Insulin Antiapoptotic Signaling Involves Insulin Activation of the Nuclear Factor κB-dependent Survival Genes Encoding Tumor Necrosis Factor Receptor-associated Factor 2 and Manganese-superoxide Dismutase*

(Received for publication, July 9, 1999)

France Bertrand‡, Christèle Desbois-Mouthon‡, Axelle Cadoret‡, Céline Prunier‡, Hélène Robin‡, Jacqueline Capeau‡, Azeddine Atfi§, and Gisèle Cherqui¶

From ‡INSERM U.402, Faculté de Médecine Saint-Antoine, 27, rue Chaligny and ¶INSERM U.482, Hôpital Saint-Antoine, 184, rue du Faubourg Saint-Antoine, 75057 Paris Cedex 12, France

We recently showed that the antiapoptotic function of insulin requires nuclear factor κB (NF-κB) activation (Bertrand, F., Atfi, A., Cadoret, A., L’Allemain, G., Robin, H., Lascols, O., Capeau, J., and Cherqui, G. (1998) J. Biol. Chem. 273, 2931–2938). Here we sought to identify the NF-κB-dependent survival genes that are activated by insulin to mediate this function. Insulin increased the expression of tumor necrosis factor receptor-associated factor 2 (TRAF2) mRNA and protein in Chinese hamster ovary cells overexpressing insulin receptors (IRs). This effect required (i) IR activation since it was abrogated by IR mutation at tyrosines 1162 and 1163 and (ii) NF-κB activation since it was abolished by overexpression of dominant-negative IRα(A32/36) and mimicked by overexpression of the NF-κB c-Rel subunit. TRAF2 contributed to insulin protection against serum withdrawal-induced apoptosis since TRAF2 overexpression mimicked insulin protection, whereas overexpression of dominant-negative TRAF2(87–501) reduced this process. Along with its protective effect, overexpressed TRAF2 increased basal and insulin-stimulated NF-κB activities. All effects were inhibited by IRα-A(A32/36), suggesting that an amplification loop involving TRAF2 activation of NF-κB is implicated in insulin antiapoptotic signaling. We also show that insulin increased manganese-superoxide dismutase (Mn-SOD) mRNA expression through NF-κB activation and that Mn-SOD contributed to insulin antiapoptotic signaling since expression of antisense Mn-SOD RNA decreased this process. This study provides the first evidence that insulin activates the NF-κB-dependent survival genes encoding TRAF2 and Mn-SOD and thereby clarifies the role of NF-κB in the antiapoptotic function of insulin.

Programmed cell death or apoptosis is a fundamental event in the developmental and homeostatic processes of all multicellular organisms. Apoptosis is inhibited by various growth factors, including insulin. The antiapoptotic function of insulin requires the integrity of the insulin receptor (IR)1 tyrosine kinase domain (1, 2) and involves several intracellular signaling molecules, including insulin receptor substrate 1 (3), Ras (2), Raf-1 (1), and phosphatidylinositol 3-kinase (1, 4). In addition, recent evidence from our laboratory (1) indicates that insulin antiapoptotic signaling involves the activation of nuclear factor κB (NF-κB), a transcription factor playing a critical role in apoptosis inhibition (5).

NF-κB is a member of the NF-κB/Rel family. This family is composed of NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB. Prototypical NF-κB is a p50/p65 heterodimer that is usually retained in the cytoplasm of unstimulated cells in an inactive form by IκB-α, one of the most important members of the IκB inhibitory protein family. In response to various stimuli, IκB-α is phosphorylated at Ser32 and Ser36 by IκB kinases α and β (6). Once phosphorylated, IκB-α is rapidly ubiquitinated and subsequently degraded by the 26 S proteasome complex (7). The degradation of IκB-α unmasks the nuclear localization signal of the NF-κB heterodimer, which then translocates into the nucleus, where it directly binds to its cognate DNA sequence to regulate gene transcription.

The antiapoptotic role played by NF-κB involves the ability of this transcription factor to induce the expression of genes that promote cell survival such as the genes coding for tumor necrosis factor (TNF) receptor-associated factors 1 and 2 (TRAF1 and TRAF2, respectively) (8), the cellular inhibitors of apoptosis 1 and 2 (c-IAP1 and c-IAP2, respectively) (8, 9), the protein A20 (10), and manganese-superoxide dismutase (Mn-SOD) (11, 12). TRAF1 and TRAF2 are cytoplasmic adapter proteins that interact directly or indirectly with the intracellular domains of members of the TNF receptor superfamily (13). Both TRAF1 and TRAF2 are able to recruit other adapter proteins such as c-IAP1 and c-IAP2 (14, 15) and the protein A20 (13). Of interest, TRAF2, the most extensively studied TRAF family member to date, was recently shown to be also localized in the nucleus (16). TRAF2 is known to activate NF-κB through its interaction with NIK, a serine/threonine-specific NF-κB-inducible kinase that activates the IκB kinase complex, thus leading to IκB phosphorylation and degradation and subsequent NF-κB activation (17). TRAF2 has a clear antiapoptotic function, as supported by the findings that thymocytes from TRAF2 null mice (18) or from transgenic mice expressing a dominant-negative form of TRAF2 (19) are highly sensitive to TNF-α-induced cell death. In addition, the overexpression of the dominant-negative TRAF2-(87–501) mutant lacking the c-IAP, cellular inhibitor of apoptosis, Mn-SOD, manganese-superoxide dismutase; CHO, Chinese hamster ovary; SFM, serum-free medium.

* This work was supported by the Association pour la Recherche sur le Cancer (Villejuif, France) and the Ligue Nationale contre le Cancer (Comité de Paris, France). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 33-1-40-01-13-56; Fax: 33-1-40-01-14-99; E-mail: cherqui@st-antoine.inserm.fr.

‡ The abbreviations used are: IR, insulin receptor; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; TRAF, TNF receptor-associated
RING finger domain increases TNF-α-induced apoptosis in MCF-7 and HeLa cells (20). The antiapoptotic function of TRAF2 is mediated by NF-κB-dependent (13, 20) and NF-κB-independent (18, 19, 21) pathways and may involve the ability of TRAF2 to interact with c-IAP1 and c-IAP2 (13). As regards Mn-SOD, it is known as an enzyme of the mitochondrial matrix functioning as a scavenger of superoxide radicals. Like TRAF2, Mn-SOD is involved in apoptosis inhibition since its overexpression suppresses TNF-α-induced apoptosis in MCF-7 cells (22).

This study was designed to investigate whether the NF-κB-dependent survival genes coding for TRAF2, c-IAP1, c-IAP2, and Mn-SOD are activated by insulin and, if so, to analyze their role in the antiapoptotic function of insulin. We used CHO cells overexpressing wild-type IRs (CHO-R and CHO-R15) or IRs made kinase-defective by mutation at Tyr1162 and Tyr1163 autophosphorylation sites (CHO-Y2). We show that insulin increased the expression of TRAF2 mRNA and protein at the cytoplasmic and nuclear levels in CHO-R15 and CHO-R cells, but not in CHO-Y2 cells. Insulin induced TRAF2 overexpression through NF-κB activation, and overexpressed TRAF2 contributed to insulin antiapoptotic signaling by exerting positive feedback control on NF-κB. We also show that insulin increased Mn-SOD (but not c-IAP1 or c-IAP2) mRNA expression in CHO-R15 cells and that Mn-SOD, like TRAF2, contributed to insulin antiapoptotic signaling. These results clarify the role of NF-κB in the antiapoptotic function of insulin and provide new insight into the nuclear mechanisms involved in this function.

EXPERIMENTAL PROCEDURES

**Plasmids**—The pCMV5lacZ, (Igκ3-conaluc, and conaluc reporter plasmids and the mutated expression plasmid hEβ-α(A32/36) were previously described (1). The pK5K-FLAG-TRAF2 and pK5K-FLAG-TRAF2(87−501) plasmids were generous gifts from Dr. D. V. Goeddel. The pCMV-c-IAP1 and pCMV-c-IAP2 plasmids were generous gifts from Dr. D. W. Ballard. The antisense Mn-SOD construct was generated by excising the Mn-SOD cDNA from pUC19 (a generous gift from Dr. S. Chouaib) and subcloning into a pCDNA3.1/neo(-) plasmid (Invitrogen).

**Cells and Transfections**—The different CHO cell lines used in this study have been previously described (1, 23). These include the CHO cell lines overexpressing wild-type human IRs (CHO-R and CHO-R15) and the CHO cell line overexpressing human IRs mutated at tyrosines 1162 and 1163 (CHO-Y2). Rel-3 cells, a clone of CHO-R cells overexpressing c-Rel, were recently described (1). CHO-R15 cells stably overexpressing wild-type human IRs (CHO-R and CHO-R15) treated with or without 10^{-7} M insulin (Ins) for 6 or 24 h were subjected to Northern blot analysis as described under “Experimental Procedures.”

**Cytosolic and nuclear extracts** (10 μg of protein) from control or insulin-treated CHO-R15 cells were immunoblotted with a TRAF2-specific antibody (22), or c-IAP2 were obtained by transfection of the corresponding expression vectors, together with a pcDNA3.1/neo(-) plasmid (Invitrogen), using the LipofectAMINE PLUS™ reagent (Life Technologies, Inc.). Cells were grown in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum.

**Northern Blot Analysis**—Total RNA and poly(A)^+ RNA were isolated from CHO-R and CHO-R15 cells using kits from QIAGEN Inc. according to the manufacturer’s instructions. Total RNA (20 μg) or poly(A)^+ RNA (2.5 μg) was electrophoresed and transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) as described (24). Membranes were hybridized (3 h at 65 °C) in Rapid-Hyb buffer (Amersham Pharmacia Biotech) to a random-primed labeled cDNA probe specific for TRAF2, c-IAP1, c-IAP2, or Mn-SOD and subsequently to a labeled human elongation factor 1 probe as a control for RNA loading. Membranes were then washed to high stringency as described (24). Signals were quantified and normalized by differential densitometric scanning of the specific bands versus the human elongation factor 1 band.

**Immunoblotting**—Total cell lysates or cytosolic and nuclear fractions prepared as described (1, 24) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were incubated with specific antibodies directed against TRAF2 (1:500), IκB-α (1:500), or c-Rel (1:100) (Santa Cruz Biotechnology, Inc.) and then with a horse-radish peroxidase-conjugated secondary antibody. Immuneoreactive proteins were visualized by the ECL system from Amersham Pharmacia Biotech.

**LipofectAMINE PLUSTM** reagent (Life Technologies, Inc.). Cells were cotransfected with the pcDNA3.1/zeo selection vector (Invitrogen), using the LipofectAMINE PLUS™ reagent (Life Technologies, Inc.). Cytosolic and nuclear extracts (10 μg of protein) from control or insulin-treated CHO-R15 cells were immunoblotted with a TRAF2-specific antibody (22). The subcellular fractions were used to measure the expression of the specific proteins of interest.

**RESULTS**

**Insulin Stimulates TRAF2 mRNA and Protein Expression in CHO Cells Overexpressing IRs**—To examine the effect of insulin on the expression of TRAF2 mRNA, total RNA, or poly(A)^+ RNA prepared from CHO-R and CHO-R15 cells, two different clones of CHO cells overexpressing wild-type human IRs (1, 23) were analyzed by Northern blotting. Insulin stimulated the expression of the 2.2-kilobase TRAF2 transcript at 6 and 24 h in total RNA from CHO-R and CHO-R15 cells (Fig. 1A). Quantification of the results by scanning densitometry of the autoradiograms after normalization to the human EF1 signal indicated that insulin increased TRAF2 mRNA expression by about 1.5- and 2.2-fold at 6 and 24 h, respectively (Fig. 1A). Higher increases (3- and 6-fold at 6 and 24 h, respectively) were measured on Northern blots performed with poly(A)^+ RNA from
Insulin Activates NF-κB-dependent Survival Genes

TABLE I
Effect of the stable or transient transfection of various expression vectors on basal and insulin-stimulated NF-κB-mediated luciferase activities

| Transfectants | Normalized luciferase activity |
|---------------|------------------------------|
|               | Basal | Insulin (10^{-7} M) |
| pCMV-zeo      | 100   | 294 ± 30          |
| IxB-(α32/36)  | 2.0 ± 0.1 | 3.0 ± 0.2 |
| c-Rel         | 220 ± 12 | 289 ± 22 |
| TRAF2         | 342 ± 36 | 674 ± 55 |
| TRAF2-(87–501)| 28 ± 2 | 76 ± 7 |
| pCMV-zeo + IxB-(α32/36) | 4.0 ± 0.2 | 5.9 ± 0.3 |
| TRAF2 + IxB-(α32/36) | 22 ± 3 | 27 ± 4 |

Cells transfected with the (Igκ3-conaluc reporter plasmid or its control counterpart conaluc in the presence of absence of the IxB-(α32/36) expression plasmid were maintained for 24 h in 0.3% fetal calf serum medium and then in SFM for a further 24 h with or without 10^{-7} M insulin. Cell extracts were analyzed for luciferase activity as described under “Experimental Procedures.” The results, presented as normalized luciferase activity (NF-κB-mediated luciferase activity minus the luciferase activity devoted to the control counterpart, normalized to the protein concentration), are the means ± S.E. of six experiments.

To examine the role of TRAF2 in insulin antiapoptotic signaling, CHO-R15 cells were transiently transfected with the expression vector coding for TRAF2 or its dominant-negative TRAF2-(87–501) mutant lacking the RING finger domain or with an empty vector, together with a β-galactosidase reporter plasmid (pCMV5/lacZ). Transfected cells were then incubated for 24 h in serum-free medium (SFM) with or without 10^{-7} M insulin. After fixation and staining, the percentage of apoptotic cells was evaluated by scoring blue β-galactosidase transfectants as healthy or apoptotic, as judged by blebbing of the membranes and shrinkage of the cell bodies. As shown in Fig. 3A, the percentage of apoptotic cells that amounted to 48 ± 2% in control cells maintained in SFM in the absence of insulin fell to 15 ± 1% in the presence of insulin. In TRAF2-transfected cells maintained for 24 h in SFM in the absence and presence of 10^{-7} M insulin, the percentages of apoptotic cells were 24 ± 2 and 16 ± 1%, respectively (Fig. 3A). In contrast, the percentages of apoptotic cells determined in TRAF2-(87–501)-transfected cells rose to 48 ± 3 and 32 ± 3% in the absence and presence of insulin, respectively.

To strengthen the above findings supporting a role for TRAF2 in insulin antiapoptotic signaling, CHO-R15 cells were stably transfected with the TRAF2 or TRAF2-(87–501) expression vector. Two clones designated as TRAF2 and TRAF2-(87–501), which exhibited TRAF2 and TRAF2-(87–501) overexpression in insulin antiapoptotic signaling, CHO-R15 cells were transiently transfected with the TRAF2 or TRAF2-(87–501) expression vector, together with a β-galactosidase reporter plasmid (pCMV5/ lacZ). Transfected cells were then incubated for 24 h in serum-free medium (SFM) with or without 10^{-7} M insulin and then analyzed for apoptosis by the DNA fragmentation assay (1). In controls, insulin almost completely abolished the DNA degradation induced by a 24-h serum deprivation (Fig. 3C). In TRAF2 cells maintained in SFM in the absence of insulin, the extent of DNA degradation was reduced as compared with controls, indicating that TRAF2 overexpression partially protected CHO-R15 cells from apoptosis. Insulin further protected TRAF2 cells from this process (Fig. 3C). In contrast, TRAF2-(87–501) cells maintained in SFM in the absence of insulin exhibited a ladder of DNA fragmentation similar to that observed in controls. Most important, Fig. 3C shows that insulin protection against apoptosis was markedly decreased in these cells. Altogether, these findings indicate that the overexpression of TRAF2 partially mimicked the antiapoptotic effect of insulin, whereas the overexpression of TRAF2-(87–501) markedly reduced this effect. They strongly argue for a role of TRAF2 in insulin antiapoptotic signaling in CHO-R15 cells.

CHO-R15 cells (Fig. 1B). Accordingly, insulin increased the expression of the 50-kDa TRAF2 immunoreactive protein in cytosolic and nuclear fractions from CHO-R15 cells (Fig. 1C). These findings provide the first evidence that insulin stimulates the expression of TRAF2 mRNA and protein at the cytoplasmic and nuclear levels in CHO cells overexpressing IRs.

Insulin Stimulation of TRAF2 Expression Is Mediated by IRs and Involves NF-κB Activation—We then evaluated the specificity of the stimulation of TRAF2 expression by insulin and investigated the role of NF-κB in this effect. As shown in Fig. 2A, the insulin-induced increase in TRAF2 protein expression observed in total cell lysates from CHO-R15 cells was lost in CHO-Y2 cells overexpressing kinase-defective IRs mutated at tyrosines 1162 and 1163. As shown in Fig. 2B, the stable expression of the dominant-negative IxB-(α32/36) mutant in CHO-R15 cells (IxB-(α32/36) cells), which almost completely abolished basal and insulin-stimulated NF-κB-mediated luciferase activities (Table I), dramatically decreased insulin stimulation of TRAF2 mRNA expression as compared with CHO-R15 cells stably transfected with an empty vector and a pcDNA3.1/zeo selection vector (pCMV-zeo cells). Reciprocally, the overexpression of the c-Rel subunit of NF-κB in CHO-R cells (Rel-3 cells) (Fig. 2B), which stimulated NF-κB-mediated activity by 2.2-fold (Table I), increased TRAF2 mRNA expression by 2.3-fold (Fig. 2B). Together, these results indicate that insulin stimulates TRAF-2 expression in CHO-R and CHO-R15 cells through IR activation and through an NF-κB-dependent pathway.

TRAF2 is Involved in Insulin Antiapoptotic Signaling—To
Insulin Activates NF-κB—Since the role played by TRAF2 in insulin antiapoptotic signaling in CHO-R15 cells could involve the well known ability of TRAF2 to activate NF-κB (13, 20), we investigated the effect of the stable overexpression of TRAF2 or TRAF2-(87–501) on basal and insulin-stimulated NF-κB activities. As reported in Table I, insulin stimulated basal NF-κB-mediated luciferase activity by 3-fold in pCMV-zeo cells. As compared with controls, TRAF2 cells exhibited 3.4- and 2.3-fold increases in basal and insulin-stimulated NF-κB activities, respectively. In contrast, TRAF2-(87–501) cells displayed 3.6- and 3.8-fold decreases in basal and insulin-stimulated NF-κB activities, respectively, as compared with controls. Noteworthy is the finding that the stimulation of NF-κB activity induced by insulin in control cells persisted in TRAF2-(87–501)-transfected cells despite the marked decrease in basal NF-κB activity exhibited by these cells. This strongly suggests that TRAF2-(87–501) did not affect NF-κB activation by insulin.

In view of the above results, we investigated the role of NF-κB in the ability of TRAF2 to mimic the antiapoptotic effect of insulin. To this end, pCMV-zeo and TRAF2 cells were transiently transfected with 2 μg of an empty vector or an expression vector coding for TRAF2 or TRAF2-(87–501), together with 0.5 μg of a pCMV5/lacZ vector. After 1 h, the medium was removed, and cells were incubated for 17 h in 10% fetal calf serum medium and then for a further 24 h in SFM with or without 10−7 M insulin (Ins). Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-galactosidase, and quantitation of apoptosis was performed by scoring β-galactosidase transfectants as healthy or apoptotic. B, total cell lysates (20 μg of protein) from pCMV-zeo, TRAF2, or TRAF2-(87–501) cells were immunoblotted with a TRAF2-specific antibody. C, pCMV-zeo, TRAF2, and TRAF2-(87–501) cells incubated for 24 h in SFM with or without 10−7 M insulin were collected for analysis of DNA degradation as described under “Experimental Procedures.” Results are the means ± S.E. of six experiments (A) or are representative of two experiments (B and C). *, p < 0.001 and †, p < 0.001 compared with control cells without or with insulin, respectively.

The Role of TRAF2 in Insulin Antiapoptotic Signaling Involves TRAF2 Activation of NF-κB—To determine whether insulin activates other antiapoptotic genes known as targets of NF-κB in mammalian cells, we examined the effect of insulin on the expression of c-IAP1, c-IAP2, and Mn-SOD mRNAs in CHO-R15 cells. To this end, total RNA or poly(A)⁺ RNA prepared from cells treated with or without 10−7 M insulin for 6 or 24 h was analyzed by Northern blotting. As shown in Fig. 5A, the 6-kilobase c-IAP2 transcript was undetectable in poly(A)⁺ RNA from CHO-R15 cells, whereas it was well detected in IAP2 cells, a clone of CHO-R15 cells stably transfected with a pCMV4-c-IAP2 expression plasmid. Insulin had no effect on c-IAP2 mRNA expression in CHO-R15 cells at either time tested (Fig. 5A). Similar results were obtained when using a c-IAP1-specific probe (data not shown). In contrast, insulin increased the expression of the 1- and 4-kilobase transcripts of Mn-SOD at 6 and 24 h, as determined by Northern blot analysis of total RNA from CHO-R15 cells (Fig. 5B). Quantification of the results showed that insulin stimulated the expression of the major 1-kilobase transcript by 1.4- and 1.7-fold at 6 and 24 h, respectively. In addition, Fig. 5B shows that the stimulation of Mn-SOD mRNA expression induced by insulin was lost in IxB-α(A32/36) cells, indicating that insulin increased Mn-SOD expression through NF-κB activation.

Role of Mn-SOD in Insulin Antiapoptotic Signaling—The role played by TRAF2 in insulin antiapoptotic signaling in CHO-R15 cells could involve the well known ability of TRAF2 to activate NF-κB (13, 20), we investigated the effect of the stable overexpression of TRAF2 or TRAF2-(87–501) on basal and insulin-stimulated NF-κB activities. As reported in Table I, insulin stimulated basal NF-κB-mediated luciferase activity by 3-fold in pCMV-zeo cells. As compared with controls, TRAF2 cells exhibited 3.4- and 2.3-fold increases in basal and insulin-stimulated NF-κB activities, respectively. In contrast, TRAF2-(87–501) cells displayed 3.6- and 3.8-fold decreases in basal and insulin-stimulated NF-κB activities, respectively, as compared with controls. Noteworthy is the finding that the stimulation of NF-κB activity induced by insulin in control cells persisted in TRAF2-(87–501)-transfected cells despite the marked decrease in basal NF-κB activity exhibited by these cells. This strongly suggests that TRAF2-(87–501) did not affect NF-κB activation by insulin.

In view of the above results, we investigated the role of NF-κB in the ability of TRAF2 to mimic the antiapoptotic effect of insulin. To this end, pCMV-zeo and TRAF2 cells were transiently transfected with 2 μg of an empty vector or an expression vector coding for TRAF2 or TRAF2-(87–501), together with 0.5 μg of a pCMV5/lacZ vector. After 1 h, the medium was removed, and cells were incubated for 17 h in 10% fetal calf serum medium and then for a further 24 h in SFM with or without 10−7 M insulin (Ins). Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-galactosidase, and quantitation of apoptosis was performed by scoring β-galactosidase transfectants as healthy or apoptotic. B, total cell lysates (20 μg of protein) from pCMV-zeo, TRAF2, or TRAF2-(87–501) cells were immunoblotted with a TRAF2-specific antibody. C, pCMV-zeo, TRAF2, and TRAF2-(87–501) cells incubated for 24 h in SFM with or without 10−7 M insulin were collected for analysis of DNA degradation as described under “Experimental Procedures.” Results are the means ± S.E. of six experiments (A) or are representative of two experiments (B and C). *, p < 0.001 and †, p < 0.001 compared with control cells without or with insulin, respectively.

To determine whether insulin activates other antiapoptotic genes known as targets of NF-κB in mammalian cells, we examined the effect of insulin on the expression of c-IAP1, c-IAP2, and Mn-SOD mRNAs in CHO-R15 cells. To this end, total RNA or poly(A)⁺ RNA prepared from cells treated with or without 10−7 M insulin for 6 or 24 h was analyzed by Northern blotting. As shown in Fig. 5A, the 6-kilobase c-IAP2 transcript was undetectable in poly(A)⁺ RNA from CHO-R15 cells, whereas it was well detected in IAP2 cells, a clone of CHO-R15 cells stably transfected with a pCMV4-c-IAP2 expression plasmid. Insulin had no effect on c-IAP2 mRNA expression in CHO-R15 cells at either time tested (Fig. 5A). Similar results were obtained when using a c-IAP1-specific probe (data not shown). In contrast, insulin increased the expression of the 1- and 4-kilobase transcripts of Mn-SOD at 6 and 24 h, as determined by Northern blot analysis of total RNA from CHO-R15 cells (Fig. 5B). Quantification of the results showed that insulin stimulated the expression of the major 1-kilobase transcript by 1.4- and 1.7-fold at 6 and 24 h, respectively. In addition, Fig. 5B shows that the stimulation of Mn-SOD mRNA expression induced by insulin was lost in IxB-α(A32/36) cells, indicating that insulin increased Mn-SOD expression through NF-κB activation.

Role of Mn-SOD in Insulin Antiapoptotic Signaling—The role played by TRAF2 in insulin antiapoptotic signaling in CHO-R15 cells could involve the well known ability of TRAF2 to activate NF-κB (13, 20), we investigated the effect of the stable overexpression of TRAF2 or TRAF2-(87–501) on basal and insulin-stimulated NF-κB activities. As reported in Table I, insulin stimulated basal NF-κB-mediated luciferase activity by 3-fold in pCMV-zeo cells. As compared with controls, TRAF2 cells exhibited 3.4- and 2.3-fold increases in basal and insulin-stimulated NF-κB activities, respectively. In contrast, TRAF2-(87–501) cells displayed 3.6- and 3.8-fold decreases in basal and insulin-stimulated NF-κB activities, respectively, as compared with controls. Noteworthy is the finding that the stimulation of NF-κB activity induced by insulin in control cells persisted in TRAF2-(87–501)-transfected cells despite the marked decrease in basal NF-κB activity exhibited by these cells. This strongly suggests that TRAF2-(87–501) did not affect NF-κB activation by insulin.

In view of the above results, we investigated the role of NF-κB in the ability of TRAF2 to mimic the antiapoptotic effect of insulin. To this end, pCMV-zeo and TRAF2 cells were transiently transfected with 2 μg of an empty vector or an expression vector coding for TRAF2 or TRAF2-(87–501), together with 0.5 μg of a pCMV5/lacZ vector. After 1 h, the medium was removed, and cells were incubated for 17 h in 10% fetal calf serum medium and then for a further 24 h in SFM with or without 10−7 M insulin (Ins). Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-galactosidase, and quantitation of apoptosis was performed by scoring β-galactosidase transfectants as healthy or apoptotic. B, total cell lysates (20 μg of protein) from pCMV-zeo, TRAF2, or TRAF2-(87–501) cells were immunoblotted with a TRAF2-specific antibody. C, pCMV-zeo, TRAF2, and TRAF2-(87–501) cells incubated for 24 h in SFM with or without 10−7 M insulin were collected for analysis of DNA degradation as described under “Experimental Procedures.” Results are the means ± S.E. of six experiments (A) or are representative of two experiments (B and C). *, p < 0.001 and †, p < 0.001 compared with control cells without or with insulin, respectively.
vestigate whether Mn-SOD participates in insulin antiapoptotic signaling, CHO-R15 cells were transiently transfected with an empty vector or an antisense Mn-SOD plasmid, together with pCMV5lacZ. After a 24-h incubation in SFM with or without 10^{-7} M insulin, cells were examined for the extent of apoptosis. In the absence of insulin, the percentage of apoptotic cells determined in CHO-R15 cells transfected with the antisense Mn-SOD plasmid was slightly but not significantly higher than that determined in control cells (Fig. 5C). In contrast, in the presence of insulin, the percentage of apoptotic cells in cells transfected with the antisense Mn-SOD plasmid was significantly increased as compared with controls (Fig. 5C). These results are consistent with the notion that Mn-SOD, like TRAF2, contributes to the antiapoptotic function of insulin in CHO-R15 cells.

**DISCUSSION**

We (25) and others (26) previously reported that insulin activates NF-κB in mammalian cells. More recently, we provided evidence for a role of NF-κB in the antiapoptotic function of insulin (1). To clarify the role of NF-κB in this function, we now investigated whether the NF-κB-dependent genes coding for the antiapoptotic proteins TRAF2, c-IAP1, c-IAP2, and Mn-SOD were activated by insulin and, if so, whether these proteins contributed to insulin antiapoptotic signaling.

Insulin caused a time-dependent increase in TRAF2 mRNA and/or protein expression in CHO-R and CHO-R15 cells. In these cells, the TRAF2 immunoreactive protein was localized in the cytoplasm and the nucleus. Accordingly, Min et al. (16) observed a cytoplasmic and nuclear localization of endogenous and transfected TRAF2 in human endothelial cells and proposed a role for nuclear TRAF2 in the regulation of gene transcription. In CHO-R15 cells, insulin increased the amount of TRAF2 protein in both the cytosolic and nuclear fractions. These findings provide the first evidence that insulin stimulates the expression of an adapter protein recruited by activated TNF receptors and thus reveal a novel aspect of the interplay between insulin and TNF-α signaling (27).

Insulin failed to increase TRAF2 expression in CHO-Y2 cells overexpressing tyrosine kinase-deficient IRs mutated at Tyr^{1162} and Tyr^{1163} residues, similar to what was found for insulin activation of NF-κB (25) and insulin antiapoptotic signaling (1). Third, the inability of insulin to induce TRAF2 expression in CHO-Y2 cells that had lost their response to insulin for the activation of NF-κB (25) suggests a role for NF-κB in insulin induction of TRAF2. This hypothesis is further supported by the following findings. (i) Insulin failed to increase TRAF2 mRNA in IκB-α(A32/36) cells, a clone of CHO-R15 cells expressing a dominant-negative form of IκB-α and exhibiting a dramatic decrease in basal and insulin-stimulated NF-κB activities; and (ii) reciprocally, the insulin-induced increase in TRAF2 mRNA found in CHO-R and CHO-R15 cells was mimicked in Rel-3 cells, a clone of CHO-R cells exhibiting a constitutive activation of NF-κB activity due to c-Rel overexpression (Ref. 1 and this study). Altogether, these results indicate that insulin increases TRAF2 expression through a pathway dependent on the IR tyrosine kinase activity and through a transcriptional mechanism involving NF-κB activation. However, the possibility that insulin may also increase TRAF2 expression through a post-transcriptional mechanism as has been shown for other insulin-responsive genes (28) cannot be excluded. Of interest, our results extend the recent findings of Wang et al. (8), who were the first to report a role for NF-κB in TNF-α induction of TRAF2 in the HT1080 fibrosarcoma cell line. In this cell line, TNF-α also increased the expression of other NF-κB-regulated genes such as the genes coding for c-IAP1 and c-IAP2 (8). In the present study, the c-IAP1 and c-IAP2 mRNAs could not be detected in CHO-R15 cells in the absence or presence of insulin.

Several lines of evidence indicate that TRAF2 has an antiapoptotic function. Thymocytes from TRAF2 null mice (18) or from transgenic mice expressing a dominant-negative form of TRAF2 (19) were highly sensitive to TNF-α-induced cell death. In addition, the overexpression of dominant-negative TRAF2-(87–501) in MCF-7 and HeLa cells potentiated TNF-α-induced apoptosis (20). In the present study, TRAF2-(87–501) overexpression in CHO-R15 cells did not modify the extent of serum...
withdrawal-induced apoptosis, as supported by the results of the β-galactosidase and DNA fragmentation assays. A possible explanation for this difference is that endogenous TRAF2 is thought to modulate TNF-α-induced apoptosis (20), whereas it is unlikely to play a role in the control of serum withdrawal-induced apoptosis (29). Of interest, the transient and stable overexpression of TRAF2-(87–501) markedly reduced basal NF-κB (25), presumably because Mn-SOD is a protective protein induced by the hormone. Together, these results strongly argue that the increase in TRAF2 expression that insulin induces in CHO-R and CHO-R15 cells serves its antiapoptotic function.

The overexpression of TRAF2 markedly stimulated NF-κB-mediated activity in CHO-R15 cells, in agreement with what was found in other cell types (20). TRAF2 activation of NF-κB involves the ability of cytoplasmic TRAF2 to bind to NIK (17), an NF-κB-inducible kinase that activates the IκB kinase complex, thus leading to IκB phosphorylation and degradation and subsequent NF-κB activation (6). In addition, nuclear TRAF2 could participate in the activation of NF-κB-regulated genes (16). Here we show that, together with increasing basal and insulin-stimulated NF-κB activities, overexpressed TRAF2 inhibited serum withdrawal-induced apoptosis in CHO-R15 cells. Moreover, NF-κB inhibition by IκB-α(A32/36) reduced both TRAF2 and insulin antiapoptotic signaling in these cells, indicating that the inhibition of apoptosis induced by TRAF2 and insulin relies, at least in part, on the capacity of these molecules to activate NF-κB. The pathways whereby insulin and TRAF2 activate NF-κB appear to be distinct. Indeed, TRAF2 was not involved in insulin stimulation of NF-κB, as supported by the failure of overexpressed TRAF2-(87–501) to affect insulin-stimulated NF-κB activity despite the fact that TRAF2-(87–501) markedly reduced basal NF-κB activity in CHO-R15 cells. Considered altogether, our results suggest that TRAF2 is not required for insulin activation of NF-κB, but that an amplification loop involving NF-κB activation by overexpressed TRAF2 is required for insulin antiapoptotic signaling. This does not exclude the possibility that overexpressed TRAF2 may also contribute to this process through an NF-κB-independent pathway (18, 19, 21). On the other hand, the finding that insulin induced TRAF2 overexpression in cytosolic and nuclear fractions in CHO-R15 cells raises the question of whether it is cytoplasmic or nuclear TRAF2 or both that play a role in insulin antiapoptotic signaling.

Besides TRAF2, insulin increased Mn-SOD mRNA expression in CHO-R15 cells. Consistent with this, insulin-like growth factor 1, a growth factor activating several signaling molecules also activated by insulin, including NF-κB (25), enhanced Mn-SOD expression in MCLM colon cancer cells (30). The finding that insulin no longer increased Mn-SOD mRNA expression in IκB-α(A32/36) cells indicates that insulin induction of Mn-SOD involves NF-κB, similar to what was reported for TNF-α (31). The overexpression of Mn-SOD, an enzyme known to function as a scavenger of superoxide radicals, conferred cell resistance to TNF-α-induced apoptosis (22). Reciprocally, the reduction in endogenous Mn-SOD levels by expression of antisense Mn-SOD RNA increased cell susceptibility to TNF-α, presumably because Mn-SOD is a protective protein induced by this factor (11). In our study, the transient expression of antisense Mn-SOD RNA did not significantly modify the sensitivity of CHO-R15 cells to serum withdrawal-induced apoptosis, as explained by the fact that this process involves a signaling apoptotic pathway distinct from that initiated by TNF-α (29). In contrast, the antisense Mn-SOD RNA significantly reduced insulin protection against apoptosis, indicating that Mn-SOD is one of the protective proteins whose synthesis is induced by insulin to mediate cell survival. In this regard, it is worth noting that the protection exerted by insulin against apoptosis induced by growth factor deprivation in primary cultures of mouse proximal tubular cells involved its ability to inhibit the production of superoxide radicals in the culture medium (32).

In conclusion, our study provides the first evidence that the antiapoptotic function of insulin involves the activation by insulin of the NF-κB-dependent survival genes coding for TRAF2 and Mn-SOD. Since a recent study reported that the bcl-2 and bcl-x survival genes were induced by TNF-α through NF-κB activation (33), it would be interesting to investigate whether these genes could also be activated by insulin and mediate this function in concert with the genes encoding TRAF2 and Mn-SOD. In addition to the NF-κB pathway, insulin-mediated cell survival involves the activation of the phosphatidylinositol 3-kinase pathway (1, 4). Recent evidence indicates that Akt, the effector of phosphatidylinositol 3-kinase, promotes cell survival through several mechanisms, including the inhibition of the nuclear translocation of FKHL1, a transcription factor of the Forkhead family that is likely to regulate the expression of apoptotic genes (34–36). Further studies will be required to examine whether the antiapoptotic function of insulin involves the inhibition of this transcription factor in addition to the activation of the NF-κB-dependent survival genes coding for TRAF2 and Mn-SOD.

Acknowledgments—We thank Dr. D. V. Goeddel for the TRAF2 and TRAF2-(87–501) plasmids. Dr. D. W. Ballard for the c-IAP1 and c-IAP2 plasmids, Dr. S. Chouaib for the Mn-SOD cDNA, and Dr. A. Israel for the IκB-α(A32/36) plasmid. We thank Prof. L. Baud and Dr. C. Horn for critical reading of the manuscript and B. Jacquin for secretarial assistance.

REFERENCES

1. Bertrand, F., Afifi A., Cadoret, A., L'Alemann, G., Robin, H., Lascols, O., Capeau, J., and Cherqui, G. (1998) J. Biol. Chem. 273, 2931–2938.
2. Lee-Kwon, W., Park, D., Baskar, P. V., Cole, S., and Bernier, M. (1998) Biochemistry 37, 15747–15757.
3. Yenush, L., Zanella, C., Uchida, T., Bernal, D., and White, M. F. (1998) Mol. Cell. Biol. 18, 6784–6794.
4. Dudek, H., Datta, S. R., Franke, T. F., Birnboum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Science 275, 661–665.
5. Van Antwerp, D. J., Martin, S. J., Verma, I. M., and Green, D. R. (1998) Trends Cell Biol. 8, 107–111.
6. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–87.
7. Finc, T. S., and Baldwin, A. S. (1995) Immunity 3, 263–272.
8. Wang, C.-Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) Science 281, 1680–1683.
9. Cha, Z.-L., McKinsey, T. A., Liu, L., Gentry, J. Y., Malim, M. H., and Ballard, D. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10057–10062.
10. Sarma, V., Lin, Z., Clark, L., Rust, B. M., Tewari, W., Noelle, R. J., and Dixit, V. M. (1995) J. Biol. Chem. 270, 13234–13246.
11. Wong, G. H. W., Ettwell, J. H., Oberley, L. W., and Goeddel, D. V. (1998) Cell 95, 923–931.
12. Hirose, K., Longo, D. L., Oppenheim, J. J., and Matsumi, K. (1993) FASEB J. 7, 361–368.
13. Arch, R. H., Gedrich, R. W., and Thompson, C. B. (1998) Gene Dev. 12, 2821–2830.
14. Rothe, M., Pan, M.-G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1997) Cell 83, 1243–1252.
15. Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K. L., and Vaux, D. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4974–4978.
16. Min, W., Bradley, J. R., Galbraith, J. J., Jones, S. E., Legderwood, E. C., and Pober, J. S. (1998) J. Immunol. 161, 319–324.
17. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544.
18. Yeh, W.-C., Shahinian, A., Speiser, D., Kraunus, J., Bilia, F., Wakeham, A., de la Pumpa, J. L., Ferrick, D., Ham, B., Iscove, N., Ohashi, P., Rothb, M., Goeddel, D. V., and Mak, T. W. (1997) Immunity 7, 715–725.
19. Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C., and Choi, Y. (1997) Immunity 7, 763–773.
Insulin Activates NF-κB-dependent Survival Genes

30602

20. Liu, Z.-g., Hsu, H., Goeddel, D. V., and Karin, M. (1996) Cell 87, 565–576
21. Natoli, G., Costanzo, A., Guido, F., Meretti F., Bernardo A., Burgio, V. L., Agresti, C., and Levrero M. (1998) J. Biol. Chem. 273, 31262–31272
22. Manna, S. K., Zang, H. J., Yan, T., Oberley, L. W., and Aggarwal, B. B. (1998) J. Biol. Chem. 273, 13245–13254
23. Desbois-Mouthon, C., Blivet-Van Eggelpoel, M.-J., Auclair, M., Cherqui, G., Capeau, J., and Caron, M. (1998) Biochem. Biophys. Res. Commun. 245, 765–770
24. Cadoret, A., Bertrand, F., Baron-Delage, S., Lévy, P., Courtois, G., Gespach, C., Capeau, J., and Cherqui, G. (1997) Oncogene 14, 1589–1600
25. Bertrand, F., Philippe, C., Antoine, P.-J., Baud, L., Groyer, A., Capeau, J., and Cherqui, G. (1995) J. Biol. Chem. 270, 24435–24441
26. Zhou, G., and Kun, M. T. (1997) J. Biol. Chem. 272, 15174–15183
27. Peraldi, P., and Spiegelman, B. (1998) Mol. Cell. Biochem. 182, 169–175
28. O’Brien, R. M., and Granner, D. K. (1996) Physiol. Rev. 76, 1109–1161
29. Dragovich, T., Rudin, C. M., and Thompson, C. B. (1998) Oncogene 17, 3207–3213
30. Guo, Y.-S., Jin, G.-F., Houston, C. W., Thompson, J. C., and Townsend, C. M. Jr. (1998) J. Cell. Physiol. 175, 141–148
31. Cui, Z., Korsner, M., Tarrantins, N., and Chouaib, S. (1997) J. Biol. Chem. 272, 96–101
32. Lieberthal, W., Triaca, V., Koh, J. S., Pagano, P. J., and Levine, J. S. (1998) Am. J. Physiol. 275, F691–F702
33. Tamatani, M., Che, Y. H., Matsuzaki, H., Ogawa, S., Okado, H., Miyake, S., Mizuno, T., and Tsuchiya, M. (1999) J. Biol. Chem. 274, 8531–8538
34. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotob, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
35. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Science 282, 1318–1321
36. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868