Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages

Matilde Caivano*

MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK

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Abstract Bacterial lipopolysaccharide (LPS) in the presence of interferon gamma (IFNγ) stimulates the synthesis of the cationic amino acid transporter 2B (CAT-2B) and inducible nitric oxide synthetase (iNOS) in RAW264 macrophages, which are thought to underlie the increased rate of arginine uptake into these cells and its conversion to nitric oxide, respectively. Here I demonstrate that the LPS- and IFNγ-induced increase in arginine uptake into RAW264 cells is partially suppressed in the presence of PD 98059, partially suppressed in the presence of SB 203580, and completely inhibited by both drugs. In contrast, the LPS- and IFNγ-induced synthesis of CAT-2B mRNA and iNOS protein is unaffected by PD 98059 and SB 203580. The results indicate that the MAPK/ERK and SAPK2/p38 cascades are both rate-limiting for LPS- and IFNγ-stimulated arginine uptake, but not for iNOS synthesis. They also suggest that PD 98059 and SB 203580 suppress CAT-2B synthesis at a post-transcriptional level.

Key words: Lipopolysaccharide; Inflammation; Arginine transport; Nitric oxide synthetase; PD 98059; SB 203580

1. Introduction

Nitric oxide (NO) is an important mediator of signal transduction in a variety of physiological systems (reviewed in [1]). However, during bacterial infection, NO is generated in particularly large amounts by monocytes, macrophages and other cells of the immune system. In these situations it is thought to be cytostatic/cytotoxic for invading bacteria, although it may also play other roles in regulating the release of interleukin-1, a proinflammatory mediator that is also produced by macrophages and monocytes during infection [2].

Nitric oxide is produced from arginine by nitric oxide synthetase (NOS) and three NOS isoforms are found in mammalian cells. Type I NOS and type III NOS are calcium-dependent enzymes that are expressed in a cell-specific manner and their activation produces the NO that mediates most of the messenger functions of this molecule. In contrast, type II NOS has a ubiquitous tissue distribution and is only expressed during cell-mediated immune responses. For this reason it is usually referred to as inducible NOS (iNOS) (reviewed in [3,4]).

During bacterial infection, the lipopolysaccharide (LPS) component of the bacterial cell wall interacts with the CD14 receptor in the plasma membranes of monocytes and macrophages [5] and activates signal transduction pathways that trigger the synthesis of the iNOS protein and ensuing production of NO. However, the formation of NO in vivo depends not only on the activity of iNOS, but also on the availability of its substrate arginine. Arginine is taken up into cells by cationic amino acid transporter (CAT) systems in the plasma membrane that also catalyse the transport of lysine and ornithine (reviewed in [6]). Three such transporters have been identified termed CAT-1, CAT-2A and CAT-2B. CAT-1 and CAT-2B are the species present in macrophages and monocytes. In RAW264 macrophages, CAT-1 is expressed in the absence of LPS and presumably accounts for the basal rate of arginine uptake, while the LPS-induced production of CAT-2B may underlie the increased rate of arginine uptake observed under these conditions. Like the LPS-induced production of iNOS, the LPS-induced synthesis of CAT-2B is greatly stimulated if interferon gamma (IFNγ) is also present in the culture medium.

The importance of CAT transporters and arginine uptake for the production of NO is well documented. For example, LPS/IFNγ-induced NO production does not occur in J774 macrophages (despite the induction of iNOS) if arginine is excluded from the culture medium or in the presence of lysine (which inhibits CAT-dependent arginine uptake by a competitive mechanism) [7]. Similarly, NO production is only sustained in brain cells if arginine is present in the extracellular cerebrospinal fluid [8–10]. In cardiac endothelial (CMEC) cells, glucocorticoids almost completely suppress LPS/IFNγ-stimulated NO production, even though the synthesis of iNOS declines by only 20% [11]. The effect of glucocorticoids in these cells appears to be mediated (at least in part) by inhibition of CAT-1 and CAT-2B synthesis, resulting in suppression of arginine uptake.

The signal transduction pathways that trigger the induction of iNOS and CAT-2B in macrophages (or any cell) have not yet been identified. In this paper, we have addressed this question by studying the effects of two drugs, PD 98059 and SB 203580, that are specific inhibitors of particular protein kinase cascades. PD 98059 prevents the activation of MAP kinase kinase-1 [12], the enzyme that activates the MAP kinase family members MAPK1/ERK1 and MAPK2/ERK2 in the 'classical' MAP kinase cascade, while SB 203580 inhibits the MAP kinase family members SAPK2a/p38 and SAPK2b/p38β2 [13] which are components of a distinct protein kinase cascade (reviewed in [14]). Here I show that LPS/IFNγ-stimulated arginine uptake is completely suppressed in the presence of both PD 98059 and SB 203580, whereas the LPS/IFNγ-stimulated induction of iNOS is unaffected by these drugs.

2. Materials and methods

2.1. Materials

LPS was a generous gift from Dr. John Lee (SmithKline Beecham, PA, USA), IFNγ, TRizol reagent and reagents for cell culture were

*Fax: (44) (1382) 223778.

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purchased from Life Technologies (Paisley, UK), PD 98059 and SB 203580 from Calbiochem (Nottingham, UK) and affinity-purified polyclonal rabbit anti-iNOS antibody from Transduction Laboratories (Lexington, KY, USA). Antibiotics, anisomycin, L-N^G-monomethylarginine (NMMA), L-lysine, L-arginine and the Griess reagent were obtained from Sigma (Poole, UK), L-[2,3,4,5-^{3}H]arginine hydrochloride from Amersham International (Bucks, UK) and the Access RT-PCR System from Promega (Southampton, UK).

2.2. Cell culture, stimulation and cell lysis

A murine macrophage cell line, RAW 264 (European Cell Culture Collection, Wilts, UK) was maintained in a 95% air/5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) heat-inactivated foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B. For measurements of iNOS and protein kinase activities, the macrophages were plated at a density of 2×10^6 cells per 6-cm plate, the day before stimulation. For measurements of arginine uptake, the macrophages were added to 24-well plates at a density of 2×10^5 cells per well. Two hours before stimulation, the medium was removed and replaced with 2 ml of DMEM. The cells were then stimulated with LPS (100 ng/ml) plus IFNγ (10 U/ml). Where indicated, SB 203580 (10 μM) or PD 98059 (50 μM) were added 1 h before the stimulation.

After stimulation, the medium was collected, immediately snap-frozen in liquid nitrogen and stored at −80°C. These samples were used to analyse nitrite production. The cells were then solubilised in 200 μl of ice-cold lysis buffer (50 mM Tris-acetate pH 7.0, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 2 μM microcystin, 1 mM benzamidine, 0.2 mM phenymethylsulphonyl fluoride, 5 μg/ml leupeptin, 0.1% (v/v) 2-mercaptoethanol). Lysates were centrifuged for 5 min at $13\,000\times g$ and the supernatants removed. After measurement of the protein concentration [15], the samples were quick-frozen in liquid nitrogen and stored in aliquots at −80°C until analysis.

2.3. Immunoprecipitation and assay of protein kinases

MAP kinase-activated protein kinase-1 (MAPKAP-K1, also known as RSK) was immunoprecipitated from cell lysates (50 μg protein) with an antibody raised against the peptide RNQSPVLEPVGRSTLAQRRGIKK corresponding to residues 605–627 of the murine MAPKAP-K1b (RSK2) isoform. This antibody immunoprecipitates MAPKAP-K1a (RSK1) as well as MAPKAP-K1b [12]. The immunoprecipitates were washed and assayed at 30°C as described [12]. One unit of MAPKAP-K1 activity was defined as that amount which catalyses the incorporation of 1 nmol of phosphate into the peptide [G245,G246]S6-(218–249) in 1 min. MAPKAP-K2 was immunoprecipitated in an identical manner using an antibody raised against the peptide MTSALATMRVDYEQIK corresponding to residues 356–371 of the human protein. This antibody immunoprecipitates MAPKAP-K3 as well as MAPKAP-K2. MAPKAP-K2 was assayed as described [13] and one unit was that amount of enzyme which catalyses the incorporation of 1 nmol of phosphate into the peptide KKLNRTLSVA in 1 min.

2.4. Assay for nitrite

Frozen cell culture medium was thawed, centrifuged for 5 min at $13\,000\times g$ to remove cellular debris, and the nitrite content of the supernatant used to measure NO production. The supernatant was incubated with the Griess reagent and the concentration of the resulting chromatophore determined spectrophotometrically at 540 nm [11]. The amount of nitrite was calculated by comparison with standard sodium nitrite solutions.

2.5. Assay for L-arginine uptake

Cells were stimulated for the times indicated in the figure legends. The medium was then removed and the cells washed with 1 ml of Dulbecco's phosphate-buffered saline (pH 7.4) comprising 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4 plus 0.1% (w/v) glucose. Cells were then incubated for 10 min at 37°C in 200 μl of the same medium containing L-[^{3}H]arginine (100 μM, 50.4 Ci/mmol; Amersham). In selected experiments, 10 mM L-lysine was also present. Incubations were terminated by immediately washing the cells three times with 1 ml ice-cold Dulbecco's phosphate-buffered saline containing 10 mM unlabelled L-arginine. Cells were solubilised in 0.5% (w/v) Nonidet P-40 and 0.1% (w/v) SDS and the radioactivity in the extract was quantified by liquid scintillation counting [16].

2.6. Reverse transcriptase polymerase chain reactions

Total RNA was prepared from RAW264 cells using the TRizol reagent according to the manufacturer's protocol. Total RNA was reverse-transcribed using Promega AMV reverse transcriptase (5 U/ml) with the specific antisense oligonucleotides CCAGAAACAACCTAATCTGGTATA and TGTCACCAGCATCGAGAGGA (for MCAT-1) and TTGTGTTACGAGCAGCCCAA and AGAATGACGGCAGCGCAGAG (for MCAT-2B). Conditions for PCR amplification of the resulting first-strand DNA template were 94°C denaturing, 60°C annealing, 68°C extension, 30 cycles using thermostable Tfl DNA polymerase (5 U/ml), and 1 mM $MgSO_4$. The PCR products of MCAT-1 (329 bp) and MCAT-2B (314 bp) were subcloned into pCR-2.1 Topovector (Invitrogen) and the sequences confirmed using an applied Biosystems model 373A DNA sequencer.

3. Results

3.1. Stimulation of the MAPK/ERK cascades and the SAPK2/p38 pathway by LPS in RAW264 macrophages

In order to examine the effect of LPS on the MAPK/ERK

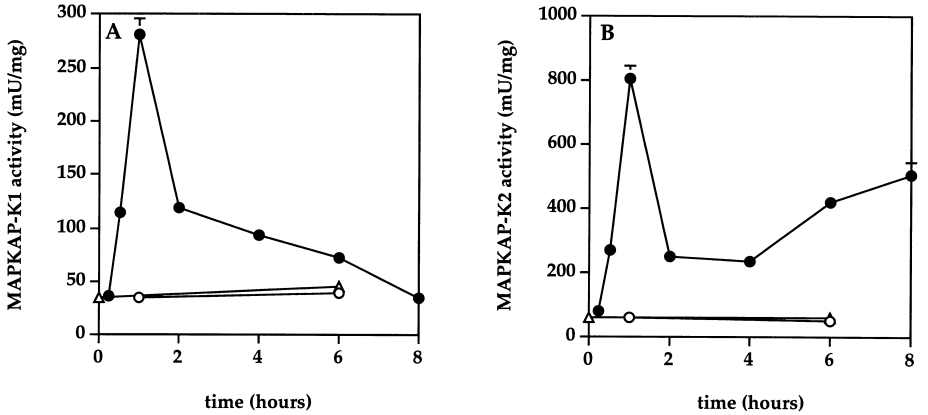

Fig. 1. Activation of MAPKAP-K1 and MAPKAP-K2 by LPS in RAW264 macrophages. Macrophages were incubated for 1 h in the presence (open circles) or absence (closed circles) of 50 μM PD 98059 (A) or 10 μM SB 203580 (B), then stimulated for the times indicated with LPS (100 ng/ml) plus IFNγ (10 U/ml) in the continued presence or absence of the inhibitors. MAPKAP-K1 activity (A) or MAPKAP-K2 activity (B) was then measured after immunoprecipitation from the lysates. The results are presented as means ± S.E.M. for two determinations from two separate dishes. Similar results were obtained in three further experiments.

and SAPK2/p38 cascades we studied the ability of this endotoxin to activate MAPKAP-K1 (a downstream target of MAPK/ERK) and MAPKAP-K2 (a physiological substrate for SAPK2/p38) (reviewed in [14]). LPS was found to activate both MAPKAP-K1 and MAPKAP-K2 with a half-life of just over 30 min. In both cases activation peaked after 1 h and then declined sharply. However, in the case of MAPKAP-K2, a second wave of activation occurred after 4 h (Fig. 1). The activation of MAPKAP-K1 was completely suppressed by PD 98059, while the activation of MAPKAP-K2 was prevented by SB 203580 (Fig. 1).

Although the experiments in Fig. 1 were performed in the presence of both LPS and IFNγ in order to achieve maximal induction of iNOS and CAT-2B, omitting IFNγ did not affect the activation of MAPKAP-K1 or MAPKAP-K2 up to 1 h, and IFNγ alone did not activate either protein kinase (data not shown).

3.2. *The MAPK/ERK and SAPK2/p38 pathways are not rate-limiting for the induction of iNOS in RAW264 macrophages*

Consistent with previous results (reviewed in [17]), LPS/IFNγ induced the appearance of iNOS activity, as judged by the appearance of nitrite in the medium (Fig. 2A), as well as the iNOS protein itself (Fig. 2B). Activity (Fig. 2A) and protein (data not shown) both started to appear after 2 h and reached a maximal level after about 6 h (Fig. 2A). The LPS-induced synthesis of iNOS protein (Fig. 2B) or activity (Fig. 2C) was unaffected by PD 98059 and/or SB 203580 under conditions where the activation of MAPKAP-K1 or MAPKAP-K2 was blocked completely (Fig. 1). Nitrite production was prevented by either NMMA, a specific inhibitor of nitric oxide synthetases, or by the protein synthesis inhibitor anisomycin (Fig. 2C).

Similar results were obtained if IFNγ was omitted, except that the induction of iNOS protein and activity was much smaller (data not shown). IFNγ alone induced hardly any nitrite production (open triangle in Fig. 2A).

3.3. *The MAPK/ERK and SAPK2/p38 cascades both contribute to the LPS-induced stimulation of arginine transport in RAW264 macrophages*

The production of nitric oxide is dependent not only on the presence of iNOS, but also on the availability of the substrate arginine (see Section 1). We confirmed that arginine uptake was stimulated by LPS/IFNγ with similar kinetics to the induction of iNOS (Fig. 3). A 2.5-fold stimulation was routinely observed after 8 h (Fig. 3A). Interestingly, and in contrast to iNOS induction, PD 98059 and SB 203580 both inhibited the LPS-stimulated uptake of arginine. Either inhibitor added alone caused a partial inhibition, but there was essentially complete inhibition when the cells were exposed to both inhibitors (Fig. 3B). Similar results were obtained if IFNγ was omitted, except that the stimulation of arginine uptake was smaller (not shown). IFNγ alone did not stimulate arginine uptake significantly (open triangles in Fig. 3A).

The LPS-stimulated increase in arginine transport was prevented by anisomycin (Fig. 3B), indicating that it resulted from increased synthesis of an arginine transporter or another protein required for transporter activity. At least two cationic amino acid transporters (CAT-1 and CAT-2B) are present in macrophages. CAT-1 is present in unstimulated cells and its

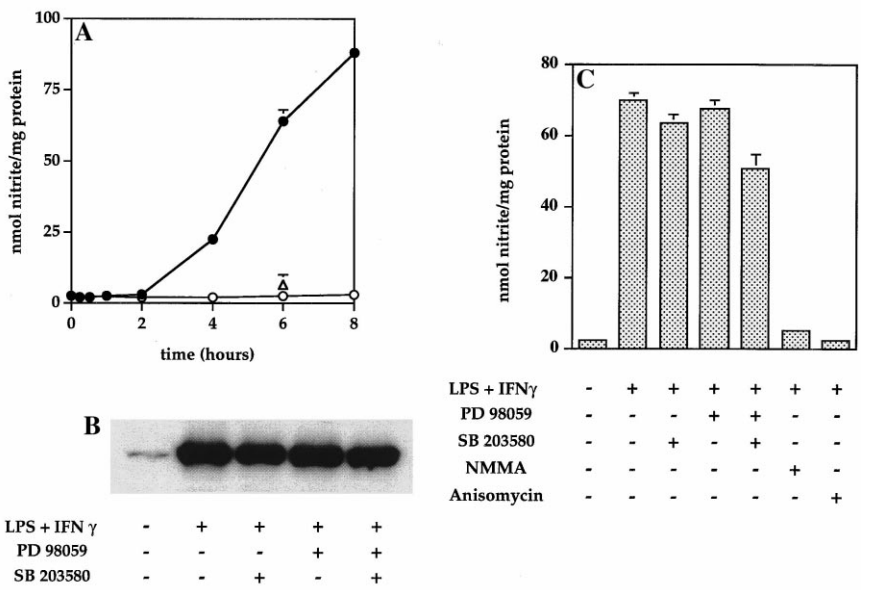

Fig. 2. Effect of different inhibitors on the LPS-induced synthesis of iNOS protein and activity in RAW264 macrophages. A: Macrophages were incubated for the times indicated in the presence (closed circles) or absence (open circles) of LPS (100 ng/ml) plus IFNγ (10 U/ml). Nitrite accumulation in the medium was then measured as described in Section 2.4. B: Macrophages were incubated for 1 h in the presence or absence of PD 98059 (50 μM) and/or SB 203580 (10 μM), then stimulated for 6 h in the absence or presence of LPS plus IFNγ in the continued presence or absence of the inhibitors. Cell lysate (30 μg protein) was denatured in SDS and electrophoresed on a 10% SDS/polyacrylamide gel. After transfer to a nitrocellulose membrane, the samples were immunoblotted with anti-iNOS antibody (1.0 μg/ml). Antibodies were visualised with the enhanced chemiluminescence detection system (Amersham, Bucks, UK). C: Macrophages were incubated for 1 h in the presence or absence of PD 98059 (50 μM) and/or SB 203580 (10 μM), or NMMA (250 μM) or anisomycin (10 μg/ml), then stimulated for 6 h in the absence or presence of LPS plus IFNγ in the continued presence or absence of the inhibitors. Nitrite accumulation in the medium was then measured as in A. The results for A and C are presented as the mean ± S.E.M. for two determinations from two separate dishes. Similar results were obtained in three further experiments.

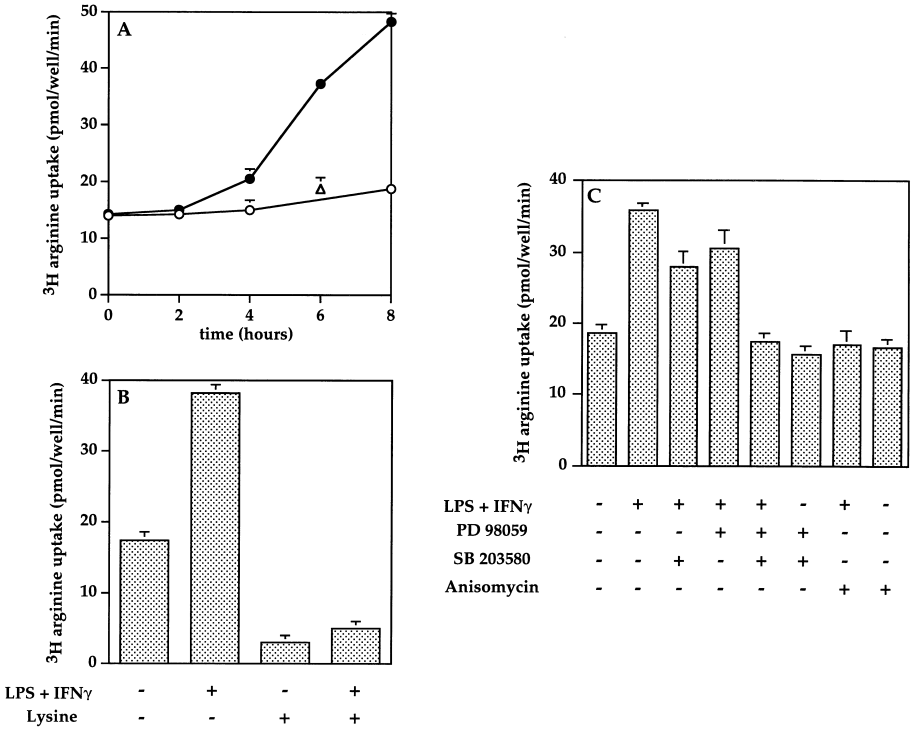

Fig. 3. Effect of different inhibitors on basal and LPS-stimulated arginine uptake in RAW264 macrophages. A: Macrophages were incubated for the times indicated in the presence (closed circles) or absence (open circles) of LPS (100 ng/ml) plus IFNγ (10 U/ml). Arginine uptake into the cells was then measured as described Section 2.5. B: Same as A except that the macrophages were incubated for 6 h in the absence or presence of 10 mM L-lysine. C: Macrophages were incubated for 1 h in the presence of PD 98059 (50 μM) and/or SB 203580 (10 μM), or anisomycin (10 μg/ml), then stimulated for 6 h in the absence or presence of LPS plus IFNγ and in the continued presence or absence of the inhibitors. Arginine uptake was then measured as in A. The results in A, B and C are presented as the mean ± S.E.M. for three determinations. Similar results were obtained in three further experiments.

level is unaffected by LPS/IFNγ. In contrast, CAT-2B is absent in unstimulated cells and only induced in the presence of LPS/IFNγ (see Section 1). These results are confirmed by the experiment presented in Fig. 4, which also shows that preincubation of macrophages with PD 98059 plus SB 203580 had no effect on the level of mRNA encoding either CAT-1 or CAT-2B in the presence or absence of LPS/IFNγ. This result is considered further in Section 4.

4. Discussion

The production of nitric oxide by macrophages depends on the availability of arginine, as well as on the activity of iNOS, but which process is rate-limiting is likely to depend on hormonal and nutritional status, as well as on other factors (see Section 1). In this paper, I have shown that LPS-stimulated arginine uptake into RAW264 macrophages is prevented by a combination of the drugs PD 98059 and SB 203580 (Fig. 3C), and at concentrations where they are thought to be specific inhibitors of the activation of MAP kinase kinase-1 and the activity of SAPK2/p38, respectively (reviewed in [14]). This is consistent with the strong activation of both the 'classical' MAP kinase cascade and the SAPK2a/p38 cascade by LPS (Fig. 1). The 30% reduction in LPS-stimulated nitric oxide

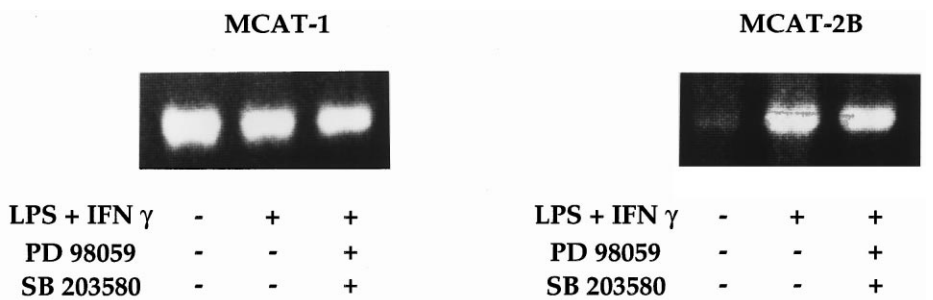

Fig. 4. Effect of PD 98059 and SB 203580 on the transcription of the cationic amino acid transporters CAT-1 and CAT-2B. Macrophages were incubated for 1 h in the presence or absence of PD 98059 (50 μM) plus SB 203580 (10 μM) then stimulated for 6 h in the absence or presence of LPS plus IFNγ, and in the continued presence or absence of the inhibitors. Total RNA was extracted from the cells and the level of mRNA encoding CAT-1 (A) and CAT-2B (B) was determined by using the reverse transcriptase polymerase chain reaction as described in Section 2.6. Similar results were obtained in two further experiments.

accumulation in the presence of both PD 98059 and SB 203580 (Fig. 2C) may reflect the contribution of arginine transport to overall nitric oxide production under the conditions studied.

The LPS-induced stimulation of arginine transport was prevented by L-lysine, indicating that the endotoxin was stimulating a cationic amino acid transporter. Arginine uptake was also suppressed by anisomycin, indicating a requirement for de novo protein synthesis (Fig. 3B). Since the mRNA encoding the CAT-2B transporter is induced by LPS, this suggested that LPS may stimulate arginine transport by inducing the synthesis of the CAT-2B protein. However, PD 98059 plus SB 203580 did not affect the LPS-stimulated CAT-2B mRNA production (Fig. 4). Thus, if CAT-2B synthesis underlies the stimulation of arginine uptake, these drugs must be exerting their effects at a post-transcriptional level. Nevertheless, it cannot be excluded that PD 98059 and SB 203580 exert their effects by another mechanism. For example, they may suppress the synthesis of another protein that is essential for CAT-2B activity, or another (as yet unidentified) CAT isoform.

In contrast to arginine transport, the induction of iNOS was unaffected by PD 98059 and/or SB 203580 (Fig. 2B), demonstrating that neither the MAP kinase cascade nor the SAPK2a/p38 pathway is rate-limiting for this process in RAW264 macrophages. However, in primary glial cultures, LPS-induced iNOS synthesis was recently reported to be partially suppressed by either PD 98059 and SB 203580 and almost completely prevented in the presence of both compounds [18]. Thus the protein kinases that are rate-limiting for iNOS gene transcription appear to vary from cell to cell. The signal transduction pathway(s) which mediates the LPS-stimulated induction of iNOS in RAW264 macrophages remains to be identified. It could involve one or more of the other MAP kinase family members whose activity or activation is unaffected by PD 98059 or SB 203580, such as SAPK1/JNK, SAPK3/ERK6, SAPK4 or SAPK5/ERK5 (reviewed in [14]). Nevertheless it is clear that, despite the similar kinetics of LPS-stimulated arginine transport and iNOS induction, different signal transduction pathways are involved in activating both processes.

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