JNK1/2 regulates ER–mitochondrial Ca\(^{2+}\) cross-talk during IL-1\(\beta\)–mediated cell death in RINm5F and human primary \(\beta\)-cells

Gaurav Verma, Himanshi Bhatia, and Malabika Datta
CSIR—Institute of Genomics and Integrative Biology, Mall Road, Delhi 110 007, India

**ABSTRACT** Elevated interleukin-1\(\beta\) (IL-1\(\beta\)) induces apoptosis in pancreatic \(\beta\)-cells through endoplasmic reticulum (ER) stress induction and subsequent c-jun-N-terminal kinase 1/2 (JNK1/2) activation. In earlier work we showed that JNK1/2 activation is initiated before ER stress and apoptotic induction in response to IL-1\(\beta\). However, the detailed regulatory mechanisms are not completely understood. Because the ER is the organelle responsible for Ca\(^{2+}\) handling and storage, here we examine the effects of IL-1\(\beta\) on cellular Ca\(^{2+}\) movement and mitochondrial dysfunction and evaluate the role of JNK1/2. Our results show that in RINm5F cells and human primary \(\beta\)-cells, IL-1\(\beta\) alters mitochondrial membrane potential, mitochondrial permeability transition pore opening, ATP content, and reactive oxygen species production and these alterations are preceded by ER Ca\(^{2+}\) release via IP\(_3\)R channels and mitochondrial Ca\(^{2+}\) uptake. All these events are prevented by JNK1/2 small interfering RNA (siRNA), indicating the mediating role of JNK1/2 in IL-1\(\beta\)–induced cellular alteration. This is accompanied by IL-1\(\beta\)–induced apoptosis, which is prevented by JNK1/2 siRNA and the IP\(_3\)R inhibitor xestospongin C. This suggests a regulatory role of JNK1/2 in modulating the ER-mitochondrial-Ca\(^{2+}\) axis by IL-1\(\beta\) in apoptotic cell death.

**INTRODUCTION** Elevated levels of the proinflammatory cytokine interleukin 1\(\beta\) (IL-1\(\beta\)) are associated with pancreatic \(\beta\)-cell apoptosis (Corbett and McDaniel, 1994; Thomas et al., 2002; Larsen et al., 2007; Grunnet et al., 2009), and several signaling intermediates are believed to mediate this deleterious effect. IL-1\(\beta\)–mediated activation of NF-\(\kappa\)B is believed to be a significant contributor to \(\beta\)-cell death (Welsh et al., 2005), and its suppression attenuates pancreatic \(\beta\)-cell cytotoxicity (Kim et al., 2007). Nitric oxide (NO) is also believed to contribute to IL-1\(\beta\)–induced \(\beta\)-cell damage that results from an initial event of internucleosomal DNA cleavage and nuclear condensation (Thomas et al., 2002, 2004). In addition, nitric oxide destroys iron–sulfur centers of ion-containing proteins, leading to mitochondrial dysfunction (Corbett et al., 1992). IL-1\(\beta\)–mediated calcium (Ca\(^{2+}\)) influx into pancreatic \(\beta\)-cells also contributes to impaired \(\beta\)-cell function (Maedler et al., 2004; Dula et al., 2010). Although controlled flow of Ca\(^{2+}\) is required for normal functioning of the \(\beta\)-cell and for insulin release, aberrant Ca\(^{2+}\) influx is frequently encountered in the presence of IL-1\(\beta\). This uninhibited increase in cellular Ca\(^{2+}\) is linked to classical mitochondrial dysfunction parameters, namely, loss of mitochondrial membrane potential (\(\Delta\psi\_m\)), ATP reduction, and increase in reactive oxygen species (ROS) production (Mbaya et al., 2010). Because it is responsible for Ca\(^{2+}\) storage and signaling, the endoplasmic reticulum (ER) contributes to maintaining cellular Ca\(^{2+}\) status, and therefore under conditions of ER stress, normal calcium homeostasis is severely altered (Deniaud et al., 2008). Such disruption is believed to be one of the factors underlying impaired \(\beta\)-cell function and decreased survival (Cardozo et al., 2005). Because pancreatic \(\beta\)-cells posses an intricate Ca\(^{2+}\) machinery that is especially important during insulin secretion (Chen et al., 2003), these alterations significantly contribute to decreased insulin release. IL-1\(\beta\) promotes such alterations within the pancreas (Gurzov et al., 2009), and an evident mediator is believed to be the induction of ER...
We demonstrated that IL-1β induces ER stress and apoptosis in pancreatic cells in a JNK-dependent manner (Verma and Datta, 2010). Although JNK activation by ER stress induction is widely accepted, we showed that JNK1/2 mediates IL-1β–induced ER stress induction, as inhibition of JNK prevented IL-1β–induced ER stress (Verma and Datta, 2010). Our study demonstrated that JNK activation is upstream of IL-1β–mediated ER stress induction. Having described the central initiating role of JNK during IL-1β action, we report in this study that JNK1/2 mediates IL-1β–induced ER Ca2+ release and the consequent mitochondrial dysfunction in pancreatic β-cells.

### RESULTS

**IL-1β induces mitochondrial dysfunction in pancreatic RINm5F cells via JNK1/2**

We previously showed that IL-1β mediates ER stress in a JNK1/2-dependent manner in Mia PaCa-2 cells (Verma and Datta, 2010). Here we demonstrate that also in insulin-producing rat pancreatic RINm5F cells, IL-1β–induced JNK1/2 activation precedes the induction of ER stress markers (Figure 1A and B). Whereas JNK1/2 activation in the presence of IL-1β was visible at 2 h of incubation, the up-regulation of ER stress markers was observed starting only at 4 h.

stress, which involves the participation of several key molecules, namely IRE-1α, PERK, ATF6, and their associated downstream intermediates (Nakatani et al., 2005; Marchetti et al., 2007). These act as proapoptotic signals, leading to caspase activation, which culminates in apoptosis of the pancreatic β-cells.

A very significant cellular phenomenon evident during these events is the cross-talk between the ER and mitochondria (Kornmann and Walter, 2010). These organelles exhibit direct physical contact regions that demonstrate dynamic interorganellar structural and functional correlation, and this is believed to be responsible for diverse arrays of cellular alterations that are critical during cell death (Pizzo and Pozzan, 2007; Csordas et al., 2010). In addition, both organelles are key players in buffering Ca2+ levels in general and in pancreatic β-cells in particular. Under pathological conditions, cellular Ca2+ imbalance leads to increased mitochondrial Ca2+ uptake, which initiates the apoptotic program within the cell. Reports suggest a role of Ca2+ in IL-1β–mediated pancreatic β-cell death, and the stress kinase JNK is believed to be critical in this event (Storling et al., 2005), with JNK inhibition preventing the IL-1β effect (Bonny et al., 2000; Major and Wolf, 2001). In addition, IL-1β induces ER stress, which activates JNK and initiates the apoptotic program in pancreatic β-cells (Collier et al., 2011).
of incubation. To further confirm this event, we used JNK1/2 small interfering RNA (siRNA; also written as JNK1/2 siRNA I) and evaluated the levels of ER stress markers. Figure 1C shows the dose-dependent effect of JNK1/2 siRNA I on total JNK1/2 levels. Maximum inhibition of total JNK1/2 levels was observed at 100 nM (Figure 1C). We therefore used this concentration of JNK1/2 siRNA I for all subsequent experiments. In addition, at this concentration of JNK1/2 siRNA, phosphorylation of the primary target of JNK, that is, c-jun, was markedly inhibited (Figure 1D). JNK1/2 also mediated IL-1β-induced c-jun phosphorylation, as inhibition of JNK1/2 with JNK1/2 siRNA I prevented this effect (Figure 1E). This pattern of c-jun phosphorylation by IL-1β was identical to IL-1β–induced JNK1/2 phosphorylation (Figure 1A), suggesting that such JNK1/2 phosphorylation mediates c-jun phosphorylation. Further, in RINm5F cells pretreated with the JNK1/2 siRNA I before IL-1β, up-regulation of IL-1β–induced ER stress markers was completely prevented (Figure 1F). All of this suggests that in RINm5F cells, JNK1/2 mediates IL-1β–regulated ER stress induction.

We further evaluated the effect of IL-1β on mitochondrial dysfunction and the contribution of JNK1/2–mediated ER stress to this. RINm5F cells were exposed to IL-1β for various times (0, 2, 8, 12, 24, and 36 h), and mitochondrial membrane potential, \( \Delta \psi_m \), was measured using flow cytometry analysis of 5,5′,6′,6′-tetrachloro–1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1) fluorescence. Normal JC1 aggregates are measured by red fluorescence, and nonaggregate forms under stress are measured by increasing green fluorescence. As compared with control cells, in IL-1β–treated RINm5F cells, an increase in the nonaggregate form of JC-1 (as measured by increased green fluorescence) was observed, suggesting altered mitochondrial membrane potential (Figure 2, A and B). This increase was visible only at 36 h of incubation, and, surprisingly, in cells incubated with IL-1β in the presence of JNK1/2 siRNA, this disturbance in membrane potential was completely prevented and cells showed positive membrane potential similar to that of control cells (as evident by the presence of red J aggregates), suggesting that JNK1/2 is involved in IL-1β–induced alteration of \( \Delta \psi_m \) (Figure 2, A and B). To substantiate these observed mitochondrial alterations, we evaluated the effect on mitochondrial permeability transition pore (mPTP) opening, a significant mitochondrial dysfunction event that leads to loss in \( \Delta \psi_m \) and release of cytchrome c (Green and Kroemer, 2004; Tait and Green, 2010). mPTP opening was assessed by flow cytometry analysis, and in the presence of IL-1β, mitochondrial fluorescence (as detected by calcine-AM fluorescence in the presence of CoCl\(_2\)) was significantly decreased at 36 h of incubation (Figure 2C). This suggests that IL-1β causes a significant increase in mPTP opening, which results in loss of mitochondrial fluorescence. This was prevented by the presence of JNK1/2 siRNA I, indicating a role of JNK1/2 in the increased opening of mPTP by IL-1β.

IL-1β causes ATP depletion and ROS (superoxide) generation in a JNK1/2–dependent manner

To evaluate the effects of IL-1β and JNK1/2 on other mitochondrial parameters, we assessed the effect of these on their ATP content and ROS production. As shown in Figure 2D, IL-1β led to a significant decrease in mitochondrial ATP content in RINm5F cells as measured by ATP determination bioluminescence assay. A time-dependent decrease in ATP content was observed starting from 12 h of IL-1β treatment, which further significantly decreased at 24 h and then plateaued until 36 h. However, in the presence of JNK1/2 siRNA, this decrease was significantly prevented (Figure 2D), suggesting a critical role of JNK1/2 in this mitochondrial activity.

Because mitochondria contribute to a major part of cellular free radical generation, we studied the effect of IL-1β and JNK1/2 inhibition on this mitochondrial event. We used the mitochondrial ROS-detecting agent MitoSox Red in combination with MitoTracker Green FM (which localizes to the mitochondria) to identify mitochondrial ROS generation. In cells treated with IL-1β, there was a marked increase in MitoSox fluorescence that colocalized with mitochondria (as tracked by the MitoTracker Green dye), suggesting that mitochondrial ROS generation was significantly increased in the presence of IL-1β (Figure 2E). At 24 h, mitochondrial ROS generation increased, which was further enhanced at 36 h. JNK1/2 siRNA prevented this IL-1β–mediated increase in mitochondrial ROS generation (Figure 2E). Taken together, these data suggest that IL-1β caused mitochondrial dysfunction in RINm5F cells and that JNK1/2 is a significant mediator in the effect.

IL-1β depletes ER Ca\(^{2+}\) in RINm5F cells

Because cellular Ca\(^{2+}\) levels are believed to be critical during mitochondrial dysfunction and cellular bioenergetics (Mbaya et al., 2010), we further sought to determine the cellular calcium dynamics in the presence of IL-1β and JNK1/2 siRNA I that might influence mitochondrial dysfunction as observed in Figure 2. In addition to the JNK1/2 siRNA I described earlier, we used a second JNK1/2 siRNA (JNK1/2 siRNA II) to confirm the specificity of JNK1/2 inhibition. In the presence of JNK1/2 siRNA II, there was a dose-dependent decrease in total JNK1/2 levels (Figure 3A) in RINm5F cells. Incubation of RINm5F cells with IL-1β led to marginal increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{cyt}\)]) levels at 4 h, and this greatly increased at 8 h of incubation and later decreased at 12 and 24 h (Figure 3B). This increase could be prevented by both JNK1/2 siRNA I and JNK1/2 siRNA II, and the gradual increase in cytosolic Ca\(^{2+}\) starting at 4 h is in accordance with our previous study (Verma and Datta, 2010), where we showed that induction of ER stress markers by IL-1β is evident starting from 4 h of incubation. Hence, we believe that IL-1β–induced ER stress is responsible for the release of Ca\(^{2+}\) from the ER to the cytosol. The decrease in cytosolic Ca\(^{2+}\) at later times, that is, 12 and 24 h, indicates that at these times, Ca\(^{2+}\) is either sequestered back into the ER or is taken up by the mitochondrion, which is evident during various pathophysiological conditions (Csordás et al., 2006).

To validate these results, we performed an independent experiment in RINm5F cells in which cells incubated with IL-1β for 2, 4, 8, 12, and 24 h were stained with Fluo-4 for 30 min at 37°C. Cells were then stimulated with thapsigargin (1 mM) to deplete ER Ca\(^{2+}\) stores. Cells incubated in the absence (0 h) or presence of IL-1β for 2 h showed a sharp increase in [Ca\(^{2+}\)\(_{cyt}\)] after exposure to thapsigargin, indicating that IL-1β at 2 h of incubation did not deplete the ER of endogenous Ca\(^{2+}\) levels (Figure 3C). Subsequently, at 4 h of incubation, thapsigargin induced a relatively blunt [Ca\(^{2+}\)\(_{cyt}\)] peak, which at later times plateaued, suggesting that some leakage of ER Ca\(^{2+}\) had initiated in the presence of IL-1β at 4 h of incubation. Further, at 8, 12, and 24 h of IL-1β incubation, thapsigargin failed to increase [Ca\(^{2+}\)\(_{cyt}\)] levels, although a very small peak was observed at 8 h of incubation (Figure 3C). These results indicate that IL-1β starts depleting ER Ca\(^{2+}\) at 4 h, and by 8 h, almost complete depletion is observed. At 12 and 24 h, a completely flat pattern of [Ca\(^{2+}\)\(_{cyt}\)] in the presence of thapsigargin is observed, indicating that at these times, the ER is completely depleted of Ca\(^{2+}\). In addition, this proves that the decrease in [Ca\(^{2+}\)\(_{cyt}\)] observed at 12 and 24 h of IL-1β incubation (Figure 3B) is not because of the Ca\(^{2+}\) going back to the ER.

To validate this, we evaluated mitochondrial Ca\(^{2+}\) levels ([Ca\(^{2+}\)\(_{mito}\)]) in the presence and absence of IL-1β using the specific mitochondrial Ca\(^{2+}\) dye Rhod-2. As depicted in Figure 3D, at 2–8 h of IL-1β
incubation, $[Ca^{2+}]_m$ is comparable to those of control cells; however, at 12 and 24 h of incubation, there is a significant increase. That this is also mediated by JNK1/2 is evident because JNK1/2 siRNA I and JNK1/2 siRNA II prevent these increases in mitochondrial Ca$^{2+}$ uptake (Figure 3D). Taken together, these results suggest that the decrease in cytoplasmic Ca$^{2+}$ that we observed at 12 and 24 h as shown in Figure 3B was presumably because they were taken up by the mitochondria. To further confirm the presence of calcium within the mitochondria, we incubated RINm5F cells in the presence of IL-1$\beta$ with or without JNK1/2 siRNA and double loaded them with MitoTracker Green dye (a mitochondrion-specific marker) and Rhod-2. An arrangement of mitochondrial threads was stained with MitoTracker Green dye (Figure 3E). Further, at 12 and 24 h of IL-1$\beta$ treatment, $[Ca^{2+}]_m$ staining also colocalized with these MitoTracker Green–stained mitochondrial threads (Figure 3E), suggesting the presence of Ca$^{2+}$ within the mitochondria.
Although this suggests a possible correlation between the decrease in \([\text{Ca}^{2+}]_c\) and the increase in \([\text{Ca}^{2+}]_m\), it does not establish that the fall in \([\text{Ca}^{2+}]_c\) is due to mitochondrial \([\text{Ca}^{2+}]_m\) uptake. To determine this, we preincubated RINm5F cells with Ru 360, a mitochondrial \([\text{Ca}^{2+}]_m\) uptake inhibitor, before IL-1\(\beta\) incubation. We then assessed \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_m\) levels with Fluo-4 and Rhod-2, respectively. In the absence of Ru360, IL-1\(\beta\)–mediated \([\text{Ca}^{2+}]_m\) movement demonstrated an identical pattern as in Figure 3. At 8 h of IL-1\(\beta\) incubation, there was a significant increase in \([\text{Ca}^{2+}]_c\), and this was followed by a decrease at 24 h of incubation that was accompanied by a concomitant increase in \([\text{Ca}^{2+}]_m\) (Figure 4A). However, in cells pretreated with mitochondria. This colocalized image validated our data of Figure 3D that at 12 and 24 h, the increased fluorescence of Rhod-2 reflects an increased presence of \([\text{Ca}^{2+}]_c\) within the mitochondria. This was also prevented in cells preincubated with JNK1/2 siRNA indicating that IL-1\(\beta\)–induced \([\text{Ca}^{2+}]_m\) uptake is mediated by JNK1/2 (Figure 3E, right).

**IL-1\(\beta\)–mediated decrease in \([\text{Ca}^{2+}]_c\) is due to mitochondrial \([\text{Ca}^{2+}]_m\) uptake**

As shown in Figure 3, the decrease in \([\text{Ca}^{2+}]_c\) at 12 and 24 h of IL-1\(\beta\) incubation was accompanied by a parallel increase in \([\text{Ca}^{2+}]_m\). Although this suggests a possible correlation between the decrease in \([\text{Ca}^{2+}]_c\) and the increase in \([\text{Ca}^{2+}]_m\), it does not establish that the fall in \([\text{Ca}^{2+}]_c\) is due to mitochondrial \([\text{Ca}^{2+}]_m\) uptake. To determine this, we preincubated RINm5F cells with Ru 360, a mitochondrial \([\text{Ca}^{2+}]_m\) uptake inhibitor, before IL-1\(\beta\) incubation. We then assessed \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_m\) levels with Fluo-4 and Rhod-2, respectively. In the absence of Ru360, IL-1\(\beta\)–mediated \([\text{Ca}^{2+}]_m\) movement demonstrated an identical pattern as in Figure 3. At 8 h of IL-1\(\beta\) incubation, there was a significant increase in \([\text{Ca}^{2+}]_m\), and this was followed by a decrease at 24 h of incubation that was accompanied by a concomitant increase in \([\text{Ca}^{2+}]_m\) (Figure 4A). However, in cells pretreated with...
Ru360, [Ca\(^{2+}\)]\(_m\) levels demonstrated a similar pattern of increase at 8 h of incubation; at 24 h, the [Ca\(^{2+}\)]\(_m\) levels did not increase, and the elevated levels of [Ca\(^{2+}\)]\(_c\) were persistent until 24 h of incubation (Figure 4B). These results suggest that the decrease in [Ca\(^{2+}\)]\(_c\) observed at 12 and 24 h of IL-1\(\beta\) incubation is due to the Ca\(^{2+}\) being taken up by the mitochondria.

IL-1\(\beta\) increases [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\) by ER Ca\(^{2+}\) release via IP\(_3\)R

To further decipher the details of the contribution of the ER in these calcium movements, we studied ER Ca\(^{2+}\) release using specific ER Ca\(^{2+}\)-channel blockers. Two types of Ca\(^{2+}\) channels are responsible for the release of Ca\(^{2+}\) from the ER: the ryanodine receptor (RyR) and the inositol triphosphate receptor (IP\(_3\)R). We used channel-specific inhibitors, namely dantrolene and ryanodine (for RyR) and xestospongin C (for IP\(_3\)R), to study their effects on [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\). Incubation of cells with either dantrolene or ryanodine (10 μM) could not prevent IL-1\(\beta\)-mediated increase in either cytoplasmic or mitochondrial Ca\(^{2+}\) levels (Figure 5, A and B). However, in the presence of xestospongin C (1 μM), IL-1\(\beta\)-mediated increase in Ca\(^{2+}\) levels in the cytosol and mitochondria was completely abrogated (Figure 5, C and D). These results indicate that the increase in [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\) that we observed (Figure 3) was due to their increased release from the ER via the IP\(_3\)R.

IL-1\(\beta\) increases the level of IP\(_3\)R-I isoform, which increases [Ca\(^{2+}\)]\(_c\)

Because we observed that IL-1\(\beta\)-mediated increases in [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\) were due to ER Ca\(^{2+}\) release through the IP\(_3\)R receptor channels, we further sought to determine the mechanism of this increased Ca\(^{2+}\) release. As shown in Figure 5E, in the presence of IL-1\(\beta\), there was a significant increase in the level of IP\(_3\)R-I at 8 h of incubation, which increased further at 12 h. However, the levels of the other isoforms, that is, IP\(_3\)R-II and IP\(_3\)R-III, were unchanged at all the time points studied. In fact, use of IP\(_3\)R-I siRNA prevented IL-1\(\beta\)-induced increases in [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\) (unpublished data).

Taken together, these results suggest that IL-1\(\beta\) induces elevations in [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\) by up-regulating the levels of IP\(_3\)R-I on the ER membrane.

IP\(_3\)R inhibition abrogates IL-1\(\beta\)-mediated mitochondrial dysfunction

We observed that Ca\(^{2+}\) release from the ER through IP\(_3\)R leads to increased cytoplasmic and mitochondrial Ca\(^{2+}\) levels during IL-1\(\beta\) action. To evaluate the role of IP\(_3\)R inhibition in IL-1\(\beta\)-mediated
mitochondrial dysfunction, we assessed the two critical events, mPTP opening and $\Delta \psi_m$, in the presence of the IP$_3$R inhibitor xestospongin C. RINm5F cells were preincubated with xestospongin C (1 μM) and then incubated with IL-1β (2 ng/ml) at various times. On termination of the incubation, $\Delta \psi_m$ and mPTP were studied. As shown in Figure 6, xestospongin C treatment before IL-1β prevented IL-1β-mediated disturbance in $\Delta \psi_m$ and opening of the mitochondrial pore, suggesting that inhibition of Ca$^{2+}$ release from the ER could prevent IL-1β-mediated mitochondrial alterations. These results suggest that ER Ca$^{2+}$ release via IP$_3$R triggered by IL-1β leads to increased cytoplasmic and mitochondrial Ca$^{2+}$ levels associated with mitochondrial dysfunction.

**JNK1/2 siRNA and xestospongin C prevent IL-1β-mediated apoptosis**

Further, to finally correlate these events with IL-1β-mediated apoptosis, we studied the effect of JNK1/2 and IP$_3$R inhibition on these phenomena. RINm5F cells were treated with IL-1β in the absence or presence of either JNK1/2 siRNA I or xestospongin C. On termination of incubation, cells were labeled with annexin V–fluorescein isothiocyanate (FITC), and IL-1β treatment alone showed a significant increase in annexin V–positive cells in a time-dependent manner (at 24 and 36 h). As compared with the control cells, IL-1β incubation showed 80% of annexin V–positive cells, including early and late apoptotic cells (Figure 7, A and B) at 36 h. However, in cells preincubated with JNK1/2 siRNA I or xestospongin C, this increase in IL-1β–mediated annexin V–positive apoptotic cells was completely prevented, suggesting a critical role of JNK1/2 and IP$_3$R in the apoptotic effect of IL-1β. In the presence of JNK1/2 siRNA I or the IP$_3$R inhibitor, only 12 and 17%, respectively, of the cells were annexin V positive even in the presence of IL-1β, as opposed to 80% apoptotic cells in the presence of IL-1β alone.

The antiapoptotic effect of JNK1/2 siRNA I was also confirmed by caspase 3 activation assay. Whereas IL-1β led to significant caspase 3 activation at 24 and 36 h of incubation, this effect was completely prevented in cells incubated with JNK1/2 siRNA (Figure 7C). These observations suggest a protective effect of these inhibitors on IL-1β–induced apoptotic cell death.

**JNK1/2 mediate IL-1β-induced alterations in human primary β-cells, and their inhibition restores glucose-mediated insulin release**

All of the results described so far are for insulin-secreting RIN5mF cells. To validate them and substantiate their physiological relevance, we used human primary β-cells and incubated them with IL-1β (2 ng/ml) in the presence and absence of JNK1/2 siRNA I (100 nM). In the presence of JNK1/2 siRNA I, there was a significant decrease in the levels of JNK1/2 in primary human β-cells.
cantly at 36 h (Figure 8E); however, in the presence of JNK1/2 siRNA I, this decrease was completely prevented. Further, as seen in Figure 8, F and G, IL-1β significantly increased the amount of monomeric green JC1 (R2) at 36 h, which suggested altered mitochondrial membrane potential, and this was prevented in the presence of JNK1/2 siRNA I. Slight increases were observed at earlier times that significantly increased at 24 and 36 h, and they were prevented in the presence of JNK1/2 siRNA I. These results suggest that in primary β-cells, IL-1β alters cellular Ca²⁺ movement and induces mitochondrial dysfunction, specifically, altered mitochondrial membrane potential and mPTP opening, in a JNK1/2-dependent manner.

To evaluate physiological relevance, we incubated human primary β-cells with IL-1β for 36 h with and without JNK1/2 siRNA I and then stimulated them with basal or increased concentrations of glucose (25 mM). On termination of incubation, the media were collected, and the insulin released into the medium was assessed as

(Figure 8, A and B). Further, IL-1β significantly increased [Ca²⁺]c in primary β-cells at 4 h, which further increased at 8 h and then declined at 12 and 24 h (Figure 8C). This pattern of [Ca²⁺]c increase, in an identical manner as described for RINm5F cells, suggests that IL-1β–mediated ER Ca²⁺ release is initiated at 4 h of incubation. To further prove that the decrease in [Ca²⁺]c at 12 and 24 h is due to its import into the mitochondria, we evaluated human primary β-cells incubated with IL-1β in the absence of [Ca²⁺]c, and [Ca²⁺]m increases were prevented in the presence of JNK1/2 siRNA I, indicating a mediating role of JNK1/2 during IL-1β action on Ca²⁺ movement within the β-cell. In the mPTP opening assay as determined by flow cytometry analysis, IL-1β led to a modest decrease in mitochondrial fluorescence (as seen in the presence of calcein-AM) at 24 h, which further decreased significantly at 36 h (Figure 8E); however, in the presence of JNK1/2 siRNA I, this decrease was completely prevented. Further, as seen in Figure 8, F and G, IL-1β significantly increased the amount of monomeric green JC1 (R2) at 36 h, which suggested altered mitochondrial membrane potential, and this was prevented in the presence of JNK1/2 siRNA I. Slight increases were observed at earlier times that significantly increased at 24 and 36 h, and they were prevented in the presence of JNK1/2 siRNA I. These results suggest that in primary β-cells, IL-1β alters cellular Ca²⁺ movement and induces mitochondrial dysfunction, specifically, altered mitochondrial membrane potential and mPTP opening, in a JNK1/2-dependent manner.

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interacting factors (Mokhtari et al., 2008) that includes primarily NF-κB, mitogen-activated protein kinase (MAPK), NO, and so on that coordinate to lead to apoptotic cell death. Although it is well studied and reported that induction of ER stress activates JNK through activated IRE-1 (Urano et al., 2000), we reported that it is JNK1/2 activation that mediates IL-1β-mediated ER stress induction (Verma and Datta, 2010).

The present study demonstrates that JNK1/2 catalyzes IL-1β-mediated mitochondrial dysfunction. IL-1β-mediated decrease in mitochondrial ATP generation and increase in ROS production were completely prevented by JNK1/2 inhibition. The increase in mitochondrial pore opening and membrane potential disturbance also displayed similar patterns, suggesting that JNK1/2 mediates these mitochondrial alterations. IL-1β has been reported to disrupt mitochondrial membrane potential and cause ATP deprivation, leading to cell death in chondrocytes (Yasuhara et al., 2005; Kim et al., 2010).

In insulin-secreting INS-1 cells, IL-1β markedly reduces mitochondrial membrane potential and metabolic activity (Veluthakal et al., 2005). These studies suggest that IL-1β induces mitochondrial dysfunction and apoptosis.

**DISCUSSION**

A previous study from our laboratory showed that IL-1β mediates pancreatic cell death via induction of ER stress in a JNK1/2-dependent manner (Verma and Datta, 2010). In the present study, we determined the consequent cellular effects of this IL-1β action and the contribution of JNK1/2 to it. To this end, we report the effect of IL-1β and JNK1/2 on ER Ca²⁺ release, mitochondrial dysfunction, and pancreatic β-cell apoptotic death. Elevated levels of IL-1β are believed to significantly contribute to β-cell death (Maedler et al., 2002), and this involves the participation of an intricate network of interacting factors (Mokhtari et al., 2008) that includes primarily NF-κB, mitogen-activated protein kinase (MAPK), NO, and so on that coordinate to lead to apoptotic cell death. Although it is well studied and reported that induction of ER stress activates JNK through activated IRE-1 (Urano et al., 2000), we reported that it is JNK1/2 activation that mediates IL-1β-mediated ER stress induction (Verma and Datta, 2010).

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In insulin-secreting INS-1 cells, IL-1β markedly reduces mitochondrial membrane potential and metabolic activity (Veluthakal et al., 2005). These studies suggest that IL-1β induces mitochondrial dysfunction.

**FIGURE 7:** IL-1β induces apoptosis in RINm5F cells that is prevented in the presence of JNK1/2 siRNA and xestospongin C. (A) RINm5F cells were transfected with JNK1/2 siRNA I (100 nM) or preincubated with xestospongin C (1 μM) and then incubated in the absence (0 h) or presence of IL-1β (2 ng/ml) for 2, 8, 12, 24, and 36 h. On termination of incubation, cells were washed and labeled with annexin V and PI. After 15 min of incubation, cells were analyzed for apoptosis by flow cytometry. Experiments were done three times, and a representative figure is shown. (B) Quantitative data of apoptotic cells (percentage of control) of the incubation as shown in A. (C) Cells incubated with IL-1β with or without JNK1/2 siRNA I for 24 and 36 h were evaluated for apoptosis using the caspase 3 activation assay kit. Each value is the mean ± SEM of three experiments and is presented with respect to control (0 h). ***p < 0.001, **p < 0.01, and *p < 0.05 as compared with control (0-h incubation); ##p < 0.001, #p < 0.01, and $p < 0.05 as compared with the incubation with IL-1β plus scramble at the same time points.
In spite of being an independent organelle and exhibiting an independent contributory role during apoptosis, the ER also experiences stress that contributes to cell death. Many cellular disturbances that interfere with the normal functioning of the ER cause accumulation of unfolded proteins that trigger the unfolded protein response, which, when compromised, can eventually trigger apoptotic cell death. Diverse mechanisms are believed to mediate this ER stress-induced cell death, including but not limited to activation of proteases, transcription factors, kinases, and Bcl-2 family protein (Pirot et al., 2007).

In addition to the mitochondrial alterations, the ER also experiences stress that contributes to cell death. Many cellular disturbances that interfere with the normal functioning of the ER cause accumulation of unfolded proteins that trigger the unfolded protein response, which, when compromised, can eventually trigger apoptotic cell death. Diverse mechanisms are believed to mediate this ER stress-induced cell death, including but not limited to activation of proteases, transcription factors, kinases, and Bcl-2 family protein (Pirot et al., 2007).
dependent MAPK and is a critical mediator of the cellular effects of chondria occurs in a JNK1/2-dependent manner. JNK is a stress-

Our data show that IL-1β–mediated ER stress leads to increased Ca\(^{2+}\) release via the IP\(_3\)R. IP\(_3\)R\(_S\) are the primary ER Ca\(^{2+}\) release channels in several tissues (Lee and Laychock, 2000). In mammals, there are primarily three subtypes of IP\(_3\)R (IP\(_3\)R\(_{I}\), II, and III), and their genes share 70–80% similarity in primary sequence (Marchi et al., 2012). However, they differ in localization, regulation, and modulation. Our data show that IL-1β leads to significant up-regulation of the levels of IP\(_3\)R\(_I\) without altering the levels of IP\(_3\)R\(_II\) and III in RINm5F cells. Such transcriptional regulation of IP\(_3\)R by IL-1β has also been shown in osteoblasts and astrocytic cell cultures (Pita et al., 1999; Bradford et al., 2000). These increases in IP\(_3\)R levels by IL-1β are responsible for increased ER Ca\(^{2+}\) release by IL-1β, which is taken up by the mitochondria, leading to mitochondrial dysfunction. The IP\(_3\)R receptor blocker xestospongin C can prevent ER Ca\(^{2+}\) release, its uptake into the mitochondria, and subsequent mitochondrial dysfunction, indicating ER–mitochondrial interplay in the IL-1β–induced Ca\(^{2+}\) movements within the cell. The Ca\(^{2+}\) that is normally maintained at a low concentration in the cytosol increases as a result of its release from the ER (Filippin et al., 2003), which is then targeted to the mitochondria under cellular stresses, specifically through voltage-dependent anion channels and mitochondrial calcium uniporters (Rapizzi et al., 2002; Kirichok et al., 2004).

Increased Ca\(^{2+}\) release from the ER leads to the formation of microdomains on the mitochondrial membrane to additionally facilitate mitochondrial Ca\(^{2+}\) uptake (Giorgetti et al., 2009; Rizzuto et al., 2009). Of interest, our results show that in the presence of JNK1/2 siRNA, both ER Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) uptake are completely prevented. Taken together, our results indicate that IL-1β–mediated Ca\(^{2+}\) release and its consequent uptake by the mitochondria occurs in a JNK1/2-dependent manner. JNK is a stress-dependent MAPK and is a critical mediator of the cellular effects of IL-1β (Bonny et al., 2000). Three different isoforms of JNK have been identified, JNK1, JNK2, and JNK3 (Abdelli et al., 2009), and JNK1 and JNK2 are believed to be ubiquitous. The presence of JNK3 was previously believed to be restricted to the neurons (Davis, 2000), but recent reports suggest that its levels also are high in pancreatic β-cells (Abdelli et al., 2009; Abdelli and Bonny, 2012). However, whereas JNK1/2 have been involved in sensitizing β-cells to apoptotic cell death, JNK3 acts to protect β-cells against cytokine-induced cellular dysfunction and cell death mainly by maintaining a normal IRS2/Akt signaling cascade (Abdelli and Bonny, 2012). We earlier showed that JNK1/2 catalyzes IL-1β–induced ER stress. This observation was very significant in the midst of diverse reports that described the activation of JNK by ER stress–mediated activation of IRE-1 (Urano et al., 2000). The present study describes the mediating role of JNK1/2 during IL-1β induced ER stress, which is translated further into ER Ca\(^{2+}\) release, its uptake into the mitochondria, exertion of mitochondrial dysfunctions, and finally apoptotic cell death (Figure 9).

Persistent uptake of Ca\(^{2+}\) into the mitochondria leads to opening of the mPTP, disturbed mitochondrial membrane potential, and induction of apoptosis (Hajnoczky et al., 2006). On encountering any death stimuli, mitochondria release the apoptosis-inducing factor and generate ROS, which triggers cell death (Rizvi et al., 2011). Such regulatory roles of mitochondria and loss of its integrity and function are evident in diverse pathological states (Wang et al., 2008), and during pancreatic β-cell death, as is seen in the present study. IL-1β exerts its effect by inducing severe mitochondrial alterations. This event, which is accompanied by ER Ca\(^{2+}\) release and its uptake by the mitochondria, is regulated by JNK1/2; therefore JNK1/2 might be an attractive target for therapeutic intervention to prevent pancreatic β-cell death.

To conclude, our study reveals JNK1/2-dependent ER–mitochondrial Ca\(^{2+}\) cross-talk during IL-1β action in pancreatic β-cells. This identifies JNK1/2 as a central key molecule in the detrimental effects of IL-1β and therefore suggests that IL-1β–mediated JNK1/2 activation is a significant event that might be targeted to prevent the deleterious apoptotic effects of ER–mitochondrial Ca\(^{2+}\) cross-talk.

MATERIALS AND METHODS

Cell culture and incubations

All experiments were done in primary human β-cells (described later) and the rat islet-derived, insulin-producing cell line RINm5F. RINm5F cells were procured from the National Center for Cell Science (Pune, India) and were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratory, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in the presence of 10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid and 2 mM L-glutamine at 37°C and 5% CO\(_2\). On attaining confluence, cells were transferred to serum-free medium containing IL-1β (2 ng/ml) and incubated for various time periods. For
experiments with JNK1/2, cells were transfected with either the control (scramble) or JNK1/2 siRNA (25–100 nM; Cell Signaling Technology, Beverly, MA, or Sigma-Aldrich) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To confirm the specificity of JNK1/2 inhibition, two different siRNAs, JNK1/2 siRNA I and siRNA II, were used in the experiments. After growing in fresh RPMI for 36 h, cells were incubated in the absence or presence of IL-1β as described. This dose of IL-1β and JNK1/2 siRNA incubation was chosen according to an earlier report from our laboratory (Verma and Datta, 2010).

**Determination of JNK1/2 activation and ER stress induction**

Confluent RINm5F cells were incubated without or with IL-1β (2 ng/ml) for 2, 4, 8, 12, and 24 h. On termination of incubation, cells were lysed, and lysates (60 μg of protein) were evaluated for the levels of p-JNK1/2 and total JNK1/2 levels by Western blot using specific antibodies (Cell Signaling Technology). In another set of experiments, total RNA was isolated from RINm5F cells identically incubated with IL-1β, and the levels of ER stress markers namely Bip and CHOP were determined by quantitative real-time (RT)-PCR using gene-specific primers (Bip: sense, 5′TCGGACTGATGTCATGAGG3′; antisense, 5′ATATCCAGGCTATGCAATGAG3′; CHOP, sense, 5′CCAGCAGGTCCAAAGCAC3′; and antisense, 5′CGCAGCTGAACCTCTGTCTC3′). To determine the optimum concentration of JNK1/2 siRNA to be used, cells were transfected with 25–100 nM of the siRNA, and after 36 h, the levels of total JNK1/2 were determined by Western blot. The effectiveness of the JNK1/2 siRNA was corroborated by its effect on c-jun and p-c-jun, the immediate target of JNK, by Western blot using specific antibodies (Cell Signaling Technology). Immunoreactive bands were detected using 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium BCIP/NBT; the phosphorylated protein forms were detected using the ECL Western Blotting Kit (Pierce, Thermo Scientific, Rockford, IL), and the membranes were then stripped and reprobed identically for the respective total proteins. Glyceraldehyde-3-phosphate dehydrogenase was taken as the loading control.

**Mitochondrial membrane potential determination**

Mitochondrial membrane potential was measured using the mitochondrial voltage-sensitive dye JC-1 (Molecular Probes, Eugene, OR; excitation 498 nm/emission 525 nm). RINm5F cells were cultured in 12-well plates and stimulated with IL-1β as described. This dose of IL-1β and JNK1/2 siRNA incubation was chosen according to the manufacturer's instructions. Luminescence was read on an Infinite M200 multiplate reader (Tecan, Männedorf, Switzerland), and values were calculated from an ATP standard curve and normalized to the total mitochondrial protein content.

**Mitochondrial ATP generation**

Mitochondrial ROS generation was detected using the MitoSox fluorescent marker (excitation 510 nm/emission 580 nm; Molecular Probes). RINm5F cells were seeded on Ibidi culture dishes (35 mm; Ibidi, Martinsried, Germany) and stimulated with IL-1β (2 ng/ml) in the presence and absence of JNK1/2 siRNA I. On termination of incubation, mitochondrial proteins were isolated using a mitochondria isolation kit (Pierce, Rockford, IL), and equal amounts of mitochondrial proteins were assayed for ATP content according to the manufacturer's instructions. Luminescence was read on an Infinite M200 multiplete reader (Tecan, Männedorf, Switzerland), and values were calculated from an ATP standard curve and normalized to the total mitochondrial protein content.

**Intracellular Ca²⁺ concentration measurement**

Intracellular calcium, [Ca²⁺], and mitochondrial, [Ca²⁺]m, calcium were measured using the fluorescent indicator Fluo-4 direct calcium assay kit and Rhod-2, respectively (Molecular Probes). RINm5F cells were cultured in 96-well (5000 cells) plates and were stimulated in the presence and absence of 2 ng/ml IL-1β for 0, 2, 4, 8, 12, and 24 h. To evaluate the contribution of ER Ca²⁺ channels on [Ca²⁺]m, and [Ca²⁺]l, cells were also treated with ER Ca²⁺-channel blockers dantrolene and ryanoindine (ryanoindine receptor blockers; 10 μM) for 5 min at 37°C and xestospongin C (IP3R blocker; 1 μM) for 15 min at 37°C. On termination of incubation, cells were loaded with MitoTracker Dye (50 nM; Molecular Probes; excitation 488 nm/emission 516 nm) and incubated for 20 min at 37°C, followed by 5 μM MitoSox (Invitrogen) in prewarmed RPMI 1640 medium for 10 min at 37°C. Cells were then washed twice with PBS and visualized under a live-cell imaging system (Eclipse Ti; Nikon, Melville, NY).

**Mitochondrial pore formation**

Mitochondrial permeability transition pore formation was measured using the mitochondrial Transition Pore Assay Kit (Molecular Probes) according to the manufacturer’s instructions. RINm5F cells were cultured in 12-well plates and stimulated with IL-1β (2 ng/ml) for 0, 24, and 36 h in the presence and absence of JNK1/2 siRNA I. The mPTP opening was measured by monitoring calcein-AM fluorescence in the absence and presence of CoCl₂. After completion of incubation, cells were washed twice with PBS and resuspended in prewarmed RPMI 1640 medium. The cells were then loaded with calcein-AM for 15 min at 37°C as per the manufacturer’s instructions. In another set, cells incubated identically were loaded with calcein-AM in the presence of CoCl₂. Cells were then washed thoroughly and centrifuged, and cell pellets were resuspended in PBS. Cells were analyzed for calcein-AM fluorescence by flow cytometry (FACSCalibur). The change in calcein AM fluorescence between calcein-AM alone and in combination with CoCl₂ indicates the continuous activation of mitochondrial permeability transition pores.
Yvon, Edison, NJ for 10 min. For the experiments with xestospongin C on mitochondrial physiology, cells were incubated with IL-1β with or without xestospongin C and evaluated for mPTP opening and for mitochondrial membrane potential as described.

Quantitative RT-PCR for IR3R isoforms
RINm5F cells were grown to confluence in six-well plates and incubated without or with IL-1β (2 ng/ml) for 8 and 12 h. On termination of incubation, total RNA was isolated using TRIzol (Invitrogen), and 2 μg of total RNA was reverse transcribed using random hexamers. The cDNA was subjected to quantitative RT-PCR using SYBR Green RT-PCR Master Mix (Applied Biosystems, Foster City, CA) and isoform-specific primers (Zhao et al., 2008; with slight modifications: IP3R-I, sense, 5′GAGATGAGCCTGCTGAAGTCAA3′, and antisense, 5′TGTGTGCTCTTCTGAGAAATGCA3′; IP3R-II, sense, 5′AGTACCTGGCCATCAACGAG′, and antisense, 5′TCTTGGTG-GGGATCTCAGCT3′; IP3R-III, sense, 5′AGACCGCGGCTACTATGAGAA3′, and antisense, 5′GTGAGAAGCTGGCAGATGGACGCT3′) according to the manufacturer's instructions (StepOne Plus RT-PCR system; Applied Biosystems). Data were analyzed using the Pfaffl method (Pfaffl, 2001) and normalized to 18S rRNA.

Live-cell imaging of [Ca2+]m using Rhod-2
To further validate the increase in mitochondrial calcium levels, we performed live-cell imaging for [Ca2+]m by incubating RINm5F cells grown in Ibidi culture dishes (35 mm) in the presence of IL-1β with or without JNK1/2 siRNA. We plated 50,000 cells in each well for the experiments. Cells were then loaded with the MitoTracker Dye (50 nM) and Rhod-2 (10 μM) for 15 min at 37°C and washed twice with Ca2+-free PBS to completely remove the excess dye solution. Hank’s balanced salt solution without phenol red was used as the imaging medium, and cells were then visualized in a fluorescence microscope (ApoChromatic, Nikon Eclipse Ti). CCCP was used as a reference control to deplete mitochondrial Ca2+ content. Images were taken at 60x magnification with a numerical aperture of 1.4 using a Nikon camera (DSQi1MC). Images were processed and quantified using Nikon Advanced element AR3.2 software.

In another experiment, cells were preincubated with Ru360 (a mitochondrial Ca2+ uptake inhibitor) at a dose of 10 μM and then incubated with IL-1β (2 ng/ml) for 8 and 24 h. On termination of incubation, cells were stained with Fluo-4 and Rhod-2, and [Ca2+]m and [Ca2+]i were imaged and their intensities quantified as described.

Detection of apoptosis by annexin V and caspase 3 activation
RINm5F cells were stimulated with IL-1β (2 ng/ml) for 0, 2, 8, 12, 24, and 36 h in the presence and absence of JNK1/2 siRNA I. They were then washed with PBS, scrapped, and centrifuged at 200 × g for 1 min. Apoptosis was detected using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions. Briefly, the cell pellet from each incubation was dissolved in the supplied binding buffer and centrifuged at 200 × g for 1 min, and the pellet was resuspended in 200 ml of binding buffer containing 5 μl of annexin V FITC and 5 μl of PI conjugate. These were incubated for 15 min at 25°C and analyzed for apoptosis by flow cytometry (FACSCalibur). The effect of xestospongin C (1 μM) on IL-β-mediated cellular apoptosis was assessed in an identical manner.

RINm5F cells were transfected with either the scramble or the JNK1/2 siRNA I and then incubated without or with IL-1β (2 ng/ml) for 24 and 36 h. On termination of incubation, cells were lysed, and equal amounts of protein from each incubation were evaluated for caspase 3 activation using the Caspase 3 Assay Kit (Sigma-Aldrich).

Primary human β-cell culture
Primary human β-cells were purchased from Applied Biological Materials (Richmond, Canada) and grown in Prigrow II medium (Applied Biological Materials) containing fetal bovine serum (10%) and antibiotics according to the manufacturer’s instructions. On attaining confluence, they were incubated in the absence or presence of IL-1β (2 ng/ml) for 2, 4, 8, 12, 24, and 36 h with or without JNK1/2 siRNA I (100 nM), and on termination of incubation, cells were evaluated for the presence of Ca2+ in the cytosol and mitochondria using Fluo-4 and Rhod-2 as described. Mitochondrial pore opening and determination of mitochondrial membrane potential in these cells was also studied under these conditions using the methods as described.

Glucose-induced insulin release assay
Human primary pancreatic β-cells were grown to confluence and transfected with JNK1/2 siRNA I (100 nM) using Lipofectamine 2000. After 36 h, cells were treated with IL-1β (2 ng/ml) for 36 h and then stimulated with basal or 25 mM glucose. After 2 h of incubation, the cells and media were collected for total protein content and insulin release, respectively. Insulin content was determined using the Insulin ELISA Kit (Glory Science, Del Rio, TX), and the values were normalized to the total protein content (measured by a BCA Kit; Pierce).

Statistical analysis
All incubations were done in triplicate, and statistical significance was calculated by the Student’s t test. A value of at least p < 0.05 was considered as statistically significant.

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