in the presence of $30 \mu\text{M}$ genistein or $1 \mu\text{M}$ herbimycin A respectively. At higher concentrations, both compounds caused a significant reduction in

Figure 4 Effect of SB203580 on basal and LPS- and IFN- γ -induced rCAT mRNA expression in cultured RASMCs

Cells were pre-incubated for 30 min with DMEM alone or DMEM containing $3 \mu\text{M}$ SB203580 before a further 18 h incubation with either DMEM alone (C) or with LPS ($100 \mu\text{g} \cdot \text{ml}^{-1}$) and IFN- γ ($50 \text{ units} \cdot \text{ml}^{-1}$). RNase protection analyses were performed for rCAT-1 (A), rCAT-2A (B) and rCAT-2B (C) as described in Figure 1. The density of each band was determined by the Phospho-Imager system (Bio-Rad, Munich, Germany). The values obtained for each rCAT band were normalized to the corresponding γ -actin band and expressed as a percentage of the values in untreated cells. Columns represent densitometric analyses from three to four experiments (means $\pm$ S.E.M.). Statistical significance at ** $P < 0.01$ and * $P < 0.05$ compared with LPS and IFN- γ controls.

total cell protein and in the viability of the remaining adherent cells, which presumably reflects 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 ($30 \mu\text{M}$) or herbimycin A ($1 \mu\text{M}$) (results 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 (Figures 2B and 2D). At $30 \mu\text{M}$, genistein reduced stimulated nitrite levels by $77 \pm 5\%$ (Figure 2B). Similarly, nitrite production

Figure 5 Effects of SB203580 on LPS- and IFN- γ -induced NOS expression in cultured RASMC

Cells were pre-incubated for 30 min with DMEM alone or DMEM containing either 0.1 μ M or 3 μ M SB203580 before activation with LPS (100 μ g $\cdot$ ml $^{-1}$) and IFN- γ (50 units $\cdot$ ml $^{-1}$) for a further 24 h. Lysates obtained were separated by SDS/PAGE and probed with a selective monoclonal anti-iNOS antibody. Lanes: 1, control; 2, 3 μ M SB203580 in control cells; 3, 0.1 μ M SB203580 in control cells; 4, LPS- and IFN- γ -activated cells; 5, 3 μ M SB203580 in activated cells; and 6, 0.1 μ M SB203580 in activated cells. This Figure is representative of three independent blots.

was inhibited by $73 \pm 6\%$ in the presence of 1 μ M herbimycin A (Figure 2D). Daidzein, the inactive analogue of genistein, was without effect at concentrations of up to 30 μ M (results not shown).

Effects of PKC 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 μ M. Furthermore, RO318220 (1 μ M) did not alter mRNA levels of either rCAT-1, -2A or -2B (results not shown). Concentrations above 1 μ M 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 with genistein or herbimycin A, in inhibiting NO synthesis, reducing the latter by $\sim 22\%$ at non-cytotoxic concentrations (≤ 1 μ M).

Role of p44/42 MAPK in induction of L-arginine transport, CAT expression and NO synthesis

Neither L-arginine transport nor induced CAT expression (results not shown) was affected by PD98059 (1–100 μ M), which selectively blocks the upstream activator of p44/42 MAPK, MAPK kinase. In parallel experiments, PD98059 (1–100 μ M) 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 RASMCs, with maximum phosphorylation occurring 30 min after exposure to LPS and IFN- γ . More importantly, phosphorylation of both p44 and p42 MAPK was virtually abolished in the presence of 30 μ M PD98059 (results not shown).

Role of p38 MAPK 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 μ M (Figure 3A). The basal rate of transport in control cells was not significantly altered over the same concentration range (0.01–10 μ M). Similarly, SB203580 markedly attenuated the induced, but not basal, expression of CAT-1, -2A and -2B, (Figure 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 μ M (Figure 3B). Interestingly, inhibitions were only observed at concentrations of 1–10 μ M. At 0.01–0.1 μ M, SB203580 potentiated LPS- and IFN- γ -induced nitrite production, increasing the latter by $40 \pm 2\%$ at 0.1 μ M. 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 Figure 5 the antibody recognized 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 0.1 μ M SB203580 and virtually abolished at 3 μ M.

DISCUSSION

One of the fundamental actions of pro-inflammatory mediators, including LPS and cytokines, is the induction of iNOS. 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 demonstrates that mRNA for all three CAT isoforms may be significantly elevated (3–7-fold) by LPS and IFN- γ . Whether expression of CAT proteins reflects the increases in mRNA levels remains 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 after 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 work). 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 after treatment with IL-1 β 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 raises 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 PTKs. 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 results 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 PTK 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 rCAT-2B. These findings contrast with those reported by Caivano [53], who suggested 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 suggests that these compounds may regulate arginine transport at a post-transcriptional level. In our studies, PD98059 was without effect, whereas SB203580 caused concentration-dependent inhibition of L-arginine transport in RASMCs. In addition, at 3 μ M, 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 micromolar range potentiated NO synthesis. Both these effects were reflected by a concomitant decrease in iNOS protein expression at 3 μ M and potentiation at 0.1 μ M 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-1 β -induced NO synthesis [54], whereas in rat pancreatic islets [55] and mouse astrocytes [56] the same process

is down-regulated after 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 p38 δ and p38 γ isoforms are thought to be insensitive to inhibition by SB203580 and other pyridinylimidazoles [58,59]. Thus, it is likely that in our system p38 α and/or p38 β 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 RASMCs, 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: first, 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 bases, 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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