Nitric Oxide Triggers the Phosphatidylinositol 3-Kinase/Akt Survival Pathway in Insulin-Producing RINm5F Cells by Arousing Src to Activate Insulin Receptor Substrate-1

JUAN R. TEJEDO, GLADYS M. CAHUANA, REMEDIOS RAMÍREZ, MARGARIDA ESBERT, JUAN JIMÉNEZ, FRANCISCO SOBRINO, AND FRANCISCO J. BEDOYA

Laboratory of Biochemistry of the Immune System, Department of Medical Biochemistry and Molecular Biology, University of Sevilla, 41009 Sevilla, Spain

Mechanisms involved in the protective action of nitric oxide (NO) in insulin-producing cells are a matter of debate. We have previously shown that pharmacological inhibition of c-Src cancels the antiapoptotic action of low and sustained concentrations of exogenous NO. In this study, using insulin-producing RINm5F cells that overexpress Src either permanently active (v-Src) or dominant negative (dn-Src) forms, we determine that this tyrosine kinase is the principal mediator of the protective action of NO. We also show that Src-directed activation of insulin receptor substrate-1, phosphatidylinositol 3-kinase (PI3K), Akt, and Bad phosphorylation confound a substantial component of the survival route because pharmacological inhibition of PI3K and Akt canceled the antiapoptotic effects of NO. Studies performed with the protein kinase G (PKG) inhibitor KT-5823 revealed that NO-dependent activation of c-Src/insulin receptor substrate-1 is not affected by PKG activation. By contrast, Akt and Bad activation are partially dependent on PKG activation. Endogenous production of NO after overexpression of endothelial nitric oxide synthase in RINm5F cells mimics the effects produced by generation of low amounts of NO from exogenous diethylenetriamine/NO. In addition, we found that NO produces c-Src/PI3K- and PKG-dependent activation of ERK 1/2. The MAPK kinase inhibitor PD 98059 suppresses NO-dependent protection from DNA fragmentation induced by serum deprivation. The protective action of low and sustained concentration of NO is also observed in staurosporine- and Taxol-induced apoptosis. Finally, NO also protects isolated rat islets from DNA fragmentation induced by serum deprivation. These data strengthen the notion that NO production at physiological levels plays a role in protection from apoptosis in pancreatic β-cells. (Endocrinology 145: 2319–2327, 2004)

APOPTOTIC DEATH OF pancreatic β-cells plays an important role in the pathogenesis of diabetes (1, 2). The apoptotic cascade can be triggered by intracellular events such as metabolic dysfunction, perturbations in the cell cycle, DNA damage, and by external factors such as activation of death receptors and inflammatory cytokines or the lack of trophic factors (3). Activation of pancreatic β-cell survival pathways is crucial for facing noxious challenges that eventually lead to destruction of the endocrine pancreas in both types 1 and 2 diabetes (4). Recent studies have shown the involvement of insulin/IGF-1 signaling in the control of β-cell survival and growth (5–8). It is also established that low concentrations of nitric oxide (NO) protects from apoptosis in several cell systems including β-cells (9–13). NO-induced survival in neurons and in endothelial cells is dependent on phosphorylation of the apoptotic protein Bad through activation of GMP/protein kinase G (PKG)/phosphatidylinositol 3-kinase (PI3K)/Akt system (10, 14, 15). The protective action of NO in serum-deprived insulin producing RINm5F cells also involves the activation of c-Src (9). This soluble tyrosine kinase is directly activated by NO and regulates apoptosis and cell survival in a number of cell systems (16–21). It is entirely possible that NO generation mediates the insulin/IGF-1 survival pathway in β-cells. In the present report, we document that the protective action of NO involves the activation of PI3K/Akt/Bad system in the pancreatic β-cell line RINm5F. The survival pathway triggered by NO is dependent on activation of c-Src and phosphorylation of insulin receptor substrate-1 (IRS-1) and activation of ERK 1/2. Activation of the PI3K/Akt system is also dependent on the soluble guanylate cyclase (GC)/PKG system.

Materials and Methods

Abbreviations: DETA, Diethylenetriamine; dn-Src, dominant negative Src; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; IRS-1, insulin receptor substrate-1; L-NMMA, Nω-monomethyl L-arginine; MEK, MAPK kinase; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; PKG, protein kinase G; PVDF, polyvinyl difluoride; v-Src, permanently active Src.

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mycin, penicillin, glutamine, amphotericin B, cell death detection ELISA\textsuperscript{plus}, 4-(2-aminoethyl)-benzenesulfonyl fluoride (peflablock), peptatin A, aprotinin, and leupeptin were from Roche (Stockholm, Sweden); polyclonal anti-p-ERK 1/2 (Thr202/Tyr204) was from Cell Signaling (Beverly, MA); polyclonal anti-IRS-1 (C-20), monoclonal anti-Bcl-2 (C-20), monoclonal anti-Akt, polyclonal anti-p-Akt (Ser473), monoclonal anti-Bad, polyclonal anti-p-Bad (Ser136), and polyclonal anti-ERK-1 (provided by Dr. D. J. Stewart, Department of Medicine, University of Toronto, Toronto, Ontario, Canada). After the pulse, the mixture was incubated for 4 h at 4°C, followed by centrifugation to recover the protein G Sepharose pellets. The immunoprecipitates were washed three times with PBS and once with kinase reaction buffer [10 mM 3[3N-morpholino]propanesulfonic acid (pH 7.5), 12.5 μM β-glycerophosphate, 7.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na\textsubscript{2}VO\textsubscript{4}] and then suspended in 20 μl of kinase reaction buffer. The immunoprecipitates were used for detection of total protein and phosphorylated protein by Western blot (IRS-1, ERK 1/2, Akt, Bad) and kinase assay (c-Src).

Src kinase assay was performed in 30 μl kinase buffer containing 20 μM ATP, 1 μCi[y-\textsuperscript{32}P]ATP, and 10 μg acid-denatured enolase for 20 min at 30°C (9). Samples were analyzed using SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membranes and subjected to autoradiography.

**Results**

Protein concentration was determined by Bradford’s technique (Bio-Rad Laboratories, Hercules, CA). Data are mean ± SD of at least three independent experiments, except for results of blots and autoradiographies, in which case a representative experiment is depicted in the figures. Comparisons between group values were analyzed using one-way ANOVA. Differences were considered significant when P < 0.05.

**Immunoprecipitation and in vitro kinase assay**

Batches of cells (3 × 10\textsuperscript{6}) were placed in 50 μl of lysis buffer for 1 h at 4°C. After centrifugation, clarified supernatants were immunoprecipitated with primary antibody (2 μg/ml protein) for 4 h at 4°C. Twenty microliters of 50% (vol/vol) protein G Sepharose were 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 three times with PBS and once with kinase reaction buffer [10 mM 3[3N-morpholino]propanesulfonic acid (pH 7.5), 12.5 μM β-glycerophosphate, 7.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na\textsubscript{2}VO\textsubscript{4}] and then suspended in 20 μl of kinase reaction buffer. The immunoprecipitates were used for detection of total protein and phosphorylated protein by Western blot (IRS-1, ERK 1/2, Akt, Bad) and kinase assay (c-Src).
and the MAPK kinase (MEK) inhibitor PD 98059 (20 μM) cancel NO-protective action on serum withdrawal-induced DNA fragmentation. These findings raise the possibility that PI3K and MEK survival pathways are involved in NO protective action.

Figure 1D shows that other apoptotic indexes such as cytochrome c release to cytosol and Bcl-2 protein down-regulation are similarly affected by serum removal. v-Src-transfected cells display a full protection from the effect of serum deprivation. dn-Src-transfected cells display higher degradation of Bcl-2 protein, whereas cytochrome c release is poorly affected.

**NO activates ERK 1/2 in a PI3K-dependent manner as a part of the antiapoptotic action**

When RINm5F cells are deprived of serum for 4 h, the degree of ERK 1/2 activation decreases notably. The addition DETA/NO (10 μM) leads to ERK 1/2 activation in serum-deprived cells, and the PI3K inhibitors wortmannin and LY 294002 suppress this action (Fig 2A). When cells are transfected with the dn-Src, DETA/NO-induced activation of ERK 1/2 is abolished. Transfection with v-Src gene protects from serum withdrawal-induced ERK 1/2 inactivation, and PI3K inhibitors block this action (Fig. 2B).
Role of PKG in the actions of NO in serum-deprived cells

Figure 3A shows that DETA/NO-dependent activation of c-Src kinase in serum-deprived RINm5F cells is not affected by PKG inhibitor KT-5823 (0.5 μM). On the other hand, DETA/NO-induced phosphorylation of ERK 1/2, Akt, and Bad is partially decreased by the PKG inhibitor (Fig. 3B).

NO promotes IRS-1 phosphorylation in a PI3K- and PKG-independent manner in serum-deprived cells

Figure 4A shows that DETA/NO (10 μM) elicits IRS-1 phosphorylation in serum-deprived RINm5F cells. Inhibition of neither PKG with KT-5823 (0.5 μM) nor PI3K with wortmannin (200 nM) was able to suppress NO stimulatory
action. A stimulatory action of LY-294002 (20 μM) on DETA/NO-induced IRS-1 phosphorylation was observed.

Serum removal fails to decrease IRS-1 phosphorylation in v-Src transfected cells. Neither DETA/NO (10 μM) nor PI3K inhibitors were able to modify the high levels of IRS-1 phosphorylation in these cells. Cells transfected with dn-Src display decreased levels of IRS-1 phosphorylation. (Fig. 4B).

Akt and Bad phosphorylation are involved in the protective action of NO

When RINm5F cells are deprived of serum, activation levels of Akt are low (Fig. 5A). Exposure to DETA/NO (10 μM) restores Akt activation in a PI3K-dependent manner because wortmannin and LY-294002 were able to block DETA/NO-induced Akt activation. Bad phosphorylation is decreased in serum-deprived cells, and addition of DETA/NO (10 μM) counteracts partially this effect. In addition, experiments with PI3K and Akt inhibitors (SH6) show that DETA/NO-dependent phosphorylation is dependent on PI3K/Akt activation (Fig. 5B).

Protection from serum-deprived apoptosis by eNOS overexpression is dependent on PI3K and PKG

Figure 6A shows that RINm5F cells overexpressing eNOS display protection from serum deprivation-induced DNA fragmentation; this action is dependent on NO generation because it is blocked by the NOS inhibitor Nω-monomethyl l-arginine (L-NMMA) (500 μM). Both PKG and PI3K are involved because protection is significantly canceled by KT-5823 (0.5 μM) and LY-294002 (20 μM). Protective effect of eNOS overexpression on DNA fragmentation in serum-depleted cells is canceled by the Src tyrosine kinase inhibitor PP1 (10 μM). Abrogation of NO-dependent protection is also observed when cells are exposed to the Akt inhibitor SH6.

Apoptotic release of cytochrome c release to cytosol and Bcl-2 degradation was observed in serum-starved cells; cells overexpressing eNOS (pcDNA3.1/eNOS cells) are resistant to serum withdrawal-induced apoptosis (Fig. 6B), an effect that is canceled by L-NMMA (500 μM). Inhibition of PI3K, Akt, and PKG canceled the protection provided by eNOS overexpression.
Involvement of c-Src, IRS-1, PI3K/Akt, and PKG in the protection from apoptosis provided by eNOS overexpression

The upper and middle blots in Fig. 7A show that IRS-1 phosphorylation is dependent on NO and involves c-Src activation because L-NMMA and PP1 inhibited significantly (60 and 50%, respectively) IRS-1 phosphorylation. The blots also show that IRS-1 phosphorylation is not dependent on PKG activation. PI3K inhibition significantly increases IRS-1 phosphorylation.

eNOS overexpression leads to enhanced Akt phosphorylation in serum-deprived cells. Pharmacological inhibition of NOS, c-Src, and PI3K blocked this action. Inhibition of PKG with KT-5823 decreases partially Akt activation (Fig. 7A, middle blots).

Lower blots in Fig. 7A show that ERK 1/2 activation is dependent on eNOS, c-Src, and PI3K and partially dependent on PKG.

Bad phosphorylation induced by eNOS overexpression is significantly suppressed by eNOS, c-Src, PI3K, and Akt inhibitors (Fig. 7B).

The protective action of NO is also apparent in Taxol and staurosporin-induced apoptosis and serum-deprived rat islets

Apoptosis inducers such as Taxol and staurosporin lead to a concentration-dependent increase in the amount of DNA fragmentation in RINm5F cells, and DETA/NO (10 μM) significantly diminishes this effect (Fig. 8A). On the other hand, isolated rat islets undergo fragmentation of DNA when deprived of serum for 24 h, and the presence of DETA/NO (10 μM) reduces partially and significantly this effect (Fig. 8B).

Discussion

The results collected in the present paper show that exogenous generation of low and sustained levels of NO protects insulin producing RINm5F cells from apoptosis triggered by serum deprivation. Protection from apoptosis results from concerted activation of protein kinases, namely c-Src, Akt, and PKG and the activation of IRS-1. We have shown previously that exogenous generation of low and sustained levels of NO protects RINm5F cells from serum deprivation-induced apoptosis in a c-Src-dependent manner (9). We thus undertook in this paper the study of the nature of the signaling pathways involved in the protective action of NO. For this purpose, we study the impact of both genetic manipulation of eNOS and c-Src and pharmacological inhibition of selected protein kinases on the apoptotic response of cells to serum deprivation.

Transfection with the permanently active form of Src confers to RINm5F cells substantial protection from apoptotic fragmentation of DNA, cytochrome c release to cytosol, and Bcl-2 degradation, whereas transfection with dn-Src en-
hances apoptosis in serum-deprived cells. The finding that low concentrations (10 μM) of DETA/NO failed to confer protection from DNA fragmentation in cells transfected with the inactive form of Src whereas conferring protection in control cells and in v-Src-transfected cells suggest that this tyrosine kinase is a relevant target for NO in the antiapoptotic response. Additional actions of NO are suggested by the fact that a significant protective action is still present in cells transfected with the permanently active form of Src. The participation of Src in the antiapoptotic response has been documented in the literature (19–21). Thus, vascular endothelial growth factor-triggered protection from apoptosis in endothelial cells depends on Raf-1 activation by Src and is linked to PI3K activation in fibroblasts (20, 21).

Our results support the notion that PI3K is also implied in the antiapoptotic action of NO because the inhibitor LY 294002 blocks the protective action of NO on DNA fragmentation in serum-deprived cells. Our data show that NO also signals through MAPK pathway. Protection from DNA fragmentation conferred by NO exposure in serum-depleted cells was canceled by the MAPK kinase inhibitor PD 58059. In addition, serum-exposed cells show activated ERK 1/2 as previously reported (25, 26) and removal of serum led to substantial decrease of ERK 1/2 phosphorylation. Experiments performed with cells expressing v-Src and dn-Src show that Src activates ERK. This kinase is involved in survival responses in several cell types, including islets (25, 27, 28). It is thus entirely possible that the activation of ERK by NO is a component of the survival response in RINm5F cells as previously shown in other cell types. Controversy exists, however, on this issue. We reported the activation of ERK in RINm5F cells exposed to apoptotic concentrations of NO (23). It is conceivable that under such circumstances the protective role of activated ERK is overruled by direct apoptotic actions of NO at the mitochondria. The involvement of the sGC/PKG in the antiapoptotic action of NO has been substantiated in a variety of cell systems (10, 11, 14, 15). We previously reported that both soluble GC and c-Src mediate NO-dependent activation of PKG (9). In this work, we show that PKG activation by NO participates in the activation of ERK 1/2 and Akt and phosphorylation of Bad. On the other hand, the PKG inhibitor KT-5823 does not affect Src activation by NO. Taken together, these results suggest that concerted activation of Src and GC by NO triggers an antiapoptotic pathway in RINmF cells that involves Akt and Bad phosphorylation and the ERK 1/2 activation.

The activation of IRS-1 by c-Src as a part of the signaling system triggered by IGF and insulin receptor activation has been reported previously (29–31). We have found that the presence of serum in culture media regulates IRS-1 phos-
phorylation in RINm5F cells. Exogenously generated NO was able to induce IRS-1 phosphorylation in serum-deprived cells in a PI3K- and PKG-independent manner. Most interestingly, v-Src cells display IRS-1 phosphorylation in the absence of external serum, whereas dn-Src cells did not. Neither DETA/NO nor PI3K and PKG inhibitors modify IRS-1 phosphorylation in transfected cells cultured in the presence of serum. Apoptotic susceptibility to serum deprivation has been described in human islets carrying Arg972 IRS-1, thus suggesting an important role for this system in β-cell survival (6). Our finding that Src governs IRS-1 activation widens the scope of extracellular survival signals that might operate through the IRS-1 system.

NO antiapoptotic action implies Bad phosphorylation through PI3K/Akt-dependent activation. The relevance of the role of NO in protecting cells from serum deprivation was substantiated by the finding that eNOS transfected cells display resistance to DNA fragmentation, cytochrome c release, and Bcl-2 degradation when exposed to serum-free media. In fact, eNOS transfection was able to restore IRS-1 phosphorylation in serum-starved cells in a Src-dependent manner. PI3K/Akt and PKG transduce the antiapoptotic signaling that involves Bad phosphorylation. The participation of PI3K/Akt in survival induced by IGF-1 and other factors is well substantiated (19, 32–34). eNOS overexpression in cells has been used as a tool to study the effect of near physiological concentrations of NO on apoptosis (13, 14). The results we report here with eNOS transfectected cells strengthen the notion that NO production at physiological levels plays a role in protection from apoptosis in insulin-producing RINm5F cells. The protective action of NO was also observed in Taxol and staurosporin-apoptosis in RINm5F cells and serum-deprived isolated rat islets, thus indicating that the mechanism reported here operates in a variety of apoptotic situations and also operates in differentiated β-cells.

The scheme presented in Fig. 9 summarizes the tentative NO-induced signaling pathways in pancreatic β-cells, as derived from the results reported in the present work. The contribution of ERK 1/2 to β-cell survival deserves further exploration. With regard to the possible physiological relevance of the present data, production of NO at low levels could be involved in the activation of β-cell survival pathways. A combination of acquired and genetic alterations in these pathways may be a significant factor in the loss of β-cell mass in types 1 and 2 diabetes.

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Address all correspondence and requests for reprints to: Francisco J. Bedoya, Laboratory of Biochemistry of the Immune System, Department
of Medical Biochemistry and Molecular Biology, University of Sevilla, Avenida Sanchez Pizjuan 4, 41009 Sevilla, Spain. E-mail: bedoya@us.es.

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