Nitric oxide mediates the survival action of IGF-1 and insulin in pancreatic β cells

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Abstract

Generation of low levels of nitric oxide (NO) contributes to beta cell survival in vitro. The purpose of this study was to explore the link between NO and the survival pathway triggered by insulin-like growth factor-1 (IGF-1) and insulin in insulin producing RINm5F cells and in pancreatic islets. Results show that exposure of cells to IGF-1/insulin protects against serum deprivation-induced apoptosis. This action is prevented with inhibitors of NO generation, PI3K and Akt. Moreover, transfection with the negative dominant form of the tyrosine kinase c-Src abrogates the effect of IGF-1 and insulin on DNA fragmentation. An increase in the expression level of NOS3 protein and in the enzyme activity is observed following exposure of serum-deprived RINm5F cells to IGF-1 and insulin. Phosphorylation of IRS-1, IRS-2 and to less extent IRS-3 takes place when serum-deprived RINm5F cells and rat pancreatic islets are exposed to either IGF-1, insulin, or diethylenetriamine nitric oxide adduct (DETA/NO). In human islets, IRS-1 and IRS-2 proteins are present and tyrosine phosphorylated upon exposure to IGF-1, insulin and DETA/NO. Both rat and human pancreatic islets undergo DNA fragmentation when cultured in serum-free medium and IGF-1, insulin and DETA/NO protect efficiently from this damage. We then conclude that generation of NO participates in the activation of survival pathways by IGF-1 and insulin in beta cells.

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1. Introduction

It is well substantiated that in type 1 diabetes apoptotic death plays an instrumental role in the destruction of pancreatic β-cells during the autoimmune attack [1,2]. On the other hand, the imbalance between insulin output and metabolic requirements distinctive of type 2 diabetes, worsen by both loss of β-cell mass due to apoptosis secondary to gluco-/lipo-toxicity and by the action of cytokines which interfere with survival and growth signalling pathways [1,3]. Therefore, activation of pancreatic β-cell survival pathways is crucial for facing noxious challenges that eventually lead to destruction of the endocrine pancreas in both type 1 and type 2 diabetes [4–8]. Previously, we showed evidences of a NO-dependent protective pathway in insulin secreting RINm5F cells [9,10]. This pathway involves the activation of phosphatidylinositol 3-kinase (PI3K) and Akt in a c-Src and insulin receptor substrate (IRS)-dependent manner [9,10]. In addition, the role played by IGF-1/insulin signalling pathway in the control of β-cell survival and growth has been studied by different groups [6,8,11–17]. Indeed, these hormones activate the PI3K/Akt in a variety of cell types through phosphorylation of IRS proteins [8,12,13,18–22]. In this respect, any link between NO and IGF-1/insulin survival pathways in β cells still remained to be explored. An activation
of nitric oxide synthase 3 (NOS3) by insulin has been reported in endothelial cells, and PI3K and IRS-1 proteins plays a relevant role in the transduction of this signal inside the cell [20,22–24]. We have shown that apoptosis induced during serum deprivation in insulin secreting RINm5F cells is blocked by exposure to low concentrations of the chemical NO donor DET/NO and by NOS3 overexpression; under such circumstances activation of c-Src, PI3K/Akt and Bad phosphorylation is required [9]. It is thus feasible that NO generation mediates the activation of IGF-1/insulin survival pathway in β-cells. In this study, we investigate the regulation of NO synthesis by IGF-1/insulin and its contribution to protection from apoptosis in insulin secreting cells and in rodent and human islets.

2. Materials and methods

2.1. Materials

Monoclonal anti-Phosphotyrosine (PY20), monoclonal anti-PARP and Diaminofluorescein-2-diacetate (DAF-2-DA) were from Calbiochem; SH6 was from Alexis Biochemicals. Polyclonal anti-NOS3, monoclonal anti-IRS-1 were from LabVision Corporation, Cleaved caspase-3 (Asp175) (5A1-1) antibody was from Cell Signaling Technology. Protein G Sepharose and PVDF transfer membrane were from Amersham Pharmacia Biotech. Dithyletheramine nitric oxide adduct (DETA/NO) was from RBI. RPMI 1640 with and without phenol red were from Cambrex. CMRL 1066 medium was from Mediatech, USA. Streptomycin, penicillin, glutamine, amphotericin B, cell death detection ELISA+/plus, 4-(2-aminoethyl)-benzenesulfonyl fluoride (pefablof), phosphatase A, aprotinin, and leupeptin and were from Roche. Polyclonal anti-IRS-2, polyclonal anti-IRS-3, monoclonal anti-Akt-1 and polyclonal anti-p-Akt (Ser473) were from Santa Cruz Biotechnology. IGF-1 and c-Src (K296R/Y528F) negative dominant in pUSEamp (+) were from Upstate Biotechnology. Affinity-purified FITC-conjugated goat anti-rabbit IgG was from Biosource. Lab-tek chamber slide was from Nalge Nunc International; Insulin, 2,3-diaminonaphthalene, N4-Monomethyl-L-Arginine (L-NMMA), LY-294002, anti-mouse and anti-rabbit IgG peroxidase conjugate, trypsin, and other chemicals were from Sigma. RINm5F rat cell line was from the American Type Culture Collection.

2.2. Islet isolation, β-cell line culture and transfection

Islets were isolated from male Wistar rats weighing 220–230 g as previously described [9]. Human islets were isolated from heart-beating cadaveric organ donors (n=4), the mean age of donors was 43.78±5.04 years with a mean BMI of 27.78±1.08 kg/m². The mean pancreas weight was 110.03±16.77 g and the mean islet weight was 2.5±0.2 μg. The mean insulin content was 2.8±0.3 μg per islet. It is thus feasible that NO generation mediates the activation of IGF-1/insulin survival pathway in β-cells. In this study, we investigate the regulation of NO synthesis by IGF-1/insulin and its contribution to protection from apoptosis in insulin secreting cells and in rodent and human islets.

Cells were cultured in diverse experimental conditions for 15 h. Medium was changed for a free-serum medium and incubated for 2 h. Nitrite accumulated in the medium during this period of time was measured by fluorometry with freshly prepared 2,3-diaminonaphthalene (50 μg/L in 0.69 M HCl) [25]. Generation of fluorescent 1-(H)-naphthotriazole was measured at excitation and emission wavelengths of 365 and 450 nm, respectively. NOS enzyme activity was expressed as pmol of nitrite produced per min and mg of protein. Standard curves were constructed with known amounts of sodium nitrite.

2.5. Nitric oxide detection with diaminofluorescein-2-diacetate (DAF-2-DA)

Cells were cultured in RPMI 1640 medium without phenol red in diverse experimental conditions for 18 h. The cells were then exposed to 10 μM DAF-2-DA for 30 min in humidified atmosphere of 5% CO₂ at 37 °C. As a negative control, cells were incubated in medium lacking DAF-2-DA. As positive control, cells were incubated in medium containing 10 μM DET/NO. At the end of the incubation period, cells were washed three times with PBS and examined on an inverted fluorescence microscope (Leica CTR-6000) equipped with fluorescent attachment. The images were acquired through LAS-AF software. Color composite pictures were processed using Adobe Photoshop CS (Adobe Systems).

2.6. Western blot

Cells were collected by centrifugation at 700 g for 3 min at 4 °C, washed twice with ice-cold PBS and centrifuged at 700 g for 3 min. The expression and phosphorylation of proteins were determined for Western blotting, as previously described [10].

2.7. Immunoprecipitation and detection of phosphorylated proteins

Batches of cells (3×10⁶) were placed in 50 μL of lysis buffer (20 mM HEPES pH 7.5, 10 mM EGTA, 40 mM glycerophosphate, 140 mM NaCl, 25 mM MgCl₂, 1 mM DTT, 2% Nonidet NP-40, 2 mM sodium orthovanadate, 50 μM phenarsine oxide, 1 mM pefablof, 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 100 U/ml aprotinin). Lysis was carried out at 4 °C for 1 h. Cell lysates were sonicated 3 times for 10 s on ice (Branson sonifier; duty cycle 100%, output control 1) and centrifuged at 15,000 g. After centrifugation, clarified supernatants were immunoprecipitated with primary antibody (2 μg/mg protein) for 4 h at 4 °C. 20 μl of 50% (V/V) protein G Sepharose was then added and the mixture was incubated for 4 h at 4 °C, followed by centrifugation to recover the protein G Sepharose pellets. The immunoprecipitates were washed 3 times with PBS and once with kinase reaction buffer (10 mM MOPS pH 7.5, 12.5 μM [γ-32P]-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na₃VO₄) and then suspended in 20 μl of kinase reaction buffer. The immunoprecipitates were used for detection of IRS-1, IRS-2, IRS-3 and Akt total protein and phosphorylated protein by western blot.

2.8. Detection of caspase 3 activation

RINm5F cells were grown on Lab-Tek chamber Slide, fixed with 4% of paraformaldehyde, and stained with rabbit Cleaved Caspase-3 (Asp175) primary antibodies. Control slides were incubated with anti-rabbit immunoglobulin G (IgG). Affinity-purified FITC-conjugated goat anti-rabbit IgG were directed against primary antibody. Mounting solution (4% propyl-gallacte (P/V), 90% Glycerol (V/V) and 10% PBS (V/V) was then added to the slides. After covering...
with slips, preparations were viewed on a Leica AF6000 microscope equipped with fluorescent attachment. The images were acquired through LAS-AF software. Color composite pictures were processed using Adobe Photoshop CS (Adobe Systems).

2.9. Other analyses

Data are mean±SD of at least 3 independent experiments, except where results of blots, in which case a representative experiment is depicted in the figures. Comparisons between group’s values were analyzed using one way analysis of variance (ANOVA). p≤0.05 was considered statistically significant.

3. Results

3.1. Protective effect of IGF-1 and insulin in serum-starved cells is dependent on PI3K, Akt and NO production

We first investigated the effect of IGF-1 and insulin exposure on RINm5F cells in serum-free culture conditions. As shown in Fig. 1A, serum deprivation leads to six-fold increase in the amount of fragmented DNA in RINm5F cells. The addition of IGF-1 and insulin to these serum-deprived cells induces a significant decrease in DNA fragmentation. However this protective effect disappears when the PI3K inhibitor LY-294002 is present in the culture, thus suggesting that PI3K mediates this action. Moreover, culture of RINm5F serum-starved cells with IGF-1/insulin cells in the presence of SH6, an inhibitor of Akt, leads to enhanced DNA fragmentation. This strong stimulatory effect of SH6 on DNA fragmentation suggests that basal Akt activation may also be critical in protecting β-cells. On the other hand, the protective effect of IGF-1 and insulin in serum-deprived cells can be partially inhibited by N'G-monomethyl L-arginine (L-NMMA), an inhibitor of NO production, which indicates that NO production is involved in the signalling process. Serum starvation is a good system to study the involvement of candidate factors in the control of β-cell fate. This is demonstrated by the fact that a significant increase in the amount of β-cell apoptosis can only be observed in serum treated cells at 2–4 times higher concentrations of LY-294002, SH6 and L-NMMA (data not shown). We have also previously described that serum starvation favours apoptosis by down-regulating Bcl-2, decreasing Bad phosphorylation and enhancing cytochrome c release from mitochondria to cytoplasm in this cell type [9]. Moreover, serum starvation leads to activation of additional cellular markers of apoptosis such as PARP cleavage and caspase 3 activation (Fig. 1, panels B and C). Again, PI3K, Akt and NOS inhibitors in the presence of IGF-1 and insulin trigger the activation of these apoptotic markers, which indicates that both IGF-1 and insulin are efficient inhibitors of the apoptotic cell death program in these cells in both early and late steps of the death program.

3.2. IRS-1 phosphorylation by IGF-1 and insulin is dependent on NO generation in RINm5F cells

We previously reported that in serum treated RINm5F cells, DETA/NO induces IRS-1 phosphorylation [9], furthermore the inhibition of PI3K by LY-294002 does not affect this phosphorylation, indicating that it is independent of PI3K [9]. To prove our hypothesis that the protective effect of IGF-1/insulin depends on NO generation, we explored if inhibition of NO production in serum-deprived RINm5F cells have any detrimental effect in the phosphorylation of IRS-1 induced by both IGF-1 and insulin. As shown in Fig. 2, 100 nM IGF-1 (panel A) and 10 nM insulin (panel B) elicit IRS-1 phosphorylation in serum-deprived RINm5F cells. But if we inhibit NO production with L-NMMA (400 μM), we partially suppress IRS-1 phosphorylation with no effect whatsoever in the level of IRS-1 expression. In contrast, in serum-deprived RINm5F cells IRS-1 phosphorylation obtained by either IGF-1 or insulin is not affected by the inhibition of PI3K with LY-294002 (20 μM).
IRS-1 phosphorylation is suppressed in cells overexpressing the negative dominant (nd) form of c-Src (RINm5F/nd-Src), suggesting that c-Src activation mediates the effect of IGF-1 and insulin on IRS-1 phosphorylation.

### 3.3. Effect of IGF-1, insulin and DETA/NO on IRS-2 and IRS-3 phosphorylation in RINm5F cells

We next examined the levels of phosphorylation of IRS-2 and IRS-3 after IGF-1 and insulin stimulation. IRS-2 and IRS-3 proteins are present in RINm5F cells in both experimental conditions, with and without serum in the culture medium. A substantial degree of phosphorylation of both IRS-2 and IRS-3 proteins is observed in serum cultured RINm5F cells and culture in the absence of serum leads to a marked decrease degree of phosphorylation of these proteins. However, while IRS-2 was phosphorylated when exposed to either 10 μM DETA/NO, 100 nM IGF-1 or 10 nM insulin in serum-deprived cells at the same levels reached in serum cultured cells (Fig. 3, panel A), IRS-3 wasn’t (Fig. 3, panel B).

### 3.4. Activation of PI3K/Akt system by IGF-1 and insulin is dependent on NOS and c-Src activation in RINm5F cells

We previously reported that NO activates PI3K/Akt signaling in a c-Src-dependent manner in serum-deprived cells [9]. Thus, we next tested whether this survival pathway was also involved in the protective actions of IGF-1 and insulin. Exposure to either IGF-1 or insulin leads to substantial increase in the phosphorylation of Akt with no significant effect on the levels of this protein in serum-starved cells (Fig. 4, panels A, B). This Akt phosphorylation is dependent on NO production, as demonstrated by the fact that the inhibition of NO production leads to a suppression of the effect of IGF-1 and partially counteracts the actions of insulin. Similarly PI3K activation is involved in the actions of IGF-1 and insulin on Akt phosphorylation, since the PI3K inhibitor LY-294002 fully abrogated their actions. On the other hand, Akt activation is suppressed in RINm5F/nd-Src cells, thus suggesting that c-Src mediates the effect of IGF-1 and insulin on Akt activation. The involvement of c-Src in conveying the protective signal triggered by IGF-1 and insulin is
further substantiated by the finding that protection from DNA fragmentation conferred by IGF-1 and insulin in serum-deprived cells is suppressed in RINm5F/nd-Src cells (Fig. 4, panel C). Additionally, a substantial increase in DNA fragmentation is observed in serum-deprived RINm5F/nd-Src cells when compared with the transfected empty vector.

3.5. IGF-1 and insulin induce NOS3 expression, enhances NOS activity and nitric oxide production in a PI3K/Akt-dependent manner

To more directly demonstrate the effect of IGF-1 and insulin at the NO generating system, we studied the expression and
activity levels of NOS. Exposure of serum-deprived cells to either IGF-1 or insulin enhances the expression level of NOS3 protein (Fig. 5, panels A, B); this effect is PI3K/Akt dependent, since LY-294002 and SH6 suppressed this action. The expression of β-actin, as protein load control, is not altered. Total NOS activity and nitric oxide production decreased in serum withdrawal condition (Fig. 5, panels C and D), and the addition of IGF-1 and insulin fully restored these actions. Both PI3K and Akt activation mediate the effect of IGF-1 and insulin on NO generation, LY-294002 and SH6 significantly cancelled this action. Moreover, nitric oxide production by NOS was cancelled by L-NMMA (Fig. 5 panel D).

3.6. Effect of IGF-1, insulin and DETA/NO on IRS-1 and IRS-2 phosphorylation in human islets

Having showed that the protective effect of IGF-1, insulin and DETA/NO is associated with selective IRS-1 and IRS-2 phosphorylation in the insulin producing RINm5F cell line, we next studied the pattern of expression and phosphorylation of IRS proteins in differentiated β cells. Human islets expressed substantial amounts of IRS-1 and IRS-2 proteins (Fig. 6, panels A and B), but no IRS-3 protein was detected (data not shown). Culture of human islets in serum-free condition produces a marked decrease in the amount of phosphorylated IRS-1 and IRS-2 which is prevented by the addition of either IGF-1, insulin or DETA/NO. These results indicate that the protective IGF-1/insulin NO pathway is also operative in human islets.

3.7. Protection from apoptosis by IGF-1, insulin and DETA/NO in rat and human islets

Going further, and in order to study the effect of IGF-1, insulin and NO in an “ex-vivo” system, we used rat and human islets. As showed previously when using RINm5F cell, rat islets (Fig. 7, panel A) and human islets (Fig. 7, panel B) cultured in serum-free...
medium also undergo a substantial degree of DNA fragmentation, and the addition of IGF-1, insulin or DETA/NO conferred to the islets a complete protection against this apoptotic feature.

4. Discussion

The purpose of this study was to explore the link between NO and the survival pathways triggered by IGF-1 and insulin in both undifferentiated insulin producing cells and in differentiated β cells. The results show that exposure of cells to both IGF-1 and insulin will increase NO production by cells which in turn will protect both human and rat islets as well as RINm5F cells against serum deprivation-induced apoptosis. The protective action is caused by concerted activation of PI3K, Akt and c-Src, as well as the activation of IRS-1, IRS-2, and NOS3.

We have previously shown that endogenous and exogenous generation of low and sustained levels of NO protect RINm5F from serum deprivation-induced apoptosis in a c-Src and PI3K/Akt-dependent manner [9,10]. In fact, exposure of serum-starved RINm5F cells to IGF-1 and insulin enhances the production of NO and NOS3 protein levels. These actions are dependent on the PI3K/Akt system, as shown in Fig. 4 (panels A, B). Our results support the notion that NO production induced by IGF-1 and insulin is also involved in the anti-apoptotic response, since the inhibitor L-NMMA blocks these protective actions on DNA fragmentation (Fig. 1) and Akt activation (Fig. 4, panels A, B). Moreover, in agreement with previous results [9], when we used RINm5F/nd-Src cells, the effects of IGF-1 and insulin on DNA fragmentation and Akt activity were cancelled (Fig. 4, panel C), suggesting that these protective effects are dependent on this non-receptor tyrosine kinase. These results coincide with previous reports in other cells systems [26,27].

Activation of IRS-1 by IGF-1 and insulin is well established [23,28–31]. Putative activation of this system may play a role in protecting cells against apoptosis [32]. Recently, it has been

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**Fig. 5.** Effect of IGF-1 and insulin on NOS3 expression and NOS activity. RINm5F cells were cultured for 15 h in serum-free medium in the absence or in the presence of IGF-1 (A), insulin (B) and inhibitors. Cell homogenates were prepared and western blot analysis was performed as described in Materials and methods using anti-NOS3 antibody, β-actin protein was detected in membranes loaded with the same homogenate protein concentration. Values shown in (A,B) are means±SD and are expressed as relative NOS3 densitometry units. NOS3 signal in extracts from cells cultured in the absence of serum was considered as 1 unit. Figures are representative from three independent experiments. (C) RINm5F cells were cultured in the indicated conditions for 15 h, the NOS activity was measured as indicated in Materials and methods. Data are mean±SD of five independent experiments. *p ≤0.005 vs control cells with serum. (D) Nitric oxide detection by immunofluorescence: (a) serum, (b) serum deprived, (c) serum deprived plus 10 nM insulin, (d) serum deprived plus 10 nM insulin plus 2 μM SH6, (e) serum deprived plus 10 nM insulin plus 20 μM LY-294002, (f) serum deprived plus 10 nM insulin plus 400 μM L-NMMA, (g) serum deprived plus 100 nM of IGF-1, (h) serum deprived plus 100 nM of IGF-1 plus 2 μM SH6, (i) serum deprived plus 100 nM of IGF-1 plus 20 μM LY-294002 and (j) serum deprived plus 100 nM IGF-1 plus 400 μM L-NMMA. NO detection was measured as indicated in Materials and methods. Images are representative from three independent experiments.
reported that insulin promotes human islet survival in a process dependent on activation of Akt [17]. Our study further substantiate this finding and proposes that this action is mediated at least in part, by generation of NO following activation of IRS-1 and-2 proteins. We have reported that the presence of serum in culture media regulates IRS-1 phosphorylation in RINm5F cells [9]. Both IGF-1 and insulin were able to induce IRS-1 phosphorylation in serum-deprived cells in a PI3K independent manner (Fig. 2). Most interestingly, inhibition of NO generation with L-NMMA produced a partial reduction in both IGF-1 and insulin-dependent IRS-1 phosphorylation. These results support the critical role played by NO generation in IRS phosphorylation.

A substantial debate is being generated in the literature on the role of different IRS proteins on β cell function. Experiments with IRS-2 KO mice clearly show a role for this protein during β cell development and protection from diabetes [28]. On the other hand, islets from human donors carrying a common mutation in IRS-1 protein undergo apoptosis in vitro [12]. We have been able to detect IRS-1,-2 proteins in human islets and also that the phosphorylation of IRS-1 and 2 proteins are downregulated in serum-deprived islets (Fig. 6), being this action is abrogated partially by exposure to IGF-1, insulin and NO. Regardless of the type of IRS protein in charge of conveying the survival signalling in human islets, it is clear that in vitro human islets undergo substantial DNA fragmentation when cultured in serum-free medium (Fig. 7) and that IGF-1, insulin and NO protects efficiently from this damage.

In conclusion, our data show that the integrity of the process of β cell survival is crucial for determining the resistance to apoptosis in type 1 and type 2 diabetes. It is also plausible that local generation of NO and growth factors may be impaired during the pathogenesis of diabetes in rodents and in humans. Further studies on the role of NO/growth factors on β cell survival in rodent models of diabetes must be done to implement this hypothesis.

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Fig. 6. IRS-1 and IRS-2 phosphorylation by IGF-1, insulin and DETA/NO in human islets. Islets were cultured in complete RPMI 1640 or serum-free RPMI 1640 medium in the absence or in the presence of IGF-1, insulin and DETA/NO for 15 min. Homogenates were immunoprecipitated with anti-IRS-1 (panel A) or IRS-2 (panel B) antibodies. Immunoprecipitates were blotted with either anti-PY20 phospho-tyrosine antibody (top picture) or anti-IRS-1 or IRS-2 antibodies (bottom picture) as in Experimental procedures. Values shown are means±SD and are expressed as relative IRS phosphorylation densitometry units. IRS densitometry signal in every condition was considered as 1 unit. *p ≤ 0.005 vs cells cultured in the presence of serum. Images are representative from three independent experiments. Phosphorylation data were expressed as ratios between densitometric values of phosphorylation and the densitometric values of total proteins normalized to 1.

Fig. 7. Effect of IGF-1, insulin and DETA/NO on DNA fragmentation in rat and human islet. Islets were cultured in complete RPMI 1640 or serum-free RPMI 1640 medium in the absence or in the presence of IGF-1, insulin and DETA/NO for 24 h. Medium containing DETA/NO was changed for medium containing freshly prepared DETA/NO after 16 h. of culture. DNA fragmentation was determined as in Experimental procedures. Data are mean±SD of three or five independent experiments. *p ≤ 0.005 vs control, serum-deprived islets.
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