Interleukin-1β-induced Rat Pancreatic Islet Nitric Oxide Synthesis Requires Both the p38 and Extracellular Signal-regulated Kinase 1/2 Mitogen-activated Protein Kinases*

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Interleukin-1β (IL-1β) is cytotoxic to rat pancreatic β-cells by inhibiting glucose oxidation, causing DNA damage and inducing apoptosis. Nitric oxide (NO) is a necessary but not sufficient mediator of these effects. IL-1β-induced kinase activity toward Elk-1, activation transcription factor 2, c-Jun, and heat shock protein 25 in rat islets. By Western blotting with phosphospecific antibodies and by immunocomplex kinase assay, IL-1β was shown to activate extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (p38) in islets and rat insulinoma cells. Specific ERK1/2 and p38 inhibitors individually reduced but in combination blocked IL-1β-mediated islet NO synthesis, and reverse transcription-polymerase chain reaction of inducible NO synthase mRNA showed that ERK1/2 and p38 controlled IL-1β-induced islet inducible NO synthase expression at the transcriptional level. Hyperosmolality caused phosphorylation of Elk-1, activation transcription factor 2, and heat shock protein 25 and activation of ERK1/2 and p38 in islets comparable to that induced by IL-1β but did not lead to NO synthesis. Inhibition of p38 but not of ERK1/2 attenuated IL-1β-mediated inhibition of glucose-stimulated insulin release. We conclude that ERK1/2 and p38 activation is necessary but not sufficient for IL-1β-mediated β-cell NO synthesis and that p38 is involved in signaling of NO-independent effects of IL-1β in β-cells.

Interleukin-1β (IL-1β) is cytotoxic to rat pancreatic β-cells (1, 2), and IL-1β in combination with tumor necrosis factor-α and interferon-γ is cytotoxic to human β-cells (3, 4), implicating IL-1β as a central immune mediator of β-cell destruction leading to insulin-dependent diabetes mellitus (5). Nitric oxide (NO) produced by IL-1β-mediated expression of β-cell cytokine inducible NO synthase (iNOS) (6, 7) has been suggested as the second messenger for the β-cell cytotoxic effect of IL-1β (8). However, because inhibition of NO synthesis only partially protects rat β-cells from IL-1β-induced cytotoxicity (9), other signals for this effect must be involved. The intracellular signal transduction pathways leading to induction of iNOS and other genes contributing to IL-1β-mediated β-cell cytotoxicity are not fully elucidated. Tyrosine kinase-dependent activation of NF-κB seems to be central for IL-1β-mediated β-cell iNOS expression (10, 11).

Recently, the activation of c-Jun NH2-terminal kinase (JNK) 1 and the transcription factor activating transcription factor 2 (ATF2) by IL-1β in an insulin-producing rat insulinoma (RIN) m5F β-cell line was described (12). JNKs (or stress-activated protein kinases) are a subgroup of the mitogen-activated protein kinase (MAPK) family of threonine or serine kinases, which upon activation phosphorylate and thereby activate transcription factors leading to changes in gene expression (reviewed in Refs. 13 and 14). Additional subgroups are p38 MAPK (hereafter termed p38) and extracellular signal-regulated kinase (ERK) 1/2. p38 is activated in many cells by IL-1 and other cellular stresses, including UV irradiation, hyperosmolality, tumor necrosis factor-α, and lipopolysaccharide (15–18), generally in parallel with JNK, although JNK and p38 may also be activated independently (19). ERK1/2 are generally activated by growth factors (20, 21) and to a minor extent by cellular stress, illustrated by the fact that IL-1 activates ERK1/2 in some (22, 23) but not all (20, 24) cells investigated. Recently, specific inhibitors of ERK1/2 (25) and p38 (26) signaling pathways have been identified, providing effective tools for investigating the role of ERK1/2 and p38 in cellular signaling.

The contribution of p38 and ERK1/2 to IL-1β signal transduction in insulin-producing β-cells has not been investigated. Therefore, we examined the involvement of ERK1/2 and p38 in IL-1β signaling in intact rat islets of Langerhans and in a RIN β-cell line. We report that both ERK1/2 and p38 are activated by IL-1β in intact islets and in a RIN β-cell line and that ERK1/2 and p38 are both important steps in signaling leading to NO synthesis, but p38 is also involved in signaling of NO-independent effects of IL-1β in intact islets.

**EXPERIMENTAL PROCEDURES**

*Reagents—All reagents were from Sigma unless otherwise specified. Recombinant human IL-1β (400 units/ng) was from Novo Nordisk (Bagsvaerd, Denmark). Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were all from Bio-Rad. γ-32P]ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech, glutathione S-trans-
ferase (GST)-Elk-1 was a gift from Peter Shaw (Max-Planck-Institut für Immunobiologie, Freiburg, Germany), GST-ATF2 (1–109) was a gift from Roger J. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA); GST-e-Jun was a gift from Peter Angel (Deutre Krebsforschungszentrum, Heidelberg, Germany). The highly specific inhibitor of p38 (compound VK-19,577, identical to SB230580 and p38i) (26, 27) was from Vertex Pharmaceuticals Inc. (Cambridge, MA). Compound PD 098059 from New England Biolabs (Hitchin Hertfordshire, United Kingdom), and MAPK-activated protein kinase-2 (MAPKAP-K2) antibody was from Upstate Biotechnological (Lake Placid, NY).

In Western blotting or stored at −80 °C.

Whole Cell Lysate Kinase Assay—The GST-Elk-1, GST-ATF2, and Hsp25 phosphotransferase reactions were carried out in a final volume of 20 μl at 30 °C for 30 min after addition of 5 μl of whole cell lysate, 17 μl of reaction buffer (2 μg of GST-Elk-1, 2 μg of GST-ATF2, 1 μg of Hsp25, 25 μM Tric-HCl, pH 7.0, 0.27 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 1% Triton X-100, 10 mM Na3VO4, 1 mM cAMP-dependent protein kinase inhibitor peptide, and 10 mM Mg-acetate), and 3 μl of ATP mixture (1 mM ATP and 3 μl [γ-32P]ATP). Reactions were terminated by addition of 25 μl of SDS sample buffer (125 μM Tris-HCl, pH 6.8, 4% SDS, 1 mM dithiothreitol, 10% glycerol, and 0.02% bromphenol blue) and boiling for 5 min. The samples were then subjected to SDS-PAGE as described by Laemmli (30), using a 4% stacking gel and a 12% separating gel. After electrophoresis, the separating gel was washed for 15 min in a mixture of 10% acetic acid and 40% methanol. The gels were dried, and the proteins were visualized by autoradiography (Research Products International, Nanyang, CA). To assess the possible contamination of the used GST-ATF2, we performed Coomassie Blue staining and kinase assay with GST-ATF2 and ATF2 (1–96 and 1–505) from Santa Cruz Biotechnology (Santa Cruz, CA), which confirmed the substrate specificity and purity of the GST-ATF2.

Immunoprecipitation, Immunocomplex, and JNK Kinase Assay (Sol-
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Fig. 1. IL-1β activates ERK1/2 and p38 in islets. Lysates of islets were prepared following exposure to 150 pg/ml IL-1β for the indicated times. A, whole cell lysate kinase assay with [γ-32P]ATP and GST-Elk-1 (Elk-1), GST-ATF2 (ATF2), and Hsp25 as substrates. Phosphorylation reactions were visualized after SDS-PAGE by autoradiography. A representative autoradiogram is shown (upper panel). Phosphorylation was quantified by PhosphorImager analysis and presented as mean ± S.E. (n = 4) relative to control islets (0) treated without IL-1β (lower panel). B, lysates of IL-1β-exposed islets were analyzed by Western blotting. Lysates from pooled duplicate experiments were separated on two gels and blotted onto two membranes. One of the membranes was probed with phosphospecific ERK1/2 (pERK1/2) antibody, washed, and then reprobed with phosphospecific p38 (p38) antibody. The other membrane was probed with ERK1/2 antibody, washed, and then reprobed with p38 antibody. Results shown are representative of two individual experiments. C, ERK1/2 (upper panel) and MAPKAP-K2 (lower panel) were immunoprecipitated from lysates of IL-β-exposed islets with specific antibodies, and their activities were measured in immunocomplex kinase assay with GST-Elk-1 (Elk-1) and Hsp25 as substrates, respectively. Phosphorylation reactions were initiated by the addition of [γ-32P]ATP. Following SDS-PAGE, phosphorylated proteins were visualized by autoradiography. Each experiment was performed twice, and representative autoradiograms are shown. Phosphorylation was quantified by PhosphorImager analysis and indicated below the autoradiograms as mean ± range.

These findings were substantiated by directly measuring the activity of ERK1/2 and MAPKAP-K2 (a specific substrate of p38 (27)) (Fig. 1C) in an immunocomplex kinase assay, showing a 3.1- and 3.6-fold activation of ERK1/2 and MAPKAP-K2, respectively, after a 20-min IL-1β exposure. Following 24 h IL-1β stimulation, kinase activities were below baseline.

Detection of ERK1/2 and p38 Activity in RIN Cells—To investigate whether the ERK1/2 and p38 activities found in intact islets were due to the presence of these kinases in β-cells, the RIN β-cell line was assayed for ERK1/2 and p38 activities. The RIN β-cell line is comparable to primary β-cells in terms of IL-1β-mediated NO production, iNOS expression, and cytotoxicity; albeit at a higher concentration than is needed in islets (36, 37). Based on the IL-1β time course from intact islets (Fig. 1A), an IL-1β exposure period of 20 min was used. IL-1β-stimulated ATP2 and Hsp25 kinase activities in a dose-dependent manner, whereas IL-1β-induced Elk-1 kinase was not further activated by an increased concentration of IL-1β above 150 pg/ml (Fig. 2A). The binding of phosphospecific antibodies (pERK1/2 and pp38) showed that both ERK1/2 and p38 were phosphorylated in RIN cells after a 20-min exposure to 1500 pg/ml IL-1β (Fig. 2B). p38 and ERK1/2 activation by IL-1β was

Fig. 2. IL-1β causes phosphorylation of MAPK-substrates (A) and ERK1/2 and p38 (B) in RIN 5AH-T2B cells. A, lysates of RIN cells exposed to 0, 150, or 1500 pg/ml IL-1β for 20 min were analyzed by the whole cell lysate kinase assay using [γ-32P]ATP and GST-Elk-1 (Elk-1), GST-ATF2 (ATF2), and Hsp25. Following SDS-PAGE, the products were visualized by autoradiography. A representative autoradiogram is shown (upper panel). Phosphorylation was quantified by PhosphorImager analysis and presented as mean ± S.E. (n = 3) relative to control cells (0) treated without IL-1β (lower panel). B, lysates of RIN cells exposed to 0 (c) or 1500 (IL-1β) pg/ml IL-1β for 20 min were analyzed by Western blotting as described in Fig. 1B. Total ERK1/2 and p38 and phosphorylated ERK1/2 (pERK1/2) and p38 (pp38) are indicated. Results shown are representative of n = 2. C, ERK1/2 (upper panel) and MAPKAP-K2 (lower panel) were immunoprecipitated from lysates of RIN cells stimulated with (IL-1β) or without (c) 1500 pg/ml IL-1β, and their activities were measured in an immunocomplex kinase assay as described in Fig. 1C with GST-Elk-1 (Elk-1) and Hsp25 as substrates, respectively. Phosphorylation was quantified by PhosphorImager analysis and is indicated below the autoradiograms as ERK1/2 and MAPKAP-K2 activity relative to control cells (c) treated without IL-1β.
further demonstrated by the immunocomplex kinase assay that showed a 3.9- and 8.1-fold increase in ERK1/2 and MAPKAP-K2 activity in IL-1β-exposed RIN cells, respectively (Fig. 2C).

**Inhibition of ERK1/2 and p38 in IL-1β-exposed Islets**—To dissect the relative contributions of ERK1/2 and p38 signaling pathways in the IL-1β-induced Elk-1, ATF2, and Hsp25 kinase activities, islets were incubated with p38i and MEKi 1 h prior to IL-1β exposure (Fig. 3A). The MEKi inhibited both basal and IL-1β-stimulated Elk-1 kinase activity without affecting the ATF2 and Hsp25 kinase activities. A maximal inhibition was seen at 10 μM of MEKi. The p38i inhibited both basal and IL-1β-induced ATF2 and Hsp25 kinase activities; it was more effective in Hsp25 kinase inhibition, which was completely blocked at 10 μM, whereas this concentration of p38i inhibited IL-1β-induced ATF2 kinase activity by 71%. When the inhibitors were combined, the kinase activities toward the three substrates were completely blocked.

The specificity of the inhibitors was further evaluated in an immunocomplex kinase assay, where the activities of immunoprecipitated ERK1/2 and MAPKAP-K2 in lysates of islets that had been preincubated with MEKi and/or p38i prior to IL-1β exposure were determined. MEKi inhibited IL-1β-induced ERK1/2 activity and did not affect the activity of MAPKAP-K2, whereas p38i inhibited IL-1β-stimulated MAPKAP-K2 activity but not that of ERK1/2 (Fig. 3B, top and middle panels). Because JNK has both Elk-1 and ATP2 kinase activities (20, 38), the complete inhibition of ATF2 and Elk-1 kinase activities in whole cell lysates of IL-1β-stimulated islets preincubated with MEKi and p38i questioned the involvement of JNK in IL-1β signaling in islets. However, we found substantial IL-1β-stimulated JNK activity determined by c-Jun phosphorylation in an in vitro solid-phase kinase assay in islets, and as expected, JNK activity was unaffected by either of the inhibitors (Fig. 3B, bottom panel). This indicates that JNK is neither an IL-1β-activated Elk-1 nor ATP2 kinase in islets.

**Involvement of ERK1/2 and p38 in IL-1β-mediated β-Cell Dysfunction**—MEKi and p38i were then used to evaluate the impact of ERK1/2 and p38 on IL-1β-mediated β-cell dysfunction. NO production was not detectable from untreated islets, and neither MEKi nor p38i added alone or in combination resulted in NO production (Table I). At a low (25 pg/ml) IL-1β concentration, p38i caused a 35% decrease in the IL-1β-induced islet NO production, whereas MEKi blocked NO production at that concentration of IL-1β. At a high (150 pg/ml) IL-1β concentration, p38i and MEKi caused a 25 and 33% reduction, respectively, of IL-1β-induced islet NO production. However, in the presence of the two inhibitors, a synergistic effect was found, and IL-1β-induced NO synthesis was completely blocked.

MEKi and p38i used either alone or in combination did not affect the glucose-stimulated insulin release from untreated islets (Table II). A low (25 pg/ml) IL-1β concentration did not cause a significant decrease in glucose-stimulated insulin release, and no effect of the inhibitors was found. The 62% inhibition of glucose-stimulated insulin release mediated by a high (150 pg/ml) IL-1β concentration was attenuated by p38i by 33%, whereas MEKi was ineffective and no synergism between the two compounds was detected.

**ERK1/2 and p38 Regulate IL-1β-induced Islet NO Synthesis at the Transcriptional Level**—To investigate whether ERK1/2 and p38 controlled IL-1β-induced islet NO production at the transcriptional level, reverse transcription-PCR of iNOS mRNA in IL-1β-exposed islets preincubated with/without the inhibitors was performed. iNOS mRNA was not detectable in
untreated islets (Fig. 4). IL-1β-induced iNOS was expressed to a 3.3-fold greater magnitude at 6 h compared to 24 h of IL-1β exposure. IL-1β-induced iNOS was inhibited by both of the inhibitors; the MEKi was slightly more effective at both 6 and 24 h of IL-1β exposure. Combined, the inhibitors reduced IL-1β-induced iNOS by ~95% at both 6 and 24 h.

Noncytokine Stimulation of ERK1/2 and p38 Activity and Islet NO Production—To investigate whether noncytokine activation of ERK1/2 and p38 was able to induce islet NO production, islets were exposed to hyperosmolarity. As shown in Fig. 5A, a weak phosphorylation of Elk-1, ATF2, and Hsp25 was found in control islets (0′, baseline). When exposed to hyperosmolarity (525 mosM) a marked time-dependent phosphorylation of the three substrates was found. Hyperosmolarity induced rapid (within 0.5 min) phosphorylation with a 2.2- and 2.3-fold activation of ATF2 and Hsp25, respectively. A 3.1-fold activation of Elk-1 was evident within 2.5 min. The time points for peak values with 3.8-, 4.6-, and 3.6-fold activation of Elk-1, ATF2, and Hsp25, respectively, were very similar to those induced by IL-1β (see Fig. 1A). Hsp25 phosphorylation declined to baseline at 12 h, whereas the phosphorylation of ATF2 and Elk-1 was sustained until at least 12 h. Elk-1 phosphorylation was more sustained, and Hsp25 phosphorylation showed a more rapid descent when compared with IL-1β-induced phosphorylation.

Phosphorylation of ERK1/2 and p38 by hyperosmolarity (525 mosM) was found by Western blotting (Fig. 5A), with detectable ERK1/2 (pERK1/2) and p38 (p38p) phosphorylations of 2.5 min to 12 h and of 0.5 min to 12 h, respectively, in agreement with the kinase activities found in the whole cell lysate kinase assay (see Fig. 5A). However, islets exposed to increasing osmolarity (285–525 mosM) for 24 h did not produce detectable NO (nitrite <2 pmol/islet/24 h; n = 6).

**DISCUSSION**

Our data demonstrate that p38 and ERK1/2 are both activated by IL-1β in rat islets of Langerhans and in the rat insulinoma cell line RIN-5AH, implying that the ERK1/2 and p38 activity found in the intact islet most likely originates from the β-cells, in accordance with the recently reported p38 activation by IL-1β in the INS-1 β-cell line (39).

MEK- and p38-specific inhibitors individually reduced IL-1β stimulated islet NO synthesis (Table I) and iNOS mRNA (Fig. 4), indicating that both ERK1/2 and p38 are involved in IL-1β-mediated expression of iNOS. iNOS induction by ERK1/2 in β-cells may be explained by Elk-1-induced c-fos expression mediated through the serum response element (40). c-Fos combines with c-Jun to form the transcription factor activating protein-1 (41), and two activating protein-1 binding sites have been identified in the murine iNOS gene promoter (42). Activated p38 may induce β-cell iNOS via several transcription factors: 1) through activating protein-1, because p38-mediated expression of c-jun and c-fos has been observed (43); 2) through NF-κB, because p38 seems to be required for NF-κB-mediated transcriptional activation, but it affects neither NF-κB DNA binding nor phosphorylation of its subunits (44, 45) and the murine iNOS gene promoter region contains two NF-κB binding sites (42), and because NF-κB is involved in iNOS expression in β-cells (36, 46); and 3) through p38-mediated cAMP-responsive element-binding protein activation (47), because cAMP-responsive element-binding protein has been reported to be involved in iNOS induction via the CAAT box in the murine iNOS promoter (48).

The involvement of ERK1/2 and p38 in IL-1β regulation of iNOS seems to be cell-specific because 1) IL-1 activation of ERK1/2 is necessary for iNOS expression in rat cardiac microvascular endothelial cells (23), 2) p38, but not ERK1/2, is involved in IL-1-mediated iNOS expression in mouse astrocytes (49), and 3) IL-1 stimulation of p38 down-regulates iNOS in rat mesangial cells (50). Our data support this concept by showing that both ERK1/2 and p38 are required for IL-1-mediated iNOS expression in rat pancreatic β-cells.

ERK1/2 and p38 activation was found to be necessary for IL-1β-mediated iNOS synthesis (Table I) and iNOS induction (Fig. 4). However, the observation that hyperosmolarity caused a maximal Elk-1, ATF2, and Hsp25 phosphorylation and ERK1/2 and p38 activation (Fig. 5) similar to that caused by IL-1β without inducing islet NO synthesis suggests that ERK1/2 and p38 activation is not sufficient to cause IL-1β-mediated islet NO synthesis. Thus, other signaling events activated by IL-1β, but not provided by hyperosmolarity, are necessary. These signals could be involved in IκB degradation and NF-κB translocation to the nucleus because a recently identified IL-1β receptor associated kinase shares similarity with a protein kinase essential for activation of a NF-κB ho-
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were prepared following exposure to 525 mos M for the indicated time periods; 0 represents control islets (285 mos M). A, whole cell lysate kinase assay was performed as described in Fig. 1A. An autoradiogram is shown (upper panel). Phosphorylation was quantified by Phosphoimager analysis and presented as mean ± S.E. (n = 4) relative to control islets (lower panel). B, lysates of islets exposed to hyperosmolarity were analyzed by Western blotting as described in Fig. 1B. Total ERK1/2, p38, phosphorylated ERK1/2 (pERK1/2), and phosphorylated p38 (p38) are indicated.

In conclusion, this study suggests that ERK1/2 and p38 are involved in IL-1β signaling in β-cells, that they are necessary but not sufficient in causing IL-1β-induced β-cell NO synthesis by controlling iNOS gene transcription, and, furthermore, that p38 is involved in signaling of NO-independent effects of IL-1β in β-cells. These observations make p38 inhibition a powerful approach to further elucidate the involvement of p38 in the pathogenesis of insulin-dependent diabetes mellitus.

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injected iNOS expression by 95% and completely blocked islet NO synthesis, the IL-1β-mediated inhibition of insulin release was only attenuated by 33%, indicating that islet NO production is not sufficient in causing β-cell death and that other mediators of NO-independent signaling pathways, in addition to p38, are involved. A possible mediator could be JNK, which is activated by IL-1β in both islets (Fig. 3) and RIN-cells (12) and is involved in apoptosis (54, 57).

Even though combination of MEKi and p38i inhibited IL-1β-
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