p38 MAPK inhibits JNK2 and mediates cytokine-activated iNOS induction and apoptosis independently of NF-κB translocation in insulin-producing cells

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ABSTRACT. The signaling pathways mediating nitric oxide production and apoptosis in pancreatic β-cells are incompletely characterized. We report here that the inhibitor of p38 MAPK (p38), SB203580 (10-100 μM) inhibits interleukin-1β (IL-1β)-induced nitric oxide production in rat insulin-producing RINm5F cells. SB203580 also counteracts apoptosis induced by a combination of IL-1β and interferon-γ. However, the contribution by p38 to the induction of inducible nitric oxide synthase (iNOS) and apoptosis is independent of NF-κB nuclear translocation since SB203580 does not prevent IL-1β-induced DNA-binding of this transcription factor. Furthermore, SB203580 alone leads to phosphorylation of JNK2 which may reflect inhibition of a p38-activated phosphatase. It is concluded that p38 mediates cytokine-induced iNOS-induction and apoptosis independently of NF-κB translocation. Moreover, a preventive effect on iNOS induction and apoptosis by inhibition of p38 may be partly masked due to simultaneous activation of JNK2 in pancreatic RINm5F cells.

Keywords: β-cell, SB203580, JNK, nitric oxide, interleukin-1β, interferon-γ

It is well established that cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) exert inhibitory and cytotoxic effects on rodent pancreatic β-cells *in vitro* [1-3]. These cytokines may, alone or in combination, be important mediators of the autoimmune destruction of β-cells during the development of type-1 diabetes mellitus [1]. The cytotoxic effect of cytokines is mediated, at least in part, by the production of nitric oxide as a result of the induction of nitric oxide synthase (iNOS) [3-5]. Indeed, transgenic mice overexpressing iNOS in the insulin-producing cells develop diabetes associated with DNA fragmentation and β-cell destruction [6]. The effects of nitric oxide include inhibition of aconitase, glucose oxidation rates, ATP generation and insulin production *in vitro* [5]. Moreover, we have recently reported that cytokines also induce a nitric oxide-dependent disruption of the mitochondrial membrane potential as well as both apoptosis and necrosis via the Bcl-2-inhibitable pathway in insulin-producing cells [3,7]. IFN-γ enhances IL-1β-induced nitric oxide production, which may explain the increased β-cell death observed with the combination of these cytokines [3, 8].

Some light was shed on the signaling pathways mediating cytokine-induced nitric oxide production in 1994 when we able to demonstrate, for the first time, that induction of iNOS in response to IL-1β is dependent upon nuclear translocation of transcription factor NF-κB in insulin-producing cells [9]. In a later study, we observed that TNF-α also triggers NF-κB activation in insulin-producing cells albeit without inducing nitric oxide production, thus illustrating the importance of additional factors to achieve the induction of iNOS [10]. Besides NF-κB activation, cytokine-induced β-cell signal transduction also seems to involve pathways leading to the activation of STAT-1, as well as mitogen-activated protein kinases (MAPK) including p38 MAPK (p38), JNK and ERK [11-13]. MAPK are activated through dual phosphorylation of conserved tyrosine and threonine residues [14]. In contrast to the classic MAPK ERK1/2, JNK and p38 are preferentially stimulated by stress and certain cytokines [14].

Despite considerable effort, the exact nature of the pathways mediating IL-1β-induced gene expression and cell death have still not been completely elucidated. The aim of the present study was to investigate the possible involvement of cytokine-induced phosphorylation events and NF-κB activation in the signaling leading to β-cell iNOS induction and apoptosis. We report that inhibition of p38 using SB203580, prevents IL-1β-induced nitric oxide production, and also counteracts apoptosis in response to a combination of IL-1β and IFN-γ in insulin-producing RINm5F cells. However, cytokine-induced nuclear translocation of NF-κB is unaffected by SB203580, which may suggest that p38 contributes to cytokine-induced iNOS-induction and apoptosis independently of NF-κB in insulin-producing cells. Moreover, a preventive effect on iNOS induction and apoptosis by p38 inhibition may be partly masked due to simultaneous activation of JNK2 in pancreatic RINm5F cells.

METHODS

Materials

[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole] (SB203580) was synthesized at Smith-Kline Beecham (King of Prussia, PA, USA). Recombinant human IL-1β was from PeproTech EC Ltd. (London, UK), and recombinant mouse IFN-γ from AMS Biotechnology Ltd. (Oxon, UK).

Cell culture

Growing RINm5F cells (passage number > 120) were trypsinized every 3-5 days and subcultured (1×10^5 cells per each 10 mm well or 5×10^5 cells per each 50 mm well) in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml), at 37 °C in humidified air with 5% CO_2.

Immunoblot analysis

RINm5F cells ($2\text{-}3 \times 10^5$) were exposed to cytokines (20 minutes) and SB203580 (20 + 20 minutes) as stated in the figure legends. Cells were washed in cold PBS and directly lysed in SDS-β-mercaptoethanol sample buffer containing 1 mM phenylmethyl sulfonyl-fluoride. Samples were then run on 9% SDS-polyacrylamide gels and electroblotted onto nitrocellulose filters. The filters were then incubated with phospho-specific (Thr180/Tyr182) JNK1 or JNK2 antibodies diluted 1:1000 in TBS supplemented with 2.5% bovine serum albumin. Horseradish peroxidase-linked goat anti-rabbit Ig was used as a second layer. The immunodetection was performed as described for the ECL immunoblotting detection system (Amersham International). The intensities of the bands were quantified by densitometric scanning using Kodak Digital Science 1D software (Eastman Kodak, Rochester, NY, USA).

Nitrite determination

Different substances were added to RINm5F cell cultures as given in the figures. The next day, duplicate samples (2×80 µl) were taken for nitrite determination as previously described [5].

Detection of apoptosis by DNA-laddering

RINm5F cells were cultured in the presence of cytokines or inhibitors, for 24 h. Cells were harvested in cold PBS using a rubber policeman, pelleted and resuspended in 400 µl of lysis buffer (100 mM Tris-HCl, pH 8.5, 200 mM NaCl, 5 mM EDTA, 100 mg/ml proteinase K, 0.2% SDS) [3]. The samples were agitated at 37 °C overnight, followed by addition of 320 µl of isopropanol. High molecular weight DNA was pelleted by a brief centrifugation and to the remaining supernatants, 40 µl of 3 M NaAc, pH 5.2 and 680 µl ethanol were added. Samples were kept at – 20 °C for 1 h and then centrifuged for 10 min at 12000 g. Pellets containing low molecular weight DNA and RNA were dissolved in water and treated with RNAase for 15 min at 37 °C. Samples were then run on 1.5% agarose gels and visualized by ethidium bromide staining.

Electrophoretic mobility shift asssay

For electrophoretic mobility shift assays (EMSA) of NF-κB, the following double-stranded oligonucleotide was used: 5'AGCTTCAGAGGGGACTTTCCGAGAGG [9]. The double-stranded oligonucleotide was labelled with [^{32} P]dCTP using a Megaprime labelling kit (Amersham International, Buckinghamshire, UK). Binding reactions contained 10 mM Tris, pH 7.5, 0.2% deoxycholic acid, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 2 µg polydeoxyinosinic-deoxycytidylic acid, 0.1 ng DNA (14000 cpm) and 4 µl of nuclear protein extract (20 µg). Samples supplemented with a 100-fold excess of non-labelled, double-stranded oligonucleotide were used as negative control. Each 20 µl reaction was incubated at room temperature for 30 min. Samples were separated on 5% non-denaturing polyacrylamide gels in 0.5 × TBE. Band intensities were quantified by densitometric scanning.

Statistical analysis

Data are presented as means ± SEM. Comparisons were made using Student's t-test.

RESULTS

SB203580 inhibits IL-1-induced nitric oxide production in pancreatic RINm5F cells

To examine the role of p38 in the induction of nitric oxide production in response to IL-1β, RINm5F cells were pretreated with the inhibitor of p38, SB203580. An over-night exposure of RINm5F cells to 25 U/ml of IL-1β resulted in high levels of nitrite, as expected *(Figure 1)*. SB203580 (10-100 µM) significantly inhibited IL-1β-induced nitrite production. Nitrite levels from control cells were not above background.

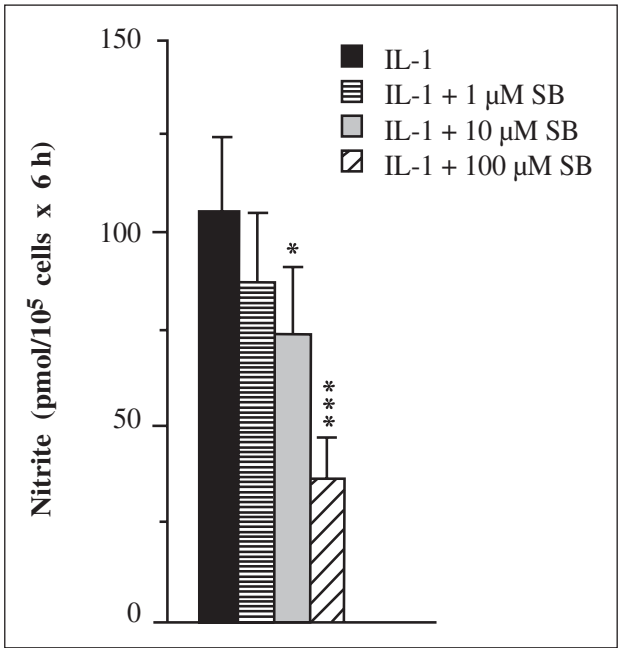

Figure 1
SB203580 inhibits IL-1β-induced nitrite production. RINm5F cells were exposed overnight to 25 U/ml of IL-1β in the presence or absence of SB203580, as given in the figure, and the formation of nitrite was determined. Results are means ± SEM for 3 observations. * denotes $p < 0.05$ and *** denotes $p < 0.001$ *versus* control cells using Student's paired t-test.

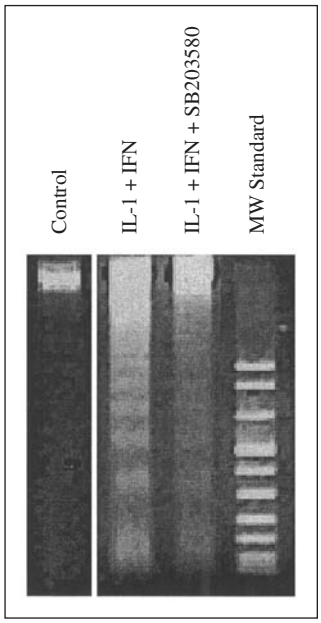

Figure 2
SB203580 counteracts cytokine-induced apoptotic DNA-ladder formation. RINm5F cell (2×10^6 cells) were exposed for 24 h to the combination of IL-1β (25 U/ml) and IFN-γ (1000 U/ml), in the presence or absence of 10 μM SB203580. Low molecular weight DNA was extracted and analyzed by agarose gel electrophoresis. The control lane represents untreated cells and the molecular weight standard is Boehringer Mannheims standard VIII. The figure is representative of two experiments.

SB203580 counteracts cytokine-induced apoptosis in RINm5F cells

Since a 24-h exposure to IL-1β, TNF-α or IFN-γ alone, in our hands, does not result in any significant β-cell apoptosis [3, 10], we used the combination of IL-1β and IFN-γ to induce RINm5F cell apoptosis. To study the role of p38 in cytokine-induced apoptosis, RINm5F cells were cultured overnight in the presence of the combination of IL-1β and IFN-γ with or without SB203580 (10 μM). The combination of cytokines clearly induced the formation of a DNA-ladder, a biochemical hallmark of apoptosis *(Figure 2)*. The apoptotic DNA-ladder formation was at least partially counteracted by SB203580 *(Figure 2)*.

Cytokine-induced nuclear translocation of NF-κB is unaffected by SB203580

To evaluate the possible role of p38 in cytokine-induced nuclear translocation of NF-κB, RINm5F cells were exposed to IL-1β in the presence or absence of SB203580. As we have previously demonstrated [9, 10], IL-1β induced a pronounced nuclear translocation of NF-κB *(Figures 3 and 4)*. However, SB203580 at 10-100 μM did not prevent IL-1β-induced activation of NF-κB *(Figures 3 and 4)*.

Effect of SB203580 on JNK1 or JNK2 phosphorylation

To study a putative effect of p38 inhibition on JNK phosphorylation events, RINm5F cells were exposed for 30 min to increasing concentrations of SB203580 followed by immunoblot analysis on cell extracts using phospho-specific JNK1 and JNK2 antibodies. SB203580 (1-100 μM) did not significantly affect JNK1 phosphorylation *(Figure 5)*. However, the phosphorylation of JNK2 was significantly enhanced by 100 μM of SB203580 *(Figure 5)*.

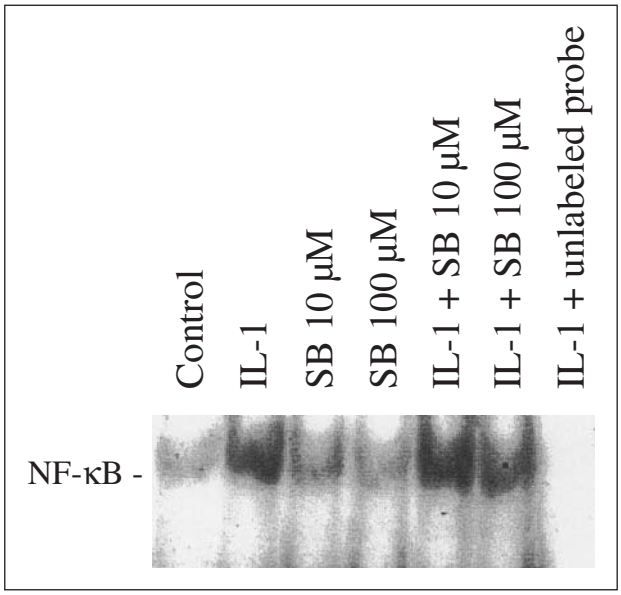

Figure 3
Effects of IL-1β and SB203580 on nuclear translocation of NF-κB. RINm5F cells were pre-incubated for 20 min with or without 0, 10 or 100 μM SB203580 and then exposed for 20 min to 25 U/ml of IL-1β. Nuclear extracts were prepared and NF-κB activity was determined by EMSA. The last lane represents IL-1β-exposed cells subjected to EMSA using an unlabelled probe as negative control.

DISCUSSION

The present study shows that the cytokine IL-1β induces activation of p38 in pancreatic RINm5F cells. This finding

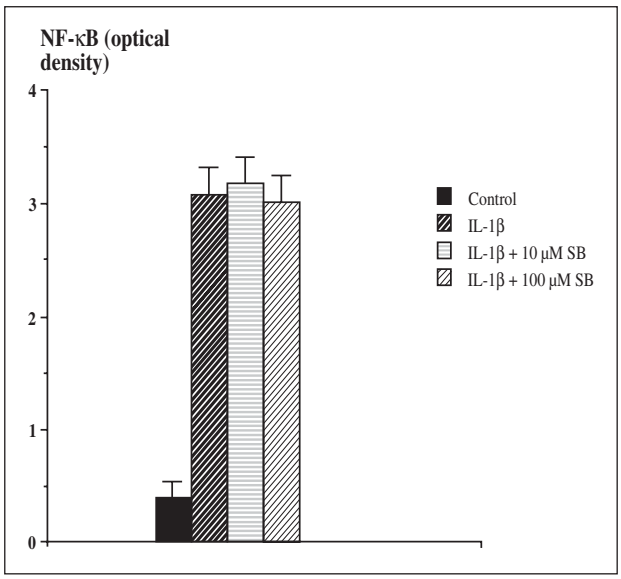

Figure 4
Densitometric scanning of results of EMSA on NF-κB activation in response to IL-1β and SB203580. RINm5F cells were exposed to IL-1β and SB203580 and subjected to EMSA as given in *Figure 3*. Densitometric scanning of shifted bands was performed. Results are expressed as arbitrary units of optical densitometry and are means ± SEM for 3-5 experiments.

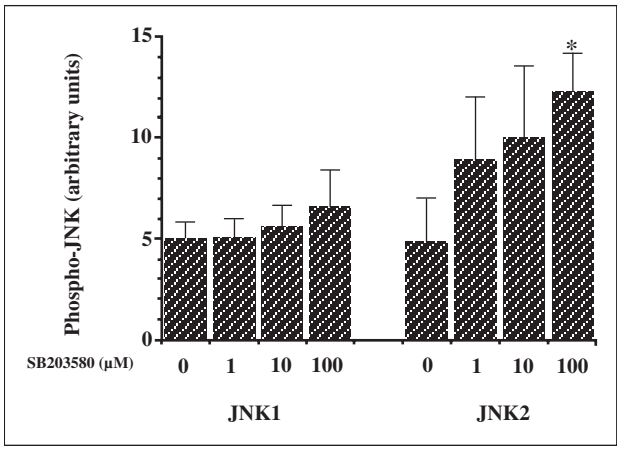

Figure 5
Effects of SB203580 on JNK1 or JNK2 phosphorylation.
RINm5F cells were exposed for 30 min to 0, 1, 10 or 100 μM SB203580 as given in the figure followed by determination of JNK1 or JNK2 phosphorylation using immuoblot analysis with densitomtric scanning of bands. Results are means ± SEM for 3 separate observations. * denotes $p < 0.05$ *versus* 0 μM SB203580.

is well in line with our previous observation that islets [Saldeen and Welsh, unpublished observation] and RINm5F cells respond similarly to IL-1β and TNF-α, with enhanced phosphorylation of both p38 and JNK2 [11]. It also indicates that the insulinoma cell line RINm5F is an appropriate model for the primary islet β-cell in this experimental system. In other cell types, exposure to proinflammatory cytokines results in modulation of the activities of intracellular molecules such as IRAK [15], TRAF2 [16], ceramide [17] or GTP-binding proteins [18] that further promote the activation of MEKK protein kinases [19]. The exact nature of the signaling pathways functioning upstream of p38 activation in β-cells, in response to cytokines, remains to be determined.

The present study also demonstrates that IL-1β-induced signal transduction mediating the induction of iNOS in rat insulin-producing cells depends, at least to some extent, on the activation of p38. However, since we have previously observed that exposure to TNF-α alone also induces p38, albeit without inducing iNOS gene expression or β-cell apoptosis [3, 11], it is evident that activation of p38 is not a sufficient event for either iNOS induction or apoptosis in insulin-producing cells. The observation that p38, at least partially, mediates IL-1β-induced iNOS induction is also in line with a previous report using neonatal rat islets where SB203580 was found to inhibit both IL-1β-induced iNOS mRNA expression and nitric oxide production [13]. Nevertheless, this finding may be seemingly contradictory to our previous finding in adult rat islets, where the p38 inhibitor was unable to counteract nitric oxide production in response to a combination of IL-1β and IFN-γ [11]. It is conceivable, however, that this difference is due to the possibility that iNOS induction by the combined action of both IL-1β and IFN-γ, is less sensitive to p38 inhibition than iNOS induction by IL-1β alone [11]. It could also be pointed out, that in the current study, we observed a dose-dependent effect by the p38 inhibitor such that nitrite formation was more potently inhibited by 100 μM as compared to 10 μM SB203580. Thus, the higher dose might possibly have exerted an inhibitory effect on nitrite formation also in response to the combination of IL-1β and IFN-γ used on rat islets in our previous study [11].

Since a 24-h exposure to IL-1β, TNF-α or IFN-γ alone, in our hands, does not induce significant β-cell apoptosis [3, 10], we exposed RINm5F cells to the combination of IL-1β and IFN-γ to induce apoptosis. The p38 inhibitor counteracted RINm5F cell apoptosis in response to this combination of cytokines. This finding supports our previous observation in isolated rat islets [11], and is also in line with numerous publications showing a pro-apoptotic role for sustained p38 activation in β-cells [20] and other cell systems [21-25]. In view of the currently observed inhibition of nitric oxide production afforded by SB203580, it is probable that the protection against cytokine-induced apoptosis by this p38 inhibitor is due, at least partially, to the inhibition of iNOS induction. Possible apoptosis signaling pathways depending directly on p38 activation in β-cells remain to be explored but may include, for example, those leading to caspase activation [26], FasL expression [27] or p53-phosphorylation [28]. However, in view of the observation that SB203580 inhibited iNOS induction while the protection against cytokine-induced apoptosis appeared merely partial, there is a possibility that p38 also induces the expression of protective genes in β-cells such as Mn-SOD and/or heme oxygenase, as has been shown in other cell types [29,30]. Indeed, such proteins may have protective effects in β-cells [31]. In the current study, IL-1β potently induced the translocation to nuclei of the transcription factor NF-κB, which is in accordance with what we have previously reported with both IL-1β and TNF-α [9,10]. There is recent evidence to support both an apoptosis-promoting effect and a protective role of this transcription factor [32-34]. The present study does not address this issue since NF-κB activity was not inhibited. However, in view of the important role of NF-κB in iNOS induction [9,10,32], it is conceivable that inhibition of NF-κB could counteract apoptosis by preventing nitric oxide formation which, as discussed above, exerts important cytotoxic effects on β-cells [3-7]. The finding that p38 activity and NF-κB nuclear translocation are dissociated in insulin-producing cells is, to our knowledge, not known before and differs from studies in other cell types where p38 may function upstream of NF-κB activation [35].

We have previously reported that IL-1β activates JNK1 in the insulin producing cell line RINm5F [11,12]. This event was paralleled by an increased phosphorylation of transcription factors c-Jun and ATF-2, as well as an enhanced binding activity to the ATF/CREB consensus element [11]. In the current study, inhibition of p38 alone by SB203580 significantly enhanced the phosphorylation of JNK2. This finding may support the results of a recent study on neutrophils where SB203580 significantly enhanced TNF-α-induced JNK activity [36]. Thus, the present data may suggest that p38, possibly through activation of phosphatase-2A, negatively regulates the JNK pathway in insulin-producing β-cells.

A scheme outlining some of the possible signaling events in cytokine-induced nitric oxide production and destruction of rat insulin-producing cells is given in *Figure 6*. In summary, the results presented herein suggest a direct role for p38 in IL-1β-mediated nitric oxide production and apoptosis in a pancreatic β-cell line. Furthermore, we have also shown that the induction of iNOS and apoptosis by

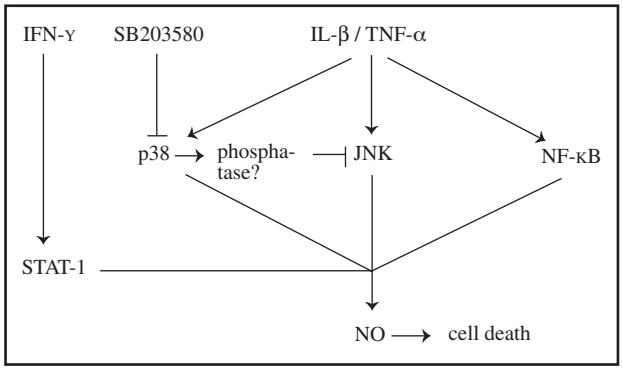

Figure 6
Scheme of some possible signaling events in cytokine-induced nitric oxide production and cell death in rat insulin-producing cells.

p38 are independent of NF-κB nuclear translocation. Moreover, a preventive effect on iNOS induction and apoptosis by p38 inhibition may be partly masked as a result of a simultaneous activation of JNK2 in pancreatic RINm5F cells. The elucidation of the molecular mechanisms and pathways will hopefully increase our understanding of the pathogenesis of type-1 diabetes and promote the development of pharmacological treatments that intervene in the autoimmune destruction of β-cells.

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