A Role for Nuclear Factor κB in the Antiapoptotic Function of Insulin*

France Bertrand, Azeddine Atti‡, Axelle Cadoret, Gilles L’Allemain, Hélène Robin, Olivier Lascols, Jacqueline Capeau, and Gisèle Cherqui

From INSERM U.402, Institut Fédératif de Recherche 65, Laboratoire de Biologie Cellulaire, Faculté de Médecine Saint-Antoine, 27 rue Chaliguy, 75571 Paris, Cedex 12, France, the INSERM U.482, Institut Fédératif de Recherche 65, Hôpital Saint-Antoine, 184 rue du Faubourg Saint-Antoine, 75571 Paris, Cedex 12 France, and the Centre de Biochimie, CNRS, Unité Mixte de Recherche 134, Faculté des Sciences, Parc Valrose, 06108 Nice, Cedex 02, France

We previously reported that insulin activates nuclear factor κB (NF-κB) in Chinese hamster ovary (CHO)-R cells overexpressing wild-type insulin receptors (IRs) through a pathway requiring IR tyrosine kinase and Raf-1 kinase activities. We now investigated whether the activation of NF-κB by insulin could serve an anti-apoptotic function. Insulin (10^{-9}-10^{-7} M) inhibited apoptosis induced by serum withdrawal in CHO-R cells in a concentration-dependent manner. Insulin anti-apoptotic signaling: (i) was dependent on IR number and IR tyrosine kinase activity since it was reduced in parental CHO cells and was abolished in CHO-Y2 cells overexpressing IRs mutated at Tyr^{1162/1163}; (ii) was, like insulin activation of NF-κB, dependent on Raf-1 but not on mitogen-activated protein kinase activity since both processes were decreased by the dominant-negative Raf-1 mutant Raf-C4 whereas they persisted in mitogen-activated protein kinase-depleted cells; and (iii) required NF-κB activation since it was decreased by proteasome inhibitors and the dominant-negative IκB-α (A32/36) mutant and was mimicked by overexpression of the NF-κB c-Rel subunit. We also show that insulin anti-apoptotic signaling but not insulin activation of NF-κB involved phosphatidylinositol 3-kinase (PI 3-kinase), as supported by the inhibition of the former but not of the latter process by the PI 3-kinase inhibitor LY294002. Inhibition of both NF-κB and PI 3-kinase totally abolished insulin anti-apoptotic signaling. Thus insulin exerts a specific anti-apoptotic function which is dependent on IR tyrosine kinase activity and is mediated by both a Raf-1-dependent pathway that leads to NF-κB activation and a PI 3-kinase-dependent pathway.

Insulin has long been known as an anabolic hormone that stimulates a number of transporters in the plasma membrane and regulates, at the mitochondrial and cytoplasmic levels, a variety of rate-limiting enzymes involved in the intermediary metabolism. In the past decade, evidence has been provided that insulin also regulates gene expression through the control of various nuclear factors (1). We (2) and others (3) recently reported that insulin activated nuclear factor κB (NF-κB) in mammalian cells through a Raf-1-dependent pathway. The NF-κB/Rel family members identified so far include NF-κB1 (p50 and its precursor, p105), NF-κB2 (p52 and its precursor, p100), p65 (Rel A), c-Rel, and Rel B. Prototypical NF-κB is a p50/p65 heterodimer which is usually retained in the cytoplasm of unstimulated cells in an inactive form by IκB-α, the best characterized member of the IκB inhibitory protein family (4–6). Upon cell stimulation, IκB-α is rapidly phosphorylated at Ser^{32} and Ser^{36} near its amino terminus (7, 8), which targets this subunit for proteolytic degradation via the ubiquitin-proteasome pathway (9, 10). The released NF-κB p50/p65 heterodimer can then translocate to the nucleus where it directly binds to its cognate DNA sequence to regulate gene transcription (9, 10). NF-κB is involved in a number of different cellular processes including the control of apoptosis, a physiological mechanism of programmed cell death that is characterized by nuclear condensation and fragmentation and degradation of DNA into oligonucleosome fragments (11–13).

The role that NF-κB plays in apoptosis appears to be complex since it has been found to depend on the cell type. Some studies have implicated NF-κB in promoting apoptosis in certain cells such as neurons (14), Schwann cells (15), prostate carcinoma cells (16), and embryonic kidney cells (17). Conversely, several recent reports provided convincing evidence that NF-κB was involved in apoptosis inhibition. Cells from transgenic mice deficient in p65/RelA are highly susceptible to TNF-α-induced apoptosis and this susceptibility is reversed by transfection of the cells with the wild-type Rel A gene (18). Inhibition of NF-κB by protease inhibitors induces apoptosis in murine B cells, a cell type expressing constitutively active NF-κB (19). Furthermore, inhibition of NF-κB nuclear translocation by expression of a dominant-negative form of IκB-α that cannot be phosphorylated (IκB-α A32/36) increased cell death induced by apoptotic stimuli known to activate NF-κB such as TNF-α (20–22), ionizing radiation and daunorubicin (21) but not by the apoptotic inducer staurosporine, a compound which does not activate NF-κB (21).

Like insulin-like growth factor I (IGF-I), insulin was shown to exert an anti-apoptotic function. However, in most (23–29) but one (30) studies, this function was observed at high concentrations of insulin which are known to activate IGF-I receptors. Since the inhibition of apoptosis by IGF-I was shown to require the activation of signaling molecules such as IRS1 (31),

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§ 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.

1 The abbreviations used are: TNF, tumor necrosis factor; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; IGF-I, insulin-like growth factor I; IR, insulin receptor; LLaL, N-acetyl-L-leucyl-L-leucinal-L-norleucinal; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PI 3-kinase, phosphatidylinositol 3-kinase; SPM, serum-free medium; Z-AL, Z-Ile-Glu-(OrBu)-Ala-leucinal; PAGE, polyacrylamide gel electrophoresis.
phosphatidylinositol 3-kinase (PI 3-kinase) (29, 32), Akt (28), and less frequently mitogen-activated protein kinase (MAPK) (29), all of which are also specifically activated by insulin, we decided to investigate a specific antiapoptotic function of insu-
lin and to examine the role of NF-κB in this function. To this end, we used parental Chinese hamster ovary (CHO) cells and CHO cells overexpressing either wild-type human insulin rece-
ptors (IRs) or kinase-defective IRs mutated at Tyr1162 and Tyr1163, two autophosphorylation sites playing a crucial role in receptor activation (33–36). Our study provides evidence for the role of insulin to trigger antiapoptotic signaling in mam-
malian cells through the activation of its own receptors. Our results moreover indicate that the antiapoptotic function of insulin requires the integrity of the IR tyrosine kinase and is mediated by both a Raf-1-dependent pathway that leads to NF-κB activation and a PI 3-kinase-dependent pathway.

EXPERIMENTAL PROCEDURES

Reagents—[γ-32P]ATP (10 Ci/mmol), ECL detection kit, Hybond N+ membranes, and hyperfilm MP were from Amersham Corp. [lacte-
lyl-H]Acetyl coenzyme A (200 mCi/mmol), CAT assay grade, was obtained from NEN Life Sciences Inc. Insulin (Lot 1609) was purchased from Nove-
Labsories. IGF-I and the proteasome inhibitor I (Z-Ile-Glu-OrBu)-
Ala-leucinal (Z-LAL) were from Calbiochem. The calpain inhibitor N-
acetyl-leucinyl-leucinal-leucinal-norleucinal (LLnL), myelin basic protein, and protein A-Sepharose were obtained from Sigma. The MAPK R2 antibody was obtained from Upstate Biotechnology, Inc. The ERK1 (C-16) and the rabbit anti-c-rel polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc.

Cell Lines—The different CHO cell lines (generous gift from Professor E. Clauser, INSEMR U.36, Paris) used in this study have been previously described (2, 34, 35). These include the parental cell line (CHO), the CHO cell line transfected with a plasmid coding for the native form of human IRs (CHO-R), and the CHO cell line expressing human IRs in which tyrosines at positions 1162 and 1163 have been replaced with phenylalanine residues by site-directed mutagenesis (CHO-Y2). Cells were grown in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS).

Plasmids and Transfections—The green fluorescent protein plasmid pEFGP and the pCMV5/lacZ and pRSV-CAT plasmids were previously described (2, 37). The reporter plasmid (Igk18-conaluc) and its control counterpart conaluc, the mutated plasmid Mx and the low condensate construct 14.0 (ASK3226) (generous gifts from Dr. A. Israel, Institut Pasteur, Paris, France), and the plasmid RSV-C4 that expresses the Raf-1 dominant-negative mut-
ant Raf-C4 (generous gift from Dr. U. R. Rapp, Institute of Medical Radiobiology and Cell Biology, Wurzburg, Germany) have been de-
scribed (8, 38, 39). The full-length p44MAPK antisense construct that inhibits the expression of ERK1 (p44MAPK) and ERK2 (p42MAPK) and the full-length cRel antisense DNA (NEH-1) have been described (40). A cRel expression plasmid coding for the c-Rel subunit of NF-κB was a gener-
ous gift from Dr. N. R. Rice (Laboratory of Molecular Virology and Carcinogenesis, Frederick, MD). Transfections were performed by the calcium phosphate precipitation method. The amounts of the different reporter or expression plasmids used in each transfection assay are indicated in the legends to Table 1 and Figs. 5 and 6.

DNA Fragmentation Assay—For analysis of DNA ladderings, 3–5 × 10^5 cells were used according to the procedure of Herrmann et al. (41). Briefly, control or treated cells were harvested, collected by centrifuga-
tion, and washed twice in cold phosphate-buffered saline. Pellets were then suspended in 50 μl of lysis buffer containing 1% Nonidet P-40, 20 mM EDTA, 50 mM Tris- HCl (pH 7.5). After centrifugation for 5 min at 1,600 × g, the supernatant was collected and the extraction was re-
peated once. The supernatants were incubated with 5 mg/ml RNase A for 2 h at 56 °C in the presence of 1% SDS (w/v). Then 2.5 mg/ml proteinase K were added and the incubation continued for at least 2 h at 37 °C. DNA fragments were precipitated with 2.5 volumes of ethanol for the presence of 0.5 volume of 10 mM ammonium acetate at −20 °C overnight. After centrifugation, samples were washed with 70% ethanol and air-dried before loading buffer. Electrophoresis was performed in 1 × TBE buffer on 1% agarose gels containing ethidium bromide.

Apoptosis Assays—CHO-R cells and CHO-R cells expressing c-Rel (clone Resis-3) were maintained for 24 h in serum-free medium (SFM) with or without 10−7 M insulin. After 24 h, cells were fixed and stained with the Hoechst 33258 DNA dye. In some experiments, CHO-R cells were cotransfected with pCMV5/lacZ or pEFGP and different expres-
sion plasmids. At the indicated times after transfection, cells were maintained for 24 h in SFM in the presence or absence of 10−7 M insulin and then fixed and revealed with 5-bromo-4-chloro-3-indolyl β-D-galac-
toside or Hoechst 33258. Cells were observed microscopically and quan-
tification of apoptosis was performed as indicated in the legends to Figs. 5, 6, and 8. 

Luciferase and CAT Assays—The lysates from transfected cells were prepared and assayed for luciferase and CAT activities, as described previously (2). Results were normalized as indicated in the legend to Table 1.

Immunocomplex MAP Kinase Assay—CHO-R cells were solubilized in 6-well plates were serum-deprived for 24 h and treated for 12 min with or without 10−7 M insulin or 20% FCS. Then cells were lysed for 30 min at 4 °C in lysis buffer containing 1% (w/v) Triton X-100, 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 10 mM p-nitrophenyl phosphate). Final pellets were re-
suspended in 40 μl of kinase buffer containing 10 μg of myelin basic protein and 50 μM ATP (3 μCi of [γ-32P]ATP). After a 30-min incubation at 30 °C, the reaction was stopped by adding 40 μl of 2 × Laemmli’s buffer. The samples were treated at 95 °C for 5 min and separated by SDS-PAGE on 12% (w/v) polyacrylamide gels (29/1). The gels were then stained with Coomassie Blue, dried, and subjected to autoradiography.

Western Blot Analysis—Cells lysates prepared as above were sepa-
rated by SDS-PAGE on 12% (w/v) polyacrylamide gels and electrophoresed onto Hybond-ECL nitrocellulose membranes in 25 mM Tris, 192 mM glycine. Membranes were blocked in Tris-buffered saline (20 mM Tris-HCl (pH 7.5), 137 mM NaCl) containing 0.1% Tween 20 and 0.2% non-fat dry milk for 30 min at room temperature. The blots were then incubated overnight at 4 °C in blocking solution with the MAPK R2 (1:1,000) or c-Rel (1:500) antibodies. Thereafter, gels were washed in Tris-buffered saline and incubated with either horseradish peroxidase-
coujugated goat anti-rabbit IgG (1:10,000) or goat anti-mouse IgG (1: 
10,000) in blocking solution for 1 h. The blots were visualized by the Amersham ECL system.

Statistical Analysis—Results are given as the means ± S.E. for the indicated numbers of independently performed experiments. Differ-
ences between the mean values were evaluated by Student’s t test.

RESULTS

Insulin Inhibits Apoptosis in CHO-R Cells through Its Own Receptors—CHO-R cells maintained in SFM undergo apoptosis as indicated by characteristic nucleosomal DNA fragmentation starting at 15 h and continuing for 24 h after serum deprivation (Fig. 1A). The ability of insulin to inhibit apoptosis was studied by incubating the cells in SFM in the presence of increasing concentrations (10−8−10−5 M) of the hormone. Insulin reduced apot-
osis in CHO-R cells in a concentration-dependent manner (Fig. 1B) with a slight effect being detected at 10−8 M and submaximal and maximal protective effects being observed at 10−6 and 10−7 M, respectively. That in CHO-R cells the inhibition of apoptosis by 10−6 M insulin was equivalent to that elicited by IGF-I at 10−6 M, a concentration reported to be maximally effective on this process in different cell types (29, 32). As compared with CHO-R cells, parental CHO cells exhib-
ted decreased sensitivity to insulin for inhibition of apoptosis (Fig. 1C). When these experiments were repeated in CHO-Y2 cells which overexpress tyrosine kinase-deficient IRs mutated at Tyr1162/L1163 autophosphorylation sites, we observed that, at 10−6 and 10−7 M insulin, no protective effect at all against apoptosis (Fig. 2B).

To quantify the protective effect against apoptosis exerted by insulin in CHO-R cells, the percentage of apoptotic cells was evaluated by staining CHO-R cells with Hoechst 33258, a flu-
orescent DNA dye which visualizes the nuclear condensation
and the chromatin fragmentation characteristic of apoptosis. Quantitative evaluation of apoptotic cells by Hoechst staining was performed by counting about 2,500 cells at high magnification (×40). As shown in Fig. 3, the percentage of apoptotic cells amounted to 30.0 ± 2.4% in CHO-R cells maintained for 24 h in SFM. This value is certainly an underestimate of the percentage of cells in the population that undergo apoptosis upon serum withdrawal because cells that have progressed into the later stages of apoptosis detach from the tissue culture dish and are not scored in the assay. However, this does not challenge the validity of this assay which is based on the comparison of values obtained in the same manner. In the presence of insulin, the percentage of apoptotic cells fell to only 3.4 ± 1.0%, indicating that insulin reduces apoptosis in CHO-R cells by about 90%. Taken together, the above results provide evidence for the ability of insulin to exert an antiapoptotic function in CHO-R cells which is mediated by its own receptors and requires the IR tyrosine kinase activity.

Role of NF-κB in Insulin Antiapoptotic Signaling—We previously reported that insulin activates NF-κB in CHO-R cells (2). In view of recent findings from several groups (18, 20–22) indicating that NF-κB activation reduces programmed cell death in different cell types, we investigated the role of this transcription factor in insulin antiapoptotic signaling. To this end, we tested the effect of LLnL (5 × 10⁻⁵ M), a calpain I inhibitor, and Z-AL (3 × 10⁻⁵ M), a potent and selective proteasome inhibitor, both of which were shown to inhibit NF-κB activity by preventing IκB-α degradation (42, 43). At the maximally effective concentrations employed, LLnL and Z-AL almost completely abrogated basal and insulin-stimulated NF-κB transcriptional activity in CHO-R cells, as determined by the luciferase assay (Table I). Under these conditions, LLnL and Z-AL had no appreciable effect on the extent of apoptosis induced by a 24-h serum deprivation (Fig. 4). In contrast, LLnL and Z-AL markedly decreased the protective effect exerted by insulin against this process with the latter being more potent than the former (Fig. 4). NH₄Cl (2 × 10⁻³ M), a nonspecific lysosomal protease inhibitor which had no effect on insulin-stimulated NF-κB-mediated luciferase activity (Table I), did not modify the inhibitory effect of insulin on apoptosis (Fig. 4).

To provide further evidence for a role of NF-κB in insulin antiapoptotic signaling, CHO-R cells were transiently transfected with a β-galactosidase reporter (pCMV5/lacZ) in the presence of an empty vector (pCMV), or of an expression vector coding for the phosphorylation-negative IkB-α (A32/36) mutant, a specific inhibitor of NF-κB (8). Fifteen hours after transfection, the medium was removed and cells were incubated for a further 24 h in SFM in the presence or absence of 10⁻⁷ M insulin. Then cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside and the percentage of apoptotic cells was evaluated by scoring blue β-galactosidase transfectants as healthy or apoptotic as judged by shrinkage of the cell bodies, condensation and fragmentation of nuclei. As shown in Fig. 5A, transfection of CHO-R cells with the expression plasmid coding for the dominant-negative IkB-α (A32/36) mutant did not appreciably modify the extent of apoptosis induced by a 24-h serum deprivation (Fig. 5B).
CHO-R cells and clones of CHO-R cells expressing c-Rel or a full-length MAPK antisense (5 × 10^6 cells/60-mm dish) were transfected with 5 μg of the (luc)3-conaluc reporter plasmid or its control counterpart conaluc, together with 1 μg of PRSV-CAT (as a monitor for transfection efficiency). After transfection, cells were maintained for 24 h in 0.3% FCS-Ham’s F-12 medium and then for 24 h in the presence or absence of 10^{-7} M insulin, with or without the indicated inhibitors. In some experiments, CHO-R cells were transiently transfected with the above reporter plasmids together with the expression plasmids coding for the dominant-negative IkB-α (A32/36) or Raf-C4 mutants and were then maintained for 24 h in 0.3% FCS medium before being treated for a further 24 h in the presence or absence of 10^{-7} M insulin. Cell extracts were prepared and assayed for luciferase and CAT activities 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 CAT activity) are the mean ± S.E. of three independent experiments.

| Treatment                  | Normalized luciferase activity (% of control) |
|----------------------------|-----------------------------------------------|
| None                       | 100 ± 2                                       |
| LmL (5 × 10^{-5} M)         | 10 ± 2                                        |
| Z-AL (3 × 10^{-5} M)        | 7 ± 3                                         |
| NHCl (2 × 10^{-5} M)        | 106 ± 8                                       |
| LYS290402 (3 × 10^{-5} M)   | 88 ± 3                                        |

**Expression plasmids**

| Treatment                  | Normalized luciferase activity (% of control) |
|----------------------------|-----------------------------------------------|
| IkB-α (A32/36)             | 3 ± 0.1                                       |
| c-Rel                     | 212 ± 14                                      |
| Raf-C4                    | 103 ± 4                                       |
| MAPK antisense            | 90 ± 23                                       |

**INS (10^{-7} M)**

- + - - + - + + +

**LmL (5 × 10^{-5} M)**

- + - - + - + + +

**Z-AL (3 × 10^{-5} M)**

- + - - + - + + +

**NHCl (2 × 10^{-5} M)**

- - + - + + - + +

**Role of NF-κB in Insulin Antiapoptotic Function**

CHO-R cells grown for 2 days in 10% FCS-Ham’s F-12 medium were shifted to SFM and maintained in this medium in the presence or absence of 10^{-7} M insulin, with or without the indicated concentrations of LmL, Z-AL, or NHCl following steps as described in the legend to Fig. 1. Results are representative of three independent experiments.

Deprivation, as indicated by the closely similar percentages of apoptotic cells observed among cells cotransfected with pCMV5/lacZ and IkB-α (A32/36) (34 ± 5%) and cells cotransfected with pCMV5/lacZ and pCMV (32 ± 3%). In contrast, IkB-α (A32/36) markedly reduced the protective effect against apoptosis exerted by insulin in CHO-R cells, since the percentage of apoptotic cells observed among insulin-treated CHO-R cells cotransfected with pCMV5/lacZ and IkB-α (A32/36) (24 ± 2%) was significantly increased as compared with the percentage found in insulin-treated transfected CHO-R cells cotransfected with pCMV5/lacZ and pCMV (4 ± 1%). In accordance with these results, we found that the transient (Fig. 5B) or stable (Fig. 5C) expression of a construct coding for c-Rel, a NF-κB subunit whose overexpression leads to NF-κB activation (Table 1), protected CHO-R cells from apoptosis (Fig. 5C), as assessed by the marked decrease observed in the percentage of apoptotic cells among serum-deprived Rel-3 cells (10.2 ± 1.3%) as compared with the percentage found in serum-deprived CHO-R cells (30.0 ± 1%) (Fig. 5C). However, the insulin-like effect of overexpressed c-Rel was partial since the percentage of apoptotic cells counted among Rel-3 cells maintained for 24 h in the absence of FCS and insulin (10.2 ± 1.3%) was higher than the percentage found in serum-deprived CHO-R cells treated with insulin for 24 h (3.4 ± 1%) (Fig. 5C). In the presence of insulin, the percentages of apoptotic cells among Rel-3 cells and CHO-R cells were closely similar (3.0 ± 0.5 and 3.4 ± 1%, respectively) (Fig. 5C). These results showing that the protection exerted by insulin against apoptosis was partially suppressed by NF-κB inhibition and conversely was partially mimicked by NF-κB activation indicate that NF-κB mediates at least in part the antiapoptotic function of insulin.

**Role of Raf-1 but Not of MAPK in Insulin Activation of NF-κB and Insulin Antiapoptotic Signaling**

We (2) and others (3) recently reported that the activation of NF-κB by insulin involved a Raf-1-mediated pathway. This is confirmed in the present paper since the dominant-negative Raf-1 mutant Raf-C4 (39) abolished insulin stimulation of NF-κB-mediated luciferase activity (Table I). To investigate the role of Raf-1 in insulin antiapoptotic signaling, CHO-R cells were transiently transfected with the pCMV5/lacZ reporter plasmid together with pCMV or the RSV-C4β expression vector coding for Raf-C4. Fifteen hours after transfection, the medium was removed and cells were incubated for a further 24 h in SFM in the presence or absence of 10^{-7} M insulin prior to evaluating the percentage of apoptotic cells by scoring β-galactosidase transfectants as healthy or apoptotic. As shown in Fig. 6A, Raf-C4 did not appreciably modify the percentage of apoptosis induced by a 24-h serum deprivation as assessed by the closely similar percentages of apoptotic cells determined in CHO-R cells cotransfected with pCMV5/lacZ and Raf-C4 (35 ± 5%) and in those cotransfected with pCMV5/lacZ and pCMV (34 ± 4%). In contrast, Raf-C4 markedly reduced the protective effect exerted by insulin since the percentage of apoptotic cells among insulin-treated CHO-R cells cotransfected with pCMV5/lacZ and Raf-C4 was significantly increased (24 ± 3%) as compared with the percentage of apoptotic cells determined in insulin-treated cells cotransfected with pCMV5/lacZ and pCMV (6.0 ± 1.5%). These results indicate that insulin antiapoptotic signaling, like insulin activation of NF-κB, involves a Raf-1-mediated pathway.

To determine whether MAPK may play a role in insulin activation of NF-κB and insulin inhibition of apoptosis, we next investigated the involvement of the ERK1 and ERK2 isoforms of MAPK. We examined the effect of insulin on both processes in an acid load-selected clone of CHO-R cells transfected with a full-length p44MAPK antisense construct. This antisense construct reduced ERK1 (p44MAPK) expression in CHO-R cells transfected to a level which was about 10–15% of that observed in controls and completely abolished ERK2 (p42MAPK) expression (Fig. 6B), which is in accordance with previous results reported for fibroblasts (40). These MAPK-depleted cells exhibited a dramatic decrease in their response to insulin (10^{-7} M) and FCS (20%) for MAPK activation, as assessed by mobility shift-up of ERK1 and ERK2 and by phosphorylation of myelin basic protein after immunoprecipitation of cell lysates with a MAPK antibody that recognizes the two MAPK isoforms. In contrast, the MAPK-depleted CHO-R cells, like control CHO-R cells, were fully responsive to insulin (10^{-7} M) for NF-κB activation (Table I) and apoptosis inhibition (Fig. 6D). Essentially similar results were observed in another clone of
MAPK-depleted CHO-R cells (data not shown). Taken together, the above results argue for a role of Raf-1 kinase but not of MAPK in both insulin activation of NF-κB and insulin inhibition of apoptosis.

Role of PI 3-Kinase in Insulin Antiapoptotic Signaling but Not in Insulin Activation of NF-κB—Because we found that: (i) the inhibition of insulin-stimulated NF-κB activity by proteasome inhibitors or IκB-α (A32/36) markedly reduced but not totally prevented insulin protection from apoptosis (Figs. 4 and 5A) and (ii) that overexpressed c-Rel only partially mimicked the insulin protective effect (Fig. 5C), we examined the possible implication of another pathway in insulin antiapoptotic signaling.

We tested the involvement of PI 3-kinase since this kinase has recently emerged as an essential intermediary in the signal transduction pathway used by growth factors (26, 29, 32) and cytokines (31) to protect cells from apoptosis. For this purpose, we tested LY294002, a potent PI 3-kinase inhibitor (44). Table I shows that a 24-h treatment with a 3 × 10⁻⁵ M concentration of LY294002 did not modify either basal or insulin-stimulated NF-κB-mediated luciferase activity in CHO-R cells. Similarly, LY294002 (3 × 10⁻⁵ M, 24 h) did not affect the extent of apoptosis induced by a 24-h serum deprivation (Fig. 7A) in these cells. In contrast, LY294002 (1–3 × 10⁻⁵ M, 24 h) caused a concentration-dependent inhibition of the protective effect exerted by insulin (10⁻⁷ M) against apoptosis (Fig. 7A). This finding together with the lack of effect of LY294002 on insulin stimulation of NF-κB activity indicated that the PI 3-kinase inhibitor reduced insulin antiapoptotic signaling independently of NF-κB. Finally, since we observed that insulin protection against apoptosis was only partially abolished by the proteasome inhibitor Z-AL (Figs. 4 and 7B) or LY294002 (Fig. 7), we tested the combined effects of these compounds on this process. As shown in Fig. 7B, the profile of DNA degradation observed in serum-deprived CHO-R cells treated with insulin in the presence of a 3 × 10⁻⁵ M concentration of LY294002 and Z-AL was closely similar to that induced by a 24-h serum deprivation in control cells, indicating that inhibition of both the PI 3-kinase and the NF-κB pathways completely abolished the protective effect against apoptosis exerted by insulin in CHO-R cells.

DISCUSSION

The present study was designed to examine the ability of insulin to exert a specific antiapoptotic function in mammalian cells. Our observations indicate that: (i) insulin inhibits apoptosis through a signal transduction pathway that involves a PI 3-kinase activity and (ii) insulin antiapoptotic signaling is independent of NF-κB.

Fig. 5. Effect of IκB-α (A32/36) or c-Rel expression on insulin antiapoptotic signaling. A, CHO-R cells (2 × 10⁵ cells/3.5-mm dish) were transiently transfected with 2 μg of an empty vector (pCMV) or of an expression vector coding for the IκB-α (A32/36) mutant together with 0.5 μg of the β-galactosidase vector (pCMV5lacZ). Fifteen hours post-transfection, the medium was removed and cells were incubated for a further 24 h in SFM in the presence or absence of 10⁻⁷ M insulin. Cells were fixed and stained with the Hoechst 33342 DNA dye. Note that IκB-α transfectants (red arrows) have large nonapoptotic nuclei, while nontransfected cells are apoptotic, as indicated by nuclear condensation (white arrows). The results, expressed as a percentage of the total β-galactosidase transfectants counted (about 1,500 transfectants in either condition) are the mean ± S.E. of three experiments. B, CHO-R cells were transiently transfected with 3 μg of an empty vector (pCMV) or of an expression vector coding for the NF-κB c-Rel subunit together with 1 μg of a green fluorescence protein vector (pEFGP). Twenty-four hours after transfection, cells were fixed and stained with the Hoechst 33342 DNA dye. Note that c-Rel transfectants (red arrows) have large nonapoptotic nuclei, while nontransfected cells are apoptotic, as indicated by nuclear condensation (white arrows). The photograph with control pCMV transfectants is not shown. C, total cell extracts prepared from CHO-R cells or from an acid-load selected clone of CHO-R cells transfected with an expression vector coding for c-Rel (Rel-3) were separated by SDS-PAGE and immunoblotted with a specific c-Rel antibody (upper panel). Cells were grown for 2 days in 10% FCS medium and then shifted to SFM and maintained in this medium for 24 h in the presence or absence of 10⁻⁷ M insulin. Thereafter, cells were fixed and Hoechst-stained and quantitation of apoptosis was performed as indicated in the legend to Fig. 3. Results are the mean ± S.E. of three independent experiments.
Role of NF-κB in Insulin Antiapoptotic Function

Fig. 6. Effect of a dominant-negative Raf-1 mutant or a MAPK antisense on insulin antiapoptotic signaling. A, CHO-R cells were transiently transfected with 2 μg of pcMV or of an expression vector coding for Raf-C4 together with 0.5 μg of pcMV5/LacZ, following steps as indicated in the legend to Fig. 5A. The results, expressed as the percentage of the total β-galactosidase transfectants counted (about 1,200 transfectants in either condition) are the mean ± S.E. of three experiments. B, total cell extracts prepared from CHO-R cells or from an acid-load selected clone of CHO-R cells transfected with an expression plasmid coding for a MAPK antisense were separated by SDS-PAGE and immunoblotted with a MAPK specific antibody. C, CHO-R cells and CHO-R cells expressing a MAPK antisense were serum-deprived for 24 h and then treated for 12 min with or without 10⁻⁷ M insulin or 20% FCS. Cell extracts were separated on a high resolution SDS-polyacrylamide gel (12% acrylamide and 0.07% bisacrylamide) and the shift-ups of the p44 and p42 isoforms of MAPK were detected with the MAPK R2 antibody. In some experiments, the ERK1 (C-16) antibody was used for immunoprecipitation and the immunoprecipitates were tested for their ability to phospho-

Fig. 7. Effect of LY294002 alone or in combination with Z-AL on insulin antiapoptotic signaling. All steps were exactly as described in the legend to Fig. 4 except that CHO-R cells were treated with the indicated concentrations of LY294002 (A), LY294002, or Z-AL alone or of LY294002 in combination with Z-AL (B). Results are representative of three independent experiments.

cells and to examine the role of NF-κB in this function. To this end, we used parental CHO cells and CHO transfectants that overexpress wild-type human IRs or IRs made kinase-defective by mutation at Tyr1162/1163, two autophosphorylation sites playing a crucial role in receptor activation (33–36). Using these cell lines, we recently demonstrated that insulin is able to activate NF-κB through a pathway that requires both IR tyrosine kinase and Raf-1 kinase activities (2).

In contrast to several studies which observed the antiapoptotic function of insulin exclusively at high concentrations which are known to activate IGF-I receptors (23–29), the present study reports that insulin is able to produce a concentration-dependent inhibition of apoptosis in CHO-R cells in a low range of insulin concentrations (10⁻⁴–10⁻⁷ M). The finding that at 10⁻⁷ M insulin only slightly inhibited apoptosis is probably due to the high rate of degradation of the hormone observed in CHO-R cells (35). Several observations indicate that insulin triggered antiapoptotic signaling through its own receptors. First, insulin was potent to inhibit apoptosis at 10⁻⁸ M, a concentration at which insulin was unable to displace the binding of ¹²⁵I-labeled IGF-I to CHO-R cells (35). Second, the sensitivity of parental CHO cells to insulin for inhibition of apoptosis was markedly reduced as compared with that exhibited by CHO-R cells, indicating that this inhibition is related to IR number. Third, at 10⁻⁸ and 10⁻⁷ M, insulin had no effect at all in CHO-Y2 cells overexpressing tyrosine kinase-deficient IRs mutated at Tyr₁₁₆₂/₁₁₆₃ autophosphorylation sites. This finding which most probably reflects a dominant-negative effect of overexpressed mutated human IRs on endogenous IRs (34, 35), indicates that the antiapoptotic capacity of insulin requires the presence of the IR 1162 and 1163 tyrosine residues. In addition, this finding shows that there is a marked difference between the antiapoptotic function of insulin which is suppressed by mutation of two of the major autophosphorylation sites in the IR kinase domain and the antiapoptotic function of IGF-I which was reported to be unaffected by mutation of the corresponding autophosphorylation sites in the IGF-I receptor kinase domain (32, 45).

The role of NF-κB in insulin antiapoptotic signaling was investigated by two complementary approaches: (i) inhibiting NF-κB activity by the proteasome inhibitors LLnL and Z-AL or by IκB-α (A32/36) expression; and (ii) inducing a deliberate activation of this transcription factor by transient or stable
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expression of the c-Rel subunit of NF-κB. Noteworthy, we established that neither maximally effective concentrations of LLnL or Z-AL nor expression of the dominant-negative IκB-α (A32/36) mutant did modify the extent of apoptosis induced by a 24-h serum deprivation in CHO-R cells, indicating no intrinsic apoptotic effect of NF-κB inhibition in this cell type. Such a result could be explained by the low level of constitutive NF-κB activity expressed in CHO-R cells (2). Consistent with this explanation, Wu et al. (19) observed that NF-κB inhibition by the protease inhibitor N-tosyl-l-chloromethyl ketone induced apoptosis in WEHI 231 cells which express constitutive nuclear NF-κB, whereas it failed to induce this process in NIH 3T3 fibroblasts which exhibit an extremely low level of nuclear NF-κB. Interestingly, we demonstrated that both the proteasome inhibitors and IκB-α (A32/36) expression markedly reduced the protective effect of insulin against apoptosis, as assessed by the following; (i) DNA degradation was reproducibly observed in the lysates from CHO-R cells treated with insulin in the presence of LLnL or Z-AL whereas it was undetectable in the lysates from CHO-R cells treated with insulin only; and (ii) the percentage of apoptotic cells in insulin-treated CHO-R cells cotransfected with pCMV5/ lacZ and IκB-α (A32/36) was significantly increased as compared with that percentage evaluated in insulin-treated CHO-R cells cotransfected with pCMV5/lacZ and pCMV. Accordingly, we observed that expression of c-Rel in CHO-R cells, which mimicked the insulin-induced increase in NF-κB activity, protected these cells against apoptosis as assessed by the significantly decreased number of apoptotic cells counted after a 24-h serum deprivation in c-Rel transfectants (Rel-3 clone) as compared with the number counted in control CHO-R cells. In agreement with this finding, recent papers reported that transfection of a c-Rel expression vector in HeLa or WEHI 231 cells significantly reduced TNF-α- or anti-IgM-induced apoptosis (19, 22). Altogether, the experiments reported here strongly argue for the antiapoptotic function exerted by insulin in CHO-R cells being mediated, at least in part, through the activation of NF-κB. The mechanisms by which insulin-activated NF-κB may reduce apoptosis in CHO-R cells are completely unknown. In this regard, Liu et al. (22) reported that the activation of NF-κB by TNF-α reduced TNF-α-induced apoptosis in HeLa cells and proposed a role for NF-κB in the induction of as yet unknown antiapoptotic genes.

Consistent with the idea that the activation of NF-κB by insulin is an intermediary step in insulin antiapoptotic signaling, both processes were found to be mediated by a common pathway involving Raf-1 kinase but not the ERK1 and ERK2 isoforms of MAPK. Indeed we observed that transient expression of the dominant-negative Raf-1 mutant Raf-C4 in CHO-R cells abolished insulin-stimulated NF-κB activity and markedly reduced insulin antiapoptotic signaling. This is supported by the loss of the insulin-induced increase in NF-κB-mediated luciferase activity in CHO-R cells expressing Raf-C4 and by the increased percentage of apoptotic cells observed in these cells in the presence of insulin as compared with the percentage determined in insulin-treated cells transfected with the vector alone. The involvement of Raf-1 in ligand-induced NF-κB activation has been previously reported by several studies including ours (2, 3, 46–48). However, the question of whether Raf-1 itself is responsible for the phosphorylation of IκB-α gave rise to controversial results (49–51). In this regard, it is worth noting that thus far several kinases have been reported to be involved in the phosphorylation of IκB-α and activation of NF-κB. These include: PKCe (50), the double-stranded RNA-activated p86 protein kinase (52), casein kinase II (53), MEKK1 (54), p90rsk (55) and CHUK (for conserved helix-loop-helix ubiquituous kinase) (56) which is identical to IKKe (for IκB-α kinase) (57). In light of these data, it was recently suggested that multiple independent NF-κB activation pathways exist, each requiring distinct IκB-α kinases (55). On the other hand, the finding reported here that Raf-1 kinase activity is required for insulin antiapoptotic signaling is consistent with the notion that Raf-1 probably plays a critical role in the promotion of cell survival, as supported by several recent studies (58–60). Conversely, Raf-1 has been found to induce apoptosis in fibroblasts (61), indicating the ability of this kinase to exert opposite effects depending on the cell context.

In contrast to Raf-1, MAPK failed to be involved in insulin activation of NF-κB and insulin antiapoptotic signaling. We indeed found that the stable expression of a MAPK antisense which almost completely suppressed MAPK expression and activity in CHO-R cells, did not affect insulin-stimulated NF-κB activity nor insulin protection from apoptosis, as assessed by the persistence of the insulin-induced increase in NF-κB-mediated luciferase activity and the absence of DNA degradation in insulin-treated CHO-R cells depleted of MAPK.

The involvement of Raf-1 but not MAPK in insulin activation of NF-κB is consistent with the result of Koong et al. (62) who observed that a dominant-negative mutant of Raf-1 but not the dominant-negative mutants of ERK1 and ERK2 abrogated NF-κB activation by hypoxia in NIH 3T3 cells. However, a role for MAPK in the activation of a NF-κB-dependent promoter by PKCe and TNF-α was subsequently reported in COS cells (63). These heterogenous observations raise the possibility that the role of MAPK in NF-κB activation may be related to the stimulus applied and/or the cell type studied. This also appears to be the case for the role of MAPK in the suppression of programmed cell death. In accordance with the present finding arguing for no implication of MAPK in insulin antiapoptotic signaling, MAPK was found to have no role in cell survival promoted by insulin (30), erythropoietin (64), or v-abl (65). Conversely, MAPK activation prevented apoptosis induced by nerve growth factor withdrawal in PC12 cells (66) and was required for the suppression of ceramide-induced apoptosis by sphingosine 1-phosphate in HL-60 cells (67). In addition, the role of MAPK in ligand-induced inhibition of apoptosis is cell-type specific is illustrated by the finding that MAPK was required for IGF-I antiapoptotic signaling in PC12 cells (29) but not in neurons (68) or cerebellar granule cells (69).

Although the activation of NF-κB by insulin was demonstrated to be a critical step in insulin antiapoptotic signaling, it only partially accounted for the effect of the hormone on this process. This is supported by the finding that the inhibition of apoptosis by insulin was markedly reduced but not totally abrogated by NF-κB inhibitors and was not fully mimicked by the overexpression of the NF-κB c-Rel subunit. We thus tested the involvement of PI 3-kinase which is known to be activated by insulin and was recently shown to play a role in the promotion of cell survival by IGF-I through a mechanism involving the activation of Akt (28, 32). In CHO-R cells, LY290042 appreciably reduced the antiapoptotic capacity of insulin but had no effect on insulin-stimulated NF-κB activity, pointing to a role of PI 3-kinase in the antiapoptotic function of insulin but not in the activation of NF-κB by the hormone. This finding argued for two independent pathways mediating the antiapoptotic function of insulin. In support of this notion, we observed that the treatment of CHO-R cells with LY290042 in combination with Z-AL completely abrogated the protection exerted by insulin against apoptosis, indicating that PI 3-kinase and NF-κB play separate roles in insulin antiapoptotic signaling. Similar to what was observed here for insulin, the antiapoptotic functions of either IGF-I (29) or interleukin-3 (70) were
reported to be mediated by two independent pathways. In conclusion, our study provides evidence for the ability of insulin to trigger antiapoptotic signaling in mammalian cells through the activation of its own receptors. Our results more-over indicate that insulin antiapoptotic signaling requires the integrity of the IR tyrosine kinase and is mediated by both a pathway that includes Raf-1 kinase and leads to NF-κB activation and a pathway that is dependent on PI 3-kinase activity.

In addition to the activation of these pathways, the antiapoptotic function exerted by insulin in CHO-R cells may require involving the p38 MAP kinase, as has been recently re-ported for PC12 cells and Rat-1 fibroblasts (30). Further studies will be needed to examine this point.

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France Bertrand, Azeddine Atfi, Axelle Cadoret, Gilles L'Allemain, Hélène Robin, Olivier Lascols, Jacqueline Capeau and Gisèle Cherqui

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