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P38 mitogen-activated protein kinase inhibitor SB203580 has a bi-directional effect on iNOS expression and NO production

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Abstract

In the present study, the mediator role of p38 kinase, a member of the mitogen-activated protein kinase (MAPK) family, was studied in lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in J774 mouse macrophages and T-84 human colon epithelial cells. Two pyridinyl imidazole inhibitors of p38 MAPK, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole (SB203580) and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole (SB202190), stimulated NO production at low drug concentrations, maximal stimulation occurring at 1 μM drug concentration. In contrast, higher concentrations inhibited NO production, which was >90% at 30 μM drug concentration. The bi-directional effect was found in both cell types tested. Negative control compound SB202474, which is structurally related but does not inhibit p38, did not stimulate NO production but inhibited it at 30 μM concentration. A chemically different p38 inhibitor 2-methyl-4-phenyl-5-(4-pyridyl)oxazole (SC68376) had only a stimulatory action on NO production. Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR) analysis of iNOS showed that both stimulatory and inhibitory effects of SB203580 occurred at the level of iNOS expression. SB203580 did not alter lipopolysaccharide-induced NF-κB activation as detected by electrophoretic mobility shift assay (EMSA). The data show that pyridinyl imidazoles SB203580 and SB202190 have a bi-directional effect on NO production through iNOS pathway depending on the drug concentration used. The inhibitory effect was unrelated to inhibition of p38 MAPK, whereas the stimulatory effect is most likely mediated by p38 MAPK dependent mechanism, suggesting a novel mechanism for regulation of iNOS expression, which is common for murine and human cells.

Keywords: iNOS (inducible nitric oxide synthase); Lipopolysaccharide; p38 MAP (mitogen activated protein) kinase

1. Introduction

Nitric oxide (NO) serves as a cytotoxic and cytostatic agent and modulates immune response in inflammatory diseases (Moilanen et al., 1999). High amounts of NO are produced from L-arginine by inducible nitric oxide synthase (iNOS), which is expressed in cells in response to various inflammatory cytokines and bacterial products, e.g. lipopolysaccharide (MacMicking et al., 1997).

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine specific kinases, which together with their upstream activators form distinct but partially overlapping signalling pathways, which are activated by various inflammatory and other stimuli (Su and Karin, 1996). We have recently shown that extracellular signal-regulated kinases p42/p44, which belong to the MAPK family, are involved in the up-regulation of lipopolysaccharide-induced iNOS expression (Lahti et al., 2000). Lipopolysaccharide-induced production of pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1 is known to be controlled by p38, another kinase of the MAPK family (Su and Karin, 1996). Therefore, we wanted to investigate whether p38 MAPK participates also in the regulation of lipopolysaccharide-induced iNOS expression and NO production.

Pyridinyl imidazole SB203580 is a widely used inhibitor of p38 MAP kinase. It inhibits the activity of p38 MAPK at IC_{50} of ~ 0.3–0.6 μM (Cuenda et al., 1995) by binding in ATP site (Young et al., 1997). In the present study, SB203580 was employed as pharmacological tool to investigate the role of p38 MAPK in the lipopolysaccharide-induced NO production in two different cell lines. J774 murine macrophages

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were chosen because the mechanisms of NO production have been widely studied in this cell line. To see whether the results obtained in J774 cells are applicable to human cells, T-84 human colon epithelial cell line was used. NO production and iNOS expression in T-84 cells has been recently characterised in our laboratory (Lähde et al., 2000).

The results show that pyridinyl imidazole SB203580 has a bi-directional, concentration-dependent effect on lipopolysaccharide-induced iNOS expression and NO production and only the enhancing effect is likely to be related to inhibition of p38.

2. Materials and methods

2.1. Materials

Reagents were obtained as follows: 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole (SB203580) and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole (SB202190) (Alexis, Läufelfingen, Switzerland), 4-ethyl-2-(4-methoxyphenyl)-5-(4-pyridyl)-imidazole (SB202474) and 2-methyl-4-phenyl-5-(4-pyridyl)oxazole (SC68376) (Calbiochem, La Jolla, CA, USA), ammonium pyrrolidinedithiocarbamate (PDTC) (Tocris Cookson, Bristol, UK), rabbit polyclonal mouse and human iNOS and goat anti-rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), PhosphoPlus® p38 MAP kinase (Thr180/Tyr182) antibody kit (New England BioLabs, Beverly, MA, USA), recombinant human transforming growth factor beta (TGF-β) and anti-TGF-β neutralizing antibody (R&D systems, Minneapolis, MN, USA). All other reagents were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

J774 macrophages and human T84 colon epithelial cells were cultured at 37 °C, 5% CO_2 atmosphere, in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax-I containing 5% (T84 cells) or 10% (J774 cells) heat inactivated foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (all from Gibco, Paisley, Scotland, UK). Cells were seeded in 24-well plates for nitrite measurements and in 96-well plates for cell viability assay and in 6-well plates for Western blot and reverse transcriptase polymerase chain reaction (RT-PCR) and grown for 72 h to confluency prior to experiments. The medium was replaced with a fresh medium containing the tested compounds and incubated for 30 min before stimulation with lipopolysaccharide.

2.3. Nitrite assays

At indicated time points, the culture medium was collected for nitrite measurement, which was used as a measure of NO production. Culture medium (100 μl) was incubated with 100 μl of Griess reagent (0.1% napthalethylenediamine dihydrochloride, 1% sulphanilamine, 2.5% H_3PO_4) and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

2.4. Cell viability assay

Cell viability was tested using Cell Proliferation Kit II (Boehringer Mannheim, Indianapolis, IN, USA). Cells were incubated with the tested compounds for 20 h before addition of sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (final concentration 0,3 mg/ml) and *N*-methyl dibenzopyrazine methyl sulfate (1.25 mM). Then cells were further incubated for 4 h and the amount of accumulated formazan in growth medium was assessed spectrophotometrically.

2.5. Preparation of cell lysates

At indicated time points, cells were rapidly washed with ice cold phosphate-buffered saline (PBS) and solubilised in cold lysis buffer containing 10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM sodiumpyrophosphate and 10 mM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (12 500 rpm, 15 min) and supernatants were mixed 1:4 with SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, 1% glycerol, 2% sodium dodecyl sulphate (SDS), 0.025% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. Protein content of the samples was measured by the Coomassie blue method (Bradford, 1976).

2.6. Western blotting

Protein (20 μg) was loaded on SDS-polyacrylamide electrophoresis gel (8% and 12% separating gels for iNOS and p38, respectively) and electrophoresed for 1.5 h at 110 V in buffer containing 95 mM Tris–HCl, 960 mM glycine and 0.5% SDS. After electrophoresis, the proteins were transferred to Hybond ECL™ nitrocellulose membrane (Amersham, Buckinghamshire, UK) with semi-dry blotter at 2.5 mA/cm^2 for 30 min in buffer containing 25 mM Tris-base, 192 mM glycine and 20% methanol. After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 °C overnight. Thereafter, the membrane was washed 4 × with TBS/T for 5 min, incubated with secondary antibody in the blocking solution for 0.5 h at room temperature and washed 4 × with TBS/T for 5 min. Specific bands were detected by ECL™ detection system (Amersham) and the membrane was exposed to film. The densitometric measurements were carried out with Sigmagel software.

2.7. RNA extraction and RT-PCR

At indicated time points, cells were rapidly washed with ice cold PBS and cells were homogenised using QIAshredder™ (QIAGEN, Santa Clarita, CA). Extraction of total RNA was carried out with RNeasy® kit for isolation of total RNA (QIAGEN). Synthesis of cDNA and subsequent amplification of cDNA were performed with RobusT RT-PCR kit (Finnzymes, Helsinki, Finland). The primers for RT-PCR were as follows: 5′-TCACTGGGACAGCACAGAAT-3′, 5′-TGTGTCTGCAGATGTGCTGA-3′ (forward and reverse mouse iNOS primer) resulting a 510-bp product, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (forward and reverse mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer) resulting a 306-bp product, 5′-CCATG-GAACATCCCAAATAC-3′, 5′-TCTGCATGTACTTCAT-GAAGG-3′ (forward and reverse human iNOS primer) resulting a 375-bp product, 5′-TGACTGACTACCTCAT-GAAGATCCTCACCG-3′, 5′-CCACGTCACACTTCAT-GATGGAGTTG-3′ (forward and reverse human β-actin primer) resulting a 309-bp product. RT-PCR reaction conditions were as follows: 250 ng of total RNA in reaction mixture containing 1.5 mM Mg^{2+} was subjected to RT-reaction at 48 °C for 30 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min and 22 cycles for mouse iNOS and mouse GAPDH; 1 mM Mg^{2+}, RT-reaction at 45 °C for 30 min, amplification 94 °C for 35 s, 60 °C for 2 min, 72 °C for 2 min, 42 and 30 cycles for human iNOS and β-actin, respectively. Products were analysed on 1.5% agarose gel containing ethidium bromide and visualised with UV-light.

2.8. Preparation of nuclear extracts

J774 macrophages and T84 colon epithelial cells were seeded on 10-cm dishes and grown for 72 h to confluency prior to experiments. Cells were incubated in the presence of the tested compounds for 30 min. Thereafter, the cells were rapidly washed with ice cold PBS and solubilised in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiotreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1 mM EGTA, 1 mM Na_2VO_4, 1 mM NaF). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 °C, 15000 rpm for 10 s. Nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiotreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF) and incubated for 20 min on ice. Nuclei were vortexed 30 s and nuclear extracts were obtained by centrifugation at 4 °C, 15000 rpm for 2 min. Protein content of the nuclear extracts was measured by Coomassie blue method (Bradford, 1976).

2.9. Electrophoretic mobility shift assay (EMSA)

Single-stranded oligonucleotides (5′-AGTTGAGGGG-ACTTTCCCAGGC-3′, 3′-TCAACTCCCCTGAAAGG-GTCCG-5′) (Amersham Pharmacia Biotech) containing the NF-κB-binding sequences were annealed and 5′[^{32}P]-end labelled with DNA 5′-End Labelling Kit (Boehringer Mannheim). For binding reactions, 10 μg of nuclear extract was incubated in 20 μl of total reaction volume containing 0.1 mg/ml (poly)dI–dC, 1 mM dithiotreitol, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 200 mM KCl, 10% glycerol 20 min at room temperature. ^{32}P-labeled oligonucleotide probe of 0.2 ng was added and the reaction mixture was incubated for 10 min. Protein/DNA complexes were separated from DNA-probe by electrophoresis on a native 4% polyacrylamide gel. Gel was dried and autoradiographied using intensifying screen at −70 °C.

2.10. Statistics

Results are expressed as mean ± standard error of mean (S.E.M.). When indicated, statistical significance was calculated by analysis of variances supported by Dunnett adjusted significance levels. Differences were considered significant at $P < 0.05$.

3. Results

3.1. SB203580 and SB202190 have a concentration-dependent bi-directional effect on lipopolysaccharide-induced NO production

We tested the effects of SB203580 and SB202190 on lipopolysaccharide-induced NO production in J774 mouse macrophages and T-84 human colon epithelial cells. Nitrite accumulated in culture medium was used as a measure of NO production. Both compounds had a clear concentration-dependent effect on lipopolysaccharide-induced NO production in both cell lines (Fig. 1). Low concentrations of SB203580 and SB202190 increased NO production and maximal stimulatory effect was achieved at 1 μM drug concentrations. At concentrations higher than 1 μM, the enhancing effect was reduced and NO production was inhibited at concentrations above 5 μM as compared with control. At the highest concentration used (30 μM), SB203580 and SB202190 reduced NO production by more than 90%.

We continued by testing the effect of SB202474 as a negative control compound in T-84 cells. SB202474 is structurally related to SB203580, but does not inhibit p38 (Lee et al., 1994). We tested also the effects of another p38 MAPK inhibitor SC68376 (Guan et al., 1997), which is structurally different from SB203580. Lipopolysaccharide-induced NO production was reduced by 1 μM and 30 μM SB202474 by 26% and 87%, respectively (Fig. 2). Inter-

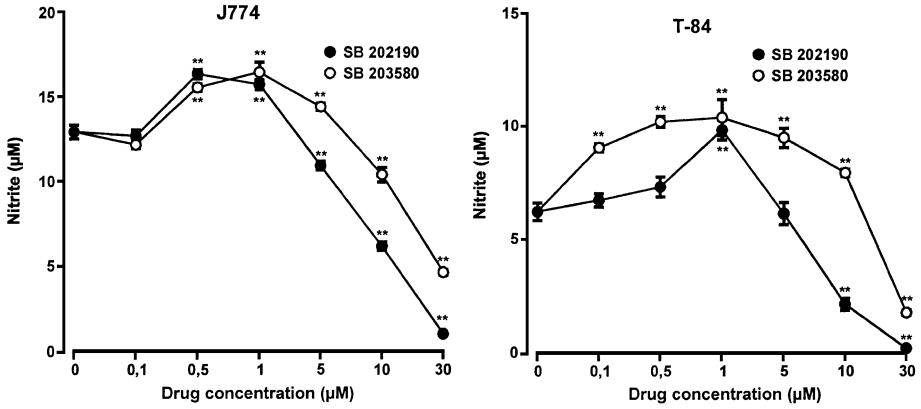

Fig. 1. Effect of SB203580 and SB202190 on NO production in lipopolysaccharide-stimulated J774 macrophages and T-84 epithelial cells. Cells were incubated for 30 min with increasing concentrations of tested compounds before stimulation with 10 ng/ml (J774) and 1 μg/ml (T-84) of lipopolysaccharide. After 24 h, nitrite accumulation in growth medium was measured as a marker of NO production. Values are mean ± S.E.M., $n=6$. $^{**}P<0.01$ as compared with the respective control. Where error bars are not visible, they are within symbols.

estingly, SC68376 (1–30 μM) had only an enhancing effect and at 30 μM concentration, it increased NO production by 37%.

3.2. The potentiating and inhibitory effects of SB203580 on NO production occur at different phases of lipopolysaccharide induction

The time dependency of the effects of SB203580 on NO production is shown in Fig. 3. J774 and T-84 cells were exposed to SB203580 30 min before lipopolysaccharide stimulation or at different time points after lipopolysaccharide. Concentrations causing maximal enhancement (1 μM) and maximal inhibition (30 μM) were used. When added to culture medium 30 min before lipopolysaccharide, 1 μM of SB203580 enhanced lipopolysaccharide-induced NO production by 70% and 110% in J774 and T-84 cells, respectively. The potentiating effect of 1 μM SB203580 was further increased when it was added into the culture medium 1–2 h after lipopolysaccharide stimulation. This effect was maximal when SB203580 was added 1 h after lipopolysaccharide stimulation producing a 120% and 160% increase in NO production in J774 and T-84 cells, respectively. The potentiating effect was reduced from the maximal value when SB203580 was added >2 h after lipopolysaccharide stimulation, but was still present even when SB203580 was added as late as 6 h after lipopolysaccharide. Inhibitory effect of 30 μM SB203580 was maximal when it was added into the culture medium 30 min before lipopolysaccharide, producing an 82% and 66% inhibition of NO production in J774 and T-84 cells, respectively. This inhibitory effect was reduced in a time-dependent manner when SB203580 was added after lipopolysaccharide and no inhibitory effect was seen in J774 cells if SB203580 was added >4 h after lipopolysaccharide stimulation (Fig. 3).

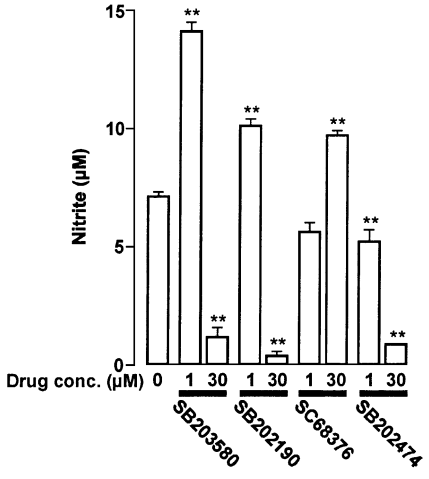

Fig. 2. Effect of SB203580, SB202190, SB202474 and SC68376 on NO production in lipopolysaccharide-stimulated T-84 epithelial cells. Cells were incubated with 1 μg/ml of lipopolysaccharide in the presence or absence of 1 and 30 μM concentrations of tested compounds for 24 h. Nitrite accumulation in growth medium was measured as a marker of NO production. Values are mean ± S.E.M., $n=6$. and $^{**}P<0.01$ as compared with the respective control.

3.3. Active p38 MAPK is present in cells several hours after lipopolysaccharide stimulation

The results showing that low concentrations of SB203580 had a clear effect even when added 4 h after lipopolysaccharide stimulation, suggest that there is an active p38 MAPK present in cells hours after lipopolysaccharide stimulation. Therefore, we studied the activation kinetics of p38 after lipopolysaccharide stimulation by using antibody recognizing the active Thr180/Tyr182 phosphory lated p38 MAPK. Equal loading of samples was controlled by using antibody against p38.

In resting cells, there were no detectable levels of active p38 MAPK. After lipopolysaccharide stimulation, p38 MAPK was activated in 15 min in both cell types and the activity was maximal in 30 min (Fig. 4). After 30 min,

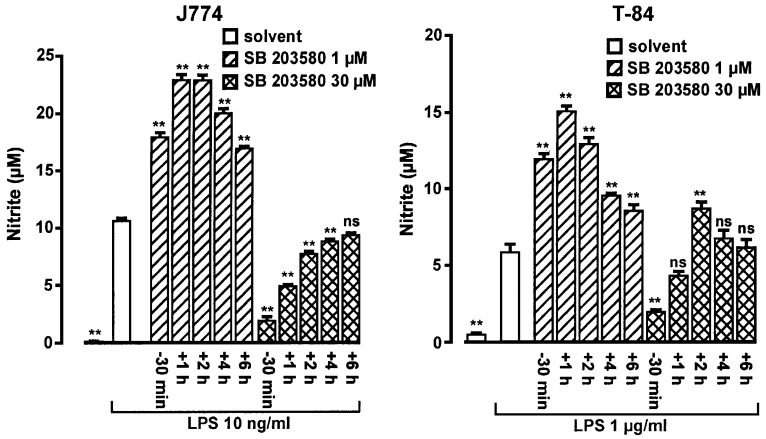

Fig. 3. The effect of SB203580 on NO production in cultures of J774 and T-84 cells when added before or at various time points after lipopolysaccharide (LPS). Nitrite concentrations in the growth medium were measured after 24-h incubation with lipopolysaccharide as a marker of NO production. Values are mean ± S.E.M. ($n = 6$). $^{**}P < 0.01$ and $^{ns}P > 0.05$ as compared with the respective control.

the level of active p38 decreased. However, lower amounts of active p38 MAPK were still present up to 5 h after lipopolysaccharide stimulation as compared with unstimulated cells (Fig. 4).

3.4. Expression of iNOS is altered by SB203580

We used Western blot analysis to investigate whether changes in NO production by SB203580 were due to altered iNOS protein expression in the cells. SB203580 (1 μM) increased iNOS protein expression in both cell types, which is consistent with the increase in NO production (Fig. 5). In contrast, 30 μM SB203580 reduced iNOS expression. iNOS expression was significantly inhibited by protein synthesis inhibitor cycloheximide and by PDTC, an inhibitor of nuclear factor κB (NF-κB), which were used as controls.

RT-PCR was used to analyse iNOS mRNA expression. Constitutively expressed GAPDH (J774) and β-actin (T-84) mRNAs were used as controls. Consistently with the Western blot data, 1 μM SB203580 potentiated and 30 μM SB203580 inhibited lipopolysaccharide-induced iNOS mRNA expression in J774 macrophages when measured 10 h after stimulation with lipopolysaccharide (Fig. 6). Expression of iNOS mRNA was increased also in T-84 cells treated with 1 μM SB203580, when measured at 10 h time point. Expression of iNOS mRNA was reduced by 30 μM SB203580 at both time points. In both cell types, PDTC totally inhibited iNOS mRNA expression.

3.5. SB203580 has no effect on NF-κB DNA binding activity

NF-κB is an essential transcription factor in lipopolysaccharide-induced iNOS expression (Kim et al., 1997), and therefore, we investigated whether the effects of SB203580 result from change in NF-κB activity. We used gel shift assay

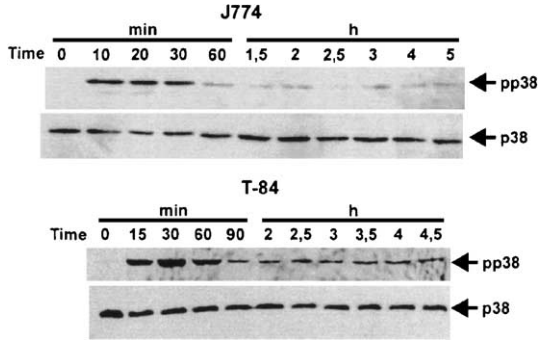

Fig. 4. The effect of lipopolysaccharide on p38 MAPK activation. J774 macrophages and T84 epithelial cells were stimulated with lipopolysaccharide (1 μg/ml). Incubations were terminated at indicated time points. Two parallel immunoblots were run from same cell lysates using antibodies against the Thr180/Tyr182 phosphorylated, i.e. activated p38 (pp38) and total p38.

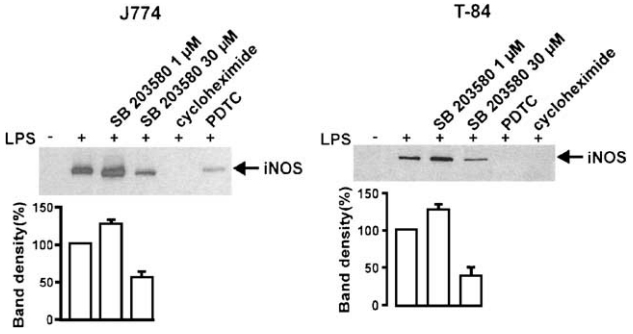

Fig. 5. The effect of SB203580 on lipopolysaccharide-induced iNOS protein expression. J774 macrophages and T-84 epithelial cells were incubated with SB203580 (30 μM), cycloheximide (1 μg/ml) or PDTC (100 μM) 30 min before and with SB203580 (1 μM) 1 h after stimulation with lipopolysaccharide (LPS) 10 ng/ml (J774) and 1 μg/ml (T-84). Incubations were terminated after 24 h. Immunoblots were run using an antibody against mouse (J774) and human (T-84) iNOS. Densitometric measurements were made from immunoblots of five (J774) and four (T-84) different cell preparations. Values are mean ± S.E.M.

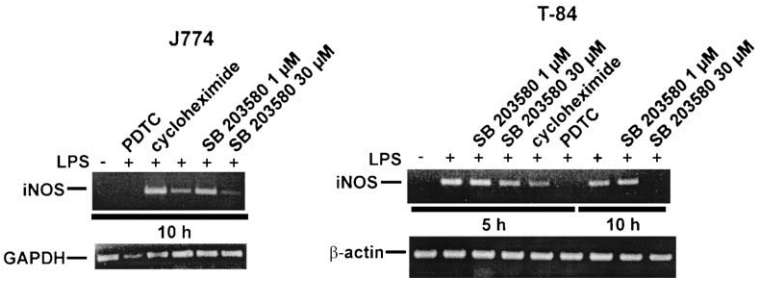

Fig. 6. The effect of SB203580 on lipopolysaccharide-induced iNOS mRNA expression. J774 macrophages and T-84 epithelial cells were incubated with SB203580 (30 μM), cycloheximide (1 μg/ml) or PDTC (100 μM) 30 min before and with SB203580 (1 μM) 1 h after stimulation with lipopolysaccharide (LPS) 10 ng/ml (J774) and 1 μg/ml (T-84). Incubations were terminated at indicated time points. RT-PCR was carried out using primers specific for mouse iNOS and GAPDH (J774) and human iNOS and β-actin (T-84). The data are representative of three separate experiments, which gave similar result.

using an oligonucleotide sequence containing NF-κB-binding sequence to test the effect of SB 203580 on NF-κB activation. In lipopolysaccharide-treated cells NF-κB DNA binding activity was increased as compared with untreated cells (Fig. 7). DNA binding complexes consisted of p50/p65 heterodimer and p50/p50 homodimer in supershift assay using antibodies against p50 and p65 (data not shown). In both cell types, low and high concentration of SB203580 had no effect on NF-κB DNA binding activity. In contrast, NF-κB inhibitor PDTC reduced lipopolysaccharide-induced NF-κB activity.

3.6. Stimulatory effect of SB203580 on NO production is not mediated by TGF-β

TGF-β regulates iNOS expression and NO production by several mechanisms (Vodovotz et al., 1993). TGF-β has also been shown to activate p38 (Hanafusa et al., 1999). Therefore, we hypothesised that 1 μM SB203580 could block auto-/paracrine effect of TGF-β produced by stimulated cells and thus increase NO production. We tested the effect of recombinant human TGF-β and anti-TGF-β neutralizing antibody on lipopolysaccharide-induced NO production in T-84 cells. None of the tested compounds alone stimulated NO production (Fig. 8). In lipopolysaccharide-treated cells, 1 μM SB203580 and 1 ng/ml TGF-β increased NO production by 50% and 105%, respectively. However, cells treated with combination of SB203580 and TGF-β produced equal amount of NO as cells treated with TGF-β alone. Furthermore, anti-TGF-β antibody had no significant effect on NO production induced by lipopolysaccharide or further stimulated by SB203580.

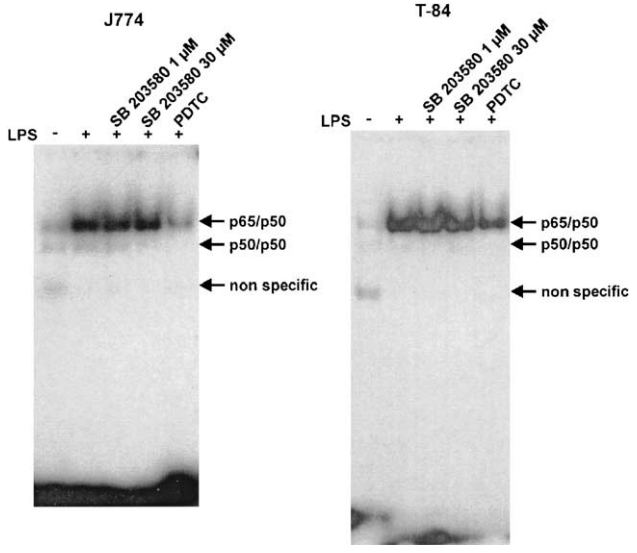

Fig. 7. The effect of SB203580 on lipopolysaccharide (LPS) induced NF-κB activation. J774 macrophages (A) and T-84 epithelial cells (B) were incubated with SB203580 (1 and 30 μM) or PDTC (100 μM) 30 min before stimulation with lipopolysaccharide (10 ng/ml in (A) and 1 μg/ml in (B)). Incubations were terminated 30 min after addition of lipopolysaccharide. NF-κB DNA binding activity was analysed by EMSA. The data are representative of three separate experiments, which gave similar result.

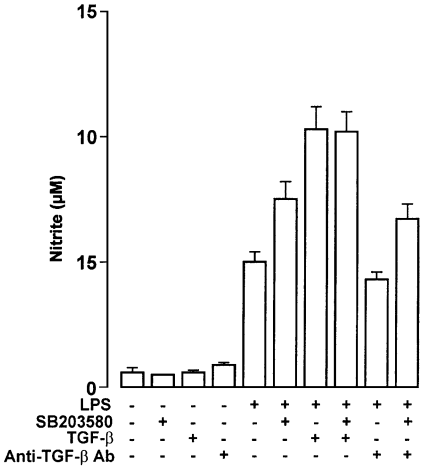

Fig. 8. Effect of SB203580, TGF-β and anti-TGF-β antibody on NO production in lipopolysaccharide-stimulated T-84 epithelial cells. Cells were incubated with or without 1 μg/ml lipopolysaccharide (LPS) and in the presence of different combinations of 1 μM SB203580, 1 ng/ml TGF-β and 1 μg/ml anti-TGF-β Ab for 24 h. Nitrite accumulation in growth medium was measured as a marker of NO production. Values are mean ± S.E.M., $n = 6$.

4. Discussion

We have shown in this study that p38 MAPK inhibitors SB203580 and SB202190 have a concentration-dependent bi-directional effect on lipopolysaccharide-induced NO production in murine J774 macrophages and T-84 human colon epithelial cells. Furthermore, we have found that both the stimulatory and inhibitory effects of SB203580 occur at the level of iNOS expression, by a NF-κB-independent mechanism.

Stimulatory effect of p38 MAPK inhibitors SB203580 and SB202190 on lipopolysaccharide-induced NO production was maximal at ~1 μM drug concentration. This concentration is high enough to efficiently inhibit p38 MAPK activity (IC_{50} ~ 0.3–0.6 μM) (Cuenda et al., 1995; Jiang et al., 1996). Interestingly, the stimulatory effect was further enhanced if the compound was added 1–4 h after lipopolysaccharide stimulation. The inhibitory effect of these two p38 MAPK inhibitors on lipopolysaccharide-induced NO production occurred at >10 μM drug concentrations. Inhibitory effect was strongest when cells were pre-treated with SB203580 before lipopolysaccharide stimulation and the effect was lost when the compound was added >2 h after lipopolysaccharide. Structurally related negative control compound SB202474, which does not inhibit p38, decreased NO production with equal potency as SB203580 and SB202190, but had no stimulatory effect. Furthermore, another structurally unrelated p38 inhibitor SC68376 (IC_{50} ~ 5 μM) also stimulated lipopolysaccharide-induced NO production at 30 μM concentrations. SC68376 has a 10-fold lower potency as p38 MAPK inhibitor as compared with SB203580, which is in agreement with the result that SC68376 was less potent to stimulate lipopolysaccharide-induced NO production than SB203580. These results suggest that the stimulation and inhibition of lipopolysaccharide-induced NO production by SB203580 and SB202190 are two distinct effects and occur at different phases of cell activation after lipopolysaccharide. Results also suggest that inhibition of NO production by high concentrations of SB203580 and SB202190 is unrelated to inhibition of p38 MAPK.

The time response curves suggest that SB203580 has distinct overlapping inhibitory and stimulatory effects on NO production. That would explain the finding that, when SB203580 (1 μM) was added to culture medium 1–2 h after lipopolysaccharide, the stimulatory effect was at it highest. At those time points, the inhibitory effect was already resolved and therefore an enhanced stimulatory effect was seen. The result also shows that the effective time frame of the stimulatory effect is later than that of the suppressive action on the lipopolysaccharide-induced iNOS expression. Western blot of activated p38 showed that, though the peak of activation was observed 30 min after the addition of lipopolysaccharide, increased levels remained for hours, which is consistent with findings by Baldassare et al. (1999) and Caivano (1998). Thus, even though the stimulatory effect was present when SB203580 was added hours after lipopolysaccharide stimulation, it may well result from inhibition of p38 MAPK.

Several members of p38 MAPKs have been characterised so far, p38 α, p38 β, p38 β2, p38 γ and p38 δ. These isoforms have differences in respect to response to activating stimuli, substrate specificity and sensitivity to inhibition by SB203580 (Kumar et al., 1997). It is possible that stimulation of iNOS expression results from an inhibition of one p38 isoform and inhibition of iNOS expression from inhibition of another isoform that is less sensitive to SB203580. However, the negative control compound SB202474 also had an inhibitory effect on NO production suggesting that inhibitory effect of SB203580 is not mediated by different p38 isoforms.

Western blot analysis showed increased iNOS protein expression when cells were treated with 1 μM SB203580. Accordingly, increased levels of iNOS mRNA were detected 10 h after lipopolysaccharide stimulation in SB203580 treated J774 macrophages and T-84 epithelial cells. These data show that stimulatory effect of SB203580 on iNOS expression occurs at the level of mRNA accumulation and might result from increased iNOS gene transcription or increased mRNA stability. However, further studies are required to reveal the exact mechanism behind this effect.

Full inhibitory effect of 30 μM SB203580 required that it was added to cells before lipopolysaccharide. This result suggests that high concentration of SB203580 blocked an essential signalling pathway for NO production in an early phase of induction. Inhibition of NO production was not due to reduced cell viability as measured by XTT-formazan assay (data not shown). Western blot and RT-PCR analysis of iNOS protein and mRNA showed that decreased NO production resulted from reduced iNOS expression. Decreased levels of mRNA were detected in SB203580 (30 μM) treated cells 5 h after lipopolysaccharide stimulation. This suggests that SB203580 inhibits iNOS expression by reducing transcription of iNOS gene. However, 30 μM SB203580 had no effect on lipopolysaccharide-induced nuclear translocation or DNA binding activity of NF-κB, a transcription factor which is essential for iNOS expression. Our results concerning the effects of SB203580 on NF-κB are in concordance with observations made by others (Zhang et al., 2000; Meja et al., 2000). However, opposite results have been reported by Chen and Wang (1999) and Jeon et al. (2000). They found that SB203580 reduced lipopolysaccharide-induced NF-κB DNA binding activity. In addition, inhibition of NF-κB-dependent transcription by SB203580, without an effect on NF-κB nuclear translocation or DNA binding activity has also been reported (Baldassare et al., 1999). Reasons for these discrepancies are currently unknown, but may be explained by cell type-specific signalling mechanisms involved in iNOS induction. Promoter regions of both murine and human iNOS genes contain binding sites for transcription factors other than NF-κB (Xie et al., 1993; Lowenstein et al., 1993; Chu et al.,

1998). Activities of interferon regulatory factor-1 (Faure et al., 1999), signal transducer and activator of transcription 1 (Kovarik et al., 1999), activator protein-1 (Garcia et al., 1998) and nuclear factor for interleukin-6 (Baldassare et al., 1999), which are present in iNOS promoter, have been shown to be affected by SB203580. Although reduced transcription is a more likely explanation for the effect of 30 μM SB203580, the possibility of reduced iNOS mRNA stability (Chen et al., 1998) cannot be excluded on the basis of the present results.

Though SB203580 is considered to be a specific inhibitor of p38 MAPK, there are reports suggesting that at higher concentrations, it also inhibits c-Jun N-terminal kinases (Clerk and Sugden, 1998), tyrosine kinase lck, c-Raf (Hall-Jackson et al., 1999) and cyclooxygenase-1 and -2 (Börsch-Haubold et al., 1998). These effects occur at considerably higher concentrations than required to inhibit p38 MAPK. SB203580 concentrations required to inhibit iNOS expression in the present study may be high enough to result in an inhibition of these effectors.

In response to lipopolysaccharide, macrophages and epithelial cells produce various cytokines (Sweet and Hume, 1996; Jung et al., 1995), which regulate NO production. Thus, the stimulatory effect of 1 μM SB203580 may result from regulation of auto-/paracrine cytokine loop. We tested the effect of TGF-β as a possible cytokine mediating this effect. Our results show that, although TGF-β had an effect on lipopolysaccharide-induced NO production, it did not mediate the effect of 1 μM SB203580. Another interesting possibility is the involvement of the newly discovered suppressor of cytokine signalling (SOCS) family of proteins, which function as feedback inhibitors of cytokine signalling (Alexander et al., 1999). SOCS3 is expressed in macrophages in response to lipopolysaccharide in p38 dependent mechanism and may be involved in the regulation of iNOS expression by SB203580 (Bode et al., 1999).

In some earlier reports, the role of p38 MAPK in induction of iNOS expression has been assessed by using compounds SB203580 and SB202190. Inhibition of iNOS expression by these compounds has been shown in murine macrophages and bovine retinal pigmented epithelial cells (Jeon et al., 2000; Chen and Wang, 1999; Faure et al., 1999), but also lack of any regulatory effect has been reported (Caivano, 1998; Chan et al., 1999). In addition, Guan et al. (1997) reported an increase in interleukin-1 β induced iNOS expression after inhibition of p38 MAPK by SC68376 in rat glomerular mesangial cells. Our results on the bi-directional effects of SB203580 and SB202190 on iNOS expression and NO production depending on the drug concentration used offer an explanation for the variable results reported earlier. The effects of p38 inhibitors may also depend on the cell type and on the stimuli used to induce iNOS expression.

In conclusion, pyridinyl imidazoles SB203580 and SB202190 had a bi-directional effect on NO production through iNOS pathway depending on the drug concentration used. At higher concentrations, SB203580 suppressed iNOS expression in a NF-κB-independent manner. At lower concentrations, SB203580 stimulated lipopolysaccharide-induced NO production at the level of iNOS mRNA expression, which most likely is a specific p38 MAPK dependent mechanism, suggesting a novel mechanism for regulation of iNOS expression common for murine and human cells.

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