

C-Peptide Signals *via* $G\alpha_i$ to Protect against TNF- α -Mediated Apoptosis of Opossum Kidney Proximal Tubular Cells

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Cell loss by apoptosis occurs in renal injury such as diabetic nephropathy. TNF- α is a cytokine that induces apoptosis and has been implicated in the pathogenesis of diabetic nephropathy. The aim was to investigate whether C-peptide or insulin could modulate TNF- α -mediated cell death in opossum kidney proximal tubular cells and to examine the mechanism(s) of any effects observed. C-peptide and insulin protect against TNF- α -induced proximal tubular cell toxicity and apoptosis. Cell viability was analyzed by methylthiazolotetrazolium assay; cell viability was reduced to $60.8 \pm 2.7\%$ of control after stimulation with 300 ng/ml TNF- α . Compromised cell viability was reversed by pretreatment with 5 nM C-peptide or 100 nM insulin. TNF- α -induced apoptosis was detected by DNA nick-end labeling and by measuring histone associated DNA fragments using ELISA. By ELISA assay, 300 ng/ml TNF- α increased apoptosis by $145.8 \pm 4.9\%$ compared with controls, whereas 5 nM C-peptide and 100 nM insulin reduced apoptosis to 81.6 ± 4.8 and $77.4 \pm 3.1\%$ of control, respectively. The protective effects of C-peptide and insulin were associated with activation of NF- κ B. Activation of NF- κ B by C-peptide was pertussis toxin sensitive and dependent on activation of $G\alpha_i$. Phosphatidylinositol 3-kinase but not extracellular signal regulated mitogen-activated protein kinase mediated C-peptide and insulin activation of NF- κ B. The cytoprotective effects of both C-peptide and insulin were related to increased expression of TNF receptor-associated factor 2, the product of an NF- κ B-dependent survival gene. These data suggest that C-peptide and/or insulin activation of NF- κ B-regulated survival genes protects against TNF- α -induced renal tubular injury in diabetes. The data further support the concept of C-peptide as a peptide hormone in its own right and suggest a potential therapeutic role for C-peptide.

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C-peptide, a cleavage product that is derived from the proinsulin molecule in the course of insulin biosynthesis (1), is stored in the secretory granules of pancreatic β cells and is released eventually into the portal circulation in an amount equimolar with that of insulin (1). Although C-peptide has an important clinical role as a surrogate marker of insulin release, it generally is regarded as biologically inert. Until recently, popular dogma held that the only significant physiologic function of C-peptide related to facilitation of proinsulin folding to allow the accurate alignment of the A and B chains of insulin (1). However, a paradigm shift in thinking is occurring. C-peptide is undergoing a reevaluation as a peptide hormone in its own right, independent of insulin, possibly acting through a G protein-coupled membrane receptor (2).

Evolving evidence suggests that C-peptide might have a protective role in diabetes by ameliorating diabetic complica-

tions (3–6). C-peptide has been shown to exert beneficial effects on both renal function and morphology in diabetic nephropathy (4,7,8). These observations are supported by the findings that pancreas transplantation may induce reversal of diabetic nephropathy (9) and that patients who have type 1 diabetes and receive combined kidney/islet cell transplants have a better renal prognosis than patients who receive a kidney transplant alone (10), irrespective of metabolic control.

The mechanisms that underlie these beneficial effects of C-peptide are incompletely understood. However, C-peptide has been found to stimulate numerous intracellular signaling pathways in proximal tubular cells such as mitogen-activated protein kinases, phosphatidylinositol 3-kinase (PI3-K)/Akt, and protein kinase C (11), resulting in increases in intracellular $[Ca^{2+}]$ (11,12). C-peptide also has been shown to stimulate endothelial nitric oxide synthase in endothelial cells (13) and Na^+, K^+ -ATPase activity in both glomerular and tubular cells (14).

TNF- α is a pleiotropic 157-amino acid peptide cytokine that is capable of eliciting a wide spectrum of cellular responses, including differentiation, proliferation, inflammation, and cell death (15), *via* interaction with two members of the TNF receptor family, TNF-R1 and TNF-R2. Predominantly produced by monocytes/macrophages but also by T and B lymphocytes and

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glomerular mesangial cells (16,17), TNF- α 's binding to TNF-R1 may simultaneously trigger apoptotic pathways by recruitment of death effector adaptor molecules with subsequent activation of caspase cascades and antiapoptotic pathways by a pathway involving TNF receptor-associated factor 2 (TRAF2) and NF- κ B (for review, see [15]). Integration of these events determines the eventual cellular response to TNF- α stimulation. In particular, NF- κ B stimulates transcription of antiapoptotic factors that modulate the caspase cascade; therefore, NF- κ B activity acts as a checkpoint in a cell's decision to survive or apoptose in response to a given stimulus.

TNF- α is a key player in diverse renal diseases. In IgA nephropathy, elevated intrarenal TNF- α production and urinary concentrations of TNF- α are seen (18). In rodent studies of diabetic nephropathy, amplified renal expression of TNF- α mRNA is observed (19). Circulating urinary and renal interstitial TNF- α levels were increased after induction of diabetes with streptozotocin, preceding the rise in urinary albumin excretion (20). Increased serum levels of TNF- α have been found in patients with type 2 diabetic nephropathy (21).

Apoptosis, an active mode of cell death, contributes to the late structural abnormalities that are seen in diabetic nephropathy (22). Ortiz *et al.* (22) found a significant increase of apoptotic cells in the tubulointerstitium but not glomeruli of diabetic mice. Increased prevailing TNF- α in the kidney in diabetes may contribute to this apoptosis. In this work, we examined the effect of TNF- α on opossum kidney proximal tubular (OK) cell viability, and we assessed whether TNF- α cytotoxicity may be modulated by administration of C-peptide and insulin.

Materials and Methods

Materials

Human 31-amino acid C-peptide and a 31-amino acid scrambled C-peptide (sC-peptide) (2) were provided by Dr. John Wahren (Karolinska Institute, Stockholm, Sweden). Recombinant human TNF- α was from Sigma (Poole, UK). Tissue culture media and plasticware were from Invitrogen Life Technologies (Paisley, UK). Wortmannin, pertussis toxin (PTX), and carbobenzoxy-L-leucyl-leucyl-L-leucinal (MG-132) were obtained from Calbiochem (Nottingham, UK). The reporter plasmid pNF- κ B-luc was from Stratagene (La Jolla, CA). Luciferase assays were performed using the LucLite assay system and LumiCount luminometer (Packard, Pangbourne, England). β -Galactosidase assay kits were from Promega (Madison, WI). Cell Death Detection ELISA and *in situ* cell death detection kit were from Roche Diagnostics (Lewes, UK). Anti-TRAF2, anti-I κ B- α , and anti-G α_i antisera were from Santa Cruz Biotechnology (Santa Cruz, CA). Fugene-6 transfection agent was from Roche Diagnostics. All other laboratory chemicals and reagents were from Sigma.

Cell Culture

OK cells, an established proximal tubular epithelial cell line that is derived from the American opossum (23), retain many characteristics of proximal tubular cells and have been used extensively to investigate proximal tubular cell function (24) and the signaling effects of C-peptide (11). OK cells that were obtained from Dr. J. Caverzasio (University Hospital, Geneva, Switzerland) were used in all experiments between passages 62 and 83. Cells were maintained in DMEM-F12 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin,

100 μ g/ml streptomycin, and 10 mM HEPES. In all experiments, cells were serum starved overnight before agonist stimulation.

Luciferase Reporter Assay

Cells were transiently transfected with pNF- κ B-luc using Fugene-6. Briefly, 2.5×10^4 cells per well were plated in 24-well plates and were grown to 50% confluence. Cells were transfected with both pNF- κ B and pSV β -Gal to control for transfection efficiency. After transfection, the cells were incubated in 1 ml of serum-free medium overnight before stimulation with various agents. When necessary, 100 ng/ml PTX was added at this stage to the serum-free medium. In some experiments, inhibitors were applied to cells 30 min before the addition of agonists, and the cells subsequently were incubated with agonists \pm inhibitors for an additional 24 h. Medium was removed, and cells were lysed in a buffer that contained of 500 mM HEPES, 2% Triton N101, 1 mM CaCl₂, and 1 mM MgCl₂ (pH 7.8). Cell lysis was allowed to proceed for 10 min, and luciferase activity in lysates was measured. In all experiments, a 50- μ l aliquot of lysate was removed for β -galactosidase assay, and luciferase activity was normalized to β -galactosidase content.

Cell Viability Assay

Cell viability was determined by methylthiazolotetrazolium assay as described previously (25).

Cell Death Detection ELISA

This assay relied on the detection by ELISA of cytoplasmic histone-associated DNA fragments to identify apoptotic cells. Cells that were cultured in 24-well plates to 70% confluence were serum starved overnight. Cells were preincubated with or without 5 nM C-peptide or 100 nM insulin for 24 h before treatment with 300 ng/ml TNF- α or other agents for an additional 24 h. When used, C-peptide and insulin were present in media during the entire period of stimulation with TNF- α and other agents. In some experiments, cells were preincubated with an NF- κ B inhibitor for 1 h before incubation with C-peptide or insulin and for the subsequent duration of the experiment. The assay then was performed according to the manufacturer's instructions. Colored reaction product was measured at 405 nm.

DNA Nick-End Labeling of OK Cells

The method of terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) was performed using an *In Situ* Cell Death Detection Kit. OK cells were plated in sterile chamber slides and allowed to adhere for 24 h. Cells were serum starved overnight, then preincubated with or without 5 nM C-peptide or 100 nM insulin for 24 h before stimulation with 300 ng/ml TNF- α for an additional 24 h. C-peptide and insulin remained during this period of stimulation. Cells were rinsed twice for 5 min with PBS (pH 7.5), fixed with 4% paraformaldehyde, and stained according to the manufacturer's instructions. Stained samples were examined by light microscopy.

Immunoblotting

OK cell membrane and cytosolic fractions were prepared as described previously (11). A total of 30 μ g of cytosolic protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were immunoblotted using 1:1000 dilutions of primary and peroxidase-conjugated secondary antisera. Bound secondary antibody was detected using enhanced chemiluminescence.

Assessment of $G\alpha_i$ Activation by [^{35}S]-Guanosine 5' Triphosphate- γS Binding Assay

Activation of $G\alpha_i$ was assessed by [^{35}S]guanosine 5' triphosphate- γS (GTP γS) binding assay as described previously by our laboratory (26). Frozen aliquots of OK cell membranes, prepared as in previous studies (26), were thawed on ice and diluted in assay buffer (10 mM HEPES, 100 mM NaCl, and 10 mM MgCl [pH 7.4]) to give a final protein concentration of 25 $\mu\text{g}/50 \mu\text{l}$. Samples that contained 25 μg of protein were placed in 1.5-ml microfuge tubes in the presence of 10 μM guanosine 5'-diphosphate sodium salt, 1 nM [^{35}S]GTP γS (1250 Ci/mmol) diluted in 10 mM tricine, 10 mM dithiothreitol, and where appropriate, 10 μM cold GTP γS to determine nonspecific binding, and 5 nM C-peptide or 5 nM sC-peptide diluted to 100 μl in assay buffer and incubated for 2 min at 37°C. Incubation was terminated by addition of 1 ml of ice-cold assay buffer, and all subsequent procedures were performed at 4°C. Cell membranes were solubilized and [^{35}S]GTP γS bound $G\alpha_i$ proteins were immunoprecipitated using anti- $G\alpha_i$ antisera and protein A Sepharose beads. After washing, radioactivity in immunoprecipitates was counted by liquid scintillation spectroscopy.

Statistical Analyses

Data are presented as mean \pm SEM. Unpaired *t* test was used for comparisons between two groups. For multiple comparisons, one-way ANOVA with Tukey range test was used. Where normalized data are shown, analysis was performed on the raw data.

Results

In line with previous C-peptide studies in which maximal activity was observed at physiologic (2) low nanomolar concentrations (11–14), we used this agent at a concentration of 5 nM in many of our experiments. In contrast, activation of cell signaling by insulin *in vitro* generally requires higher than physiologic concentrations; 100 nM insulin is commonly used and was used in our experiments.

C-Peptide and Insulin Protect against TNF- α -Induced Cytotoxicity in Proximal Tubular Cells

Incubation of OK cells with increasing concentrations of TNF- α resulted in declining viability, becoming significant at the higher concentration of 300 ng/ml TNF- α , when cell viability was reduced to approximately 60.8 \pm 2.7% of control (Figure 1). Both C-peptide and insulin treatment were able to abolish TNF- α toxicity.

C-Peptide and Insulin Protect against TNF- α -Induced Apoptosis of Proximal Tubular Cells

We hypothesized that reduced cell viability that was induced by 300 ng/ml TNF- α may be due to apoptosis; therefore, we went on to examine the ability of TNF- α to induce OK cell apoptosis using two different methods. Apoptotic cells were directly visualized and counted using the TUNEL assay (Figure 2, A and B). Serum starvation of cells was accompanied by low-level background apoptosis of 3.0 \pm 0.5% of cells. This was significantly exacerbated by 300 ng/ml TNF- α when 12.0 \pm 1.1% of cells were observed to be apoptotic. The TNF- α -induced apoptosis was completely attenuated by 5 nM C-peptide and/or 100 nM insulin when 2.3 \pm 0.7 and 2.0 \pm 0.5% of cells, respectively, were found to be apoptotic.

TNF- α significantly also increased apoptosis as judged by

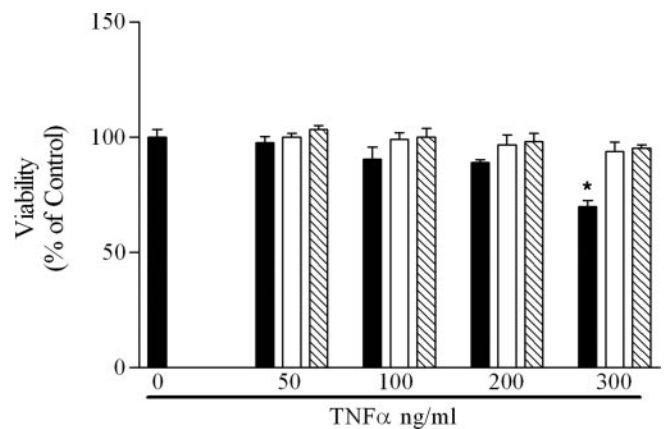


Figure 1. C-peptide and insulin protect against TNF- α -induced cytotoxicity. Methylthiazolotetrazolium assay of cells that were preincubated with 5 nM C-peptide (□), 100 nM insulin (▨), or medium alone (■) before exposure to TNF- α ; *n* = 3 independent experiments with each condition performed in triplicate; **P* < 0.01 relative to control.

ELISA of histone-associated DNA fragments (Figure 2C) by 145.8 \pm 5.9% compared with control serum-starved cells. This effect was prevented by treatment with C-peptide (81.6 \pm 4.8% compared with control serum-starved cells) or insulin (77.4 \pm 3.1% of control) serum-starved cells. As a positive control, cells were treated with 50 μM etoposide, which increased histone-associated DNA fragments to 160.8 \pm 2.5% of control serum-starved cells. Combining insulin with C-peptide did not result in any additive antiapoptotic effect in these studies (data not shown).

NF- κB Inhibitor MG-132 Antagonizes Protective Effects of C-Peptide and Insulin on TNF- α -Induced Apoptosis

We reasoned that activation of NF- κB might be responsible for the cytoprotective effects of C-peptide and insulin. To address this question, we used the proteasome inhibitor MG-132 to block NF- κB activity. This agent blocks the degradation of I κB , thus preventing the translocation of NF- κB to the nucleus (27). Treatment of serum-starved cells with MG-132 (Figure 3) induced an insignificant increase in histone-associated DNA fragments to 100.7 \pm 2.9% of control, indicating that at these concentrations, MG-132 was not toxic to OK cells. Pretreatment of cells for 1 h with 5 μM MG-132 completely abolished the ability of C-peptide and insulin to antagonize TNF- α -induced apoptosis. Compared with 80.8 \pm 3.6% of apoptotic cells seen in TNF- α /C-peptide-treated cultures, MG-132 pretreatment led to the appearance of 159.2 \pm 4.2% of apoptotic cells. Pretreatment with MG-132 resulted in an increase in apoptotic cells from 78.6 \pm 4.1 to 154.9 \pm 1.8% in TNF- α /insulin-stimulated cultures.

C-Peptide and Insulin Activate NF- κB in OK Cells

To demonstrate activation of NF- κB , the levels of I κB - α protein were determined by Western blotting of OK cell cytosol fractions (Figure 4A) 24 h after treatment of the cells with 5 nM C-peptide, 100 nM insulin, and, as a positive control, 1 μM

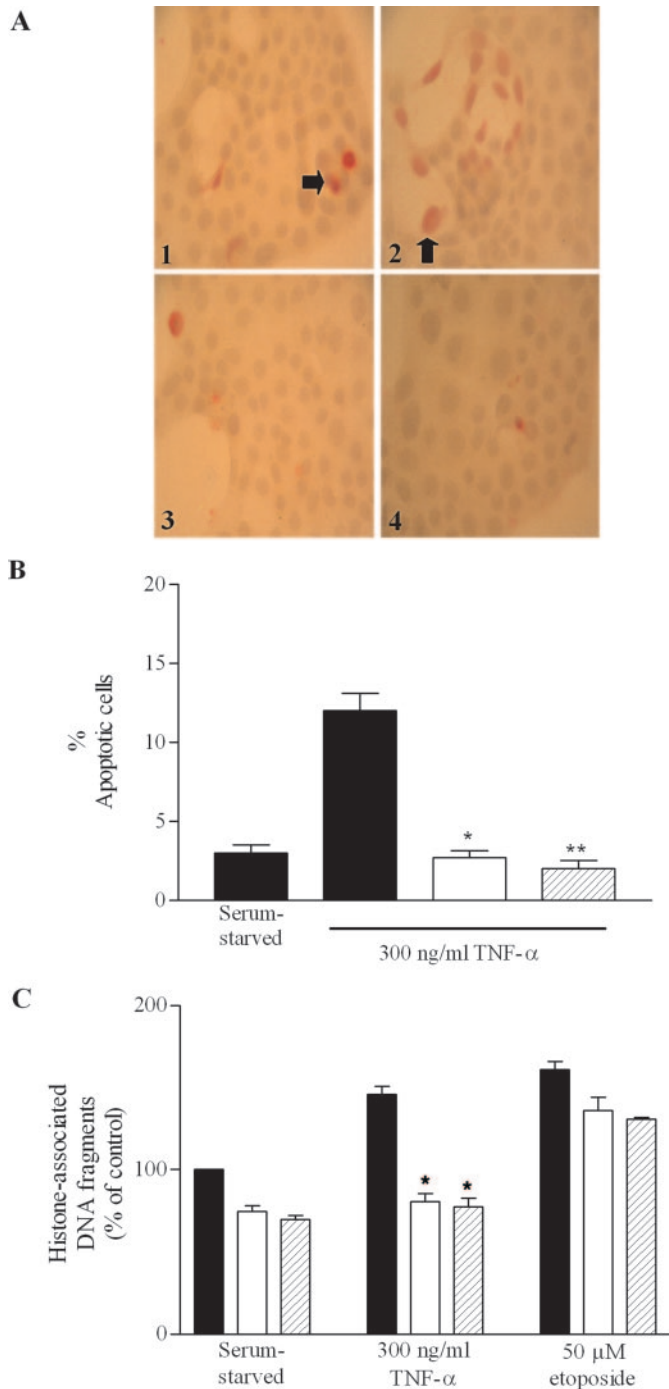


Figure 2. C-peptide and insulin protect against TNF- α -induced apoptosis. (A) Transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) assay. Some apoptotic cells are identified by arrows. 1, serum-starved control; 2, 300 ng/ml TNF- α treatment; 3, C-peptide pretreatment before 300 ng/ml TNF- α ; 4, insulin pretreatment before 300 ng/ml TNF- α . (B) Quantification of TUNEL-stained apoptotic cells. \blacksquare , cells that were not treated with either C-peptide or insulin; \square , C-peptide-stimulated cells; ▨ , insulin-stimulated cells; $n = 3$; * $P < 0.05$ and ** $P < 0.01$ versus TNF- α -treated cells. (C) ELISA apoptosis assay. Serum-starved cells were preincubated without (\blacksquare) or with 5 nM C-peptide (\square) or 100 nM insulin (▨) for 24 h before exposure to TNF- α or etoposide; $n = 3$ independent experiments with each condition performed in triplicate; * $P < 0.01$ versus corresponding control where insulin and C-peptide omitted.

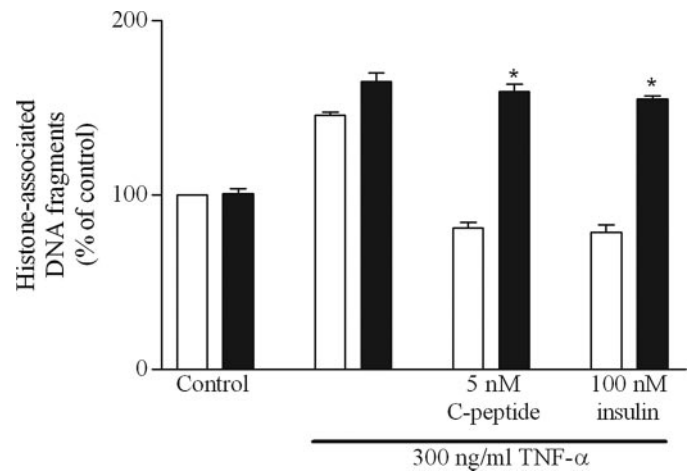


Figure 3. NF- κ B inhibitor blocks C-peptide and insulin protection against TNF- α -induced apoptosis. Cells were preincubated with (\blacksquare) or without (\square) carbobenzoxy-L-leucyl-leucyl-L-leucinal (MG-132) before incubation with or without C-peptide or insulin. Cells then were treated with TNF- α ; $n = 3$ independent experiments with each condition performed in triplicate; * $P < 0.01$ versus non-MG-132-stimulated cells. Apoptosis in cells that were not treated with MG-132, C-peptide, insulin, or TNF- α was normalized to 100%, and all other conditions were compared with this.

phorbol myristate acetate (PMA). After 24 h, levels of I κ B- α protein were less in cells that were stimulated with C-peptide, insulin, and PMA. Densitometry revealed that levels of I κ B- α protein were decreased to 0.7 ± 0.07 -, 0.55 ± 0.06 -, and 0.5 ± 0.01 -fold relative to basal by C-peptide, insulin, and PMA, respectively (Figure 4B).

To establish unequivocally NF- κ B activation, we transiently transfected OK cells with pNF- κ B-luc and stimulated them with various concentrations of C-peptide, insulin, and 1 μ M PMA for 24 h. Insulin and C-peptide induced a concentration-dependent activation of NF- κ B transcriptional activity (Figure 4C). C-peptide, at a concentration of 5 nM, maximally stimulated NF- κ B to $169.2 \pm 3.6\%$ compared with control. Similarly, insulin, at a concentration of 100 nM, maximally stimulated NF- κ B to $221.5 \pm 5.2\%$ compared with control. As expected, 1 μ M PMA was a potent stimulator of NF- κ B, eliciting maximal activation of $1999.3 \pm 15.9\%$ compared with control. Combining 5 nM C-peptide and 100 nM insulin together had no additive effects on NF- κ B activation over that observed with each agent alone (data not shown).

Role of G α_i Proteins in Activation of NF- κ B by C-Peptide

Pretreatment of cells with 100 ng/ml PTX for 18 h before stimulation with 5 nM C-peptide blocked activation of NF- κ B activity in the luciferase reporter assay (Figure 5A). Activation of NF- κ B by insulin was unaffected by PTX.

Both G α_i and G α_o G-proteins are functionally inhibited by PTX. C-peptide activation of G α_i in OK cells was assessed by immunoprecipitation of [35 S]GTP γ S-bound G α_i after agonist stimulation. Initially, expression of G α_i proteins in OK cells was assessed by Western blotting of cell membranes with

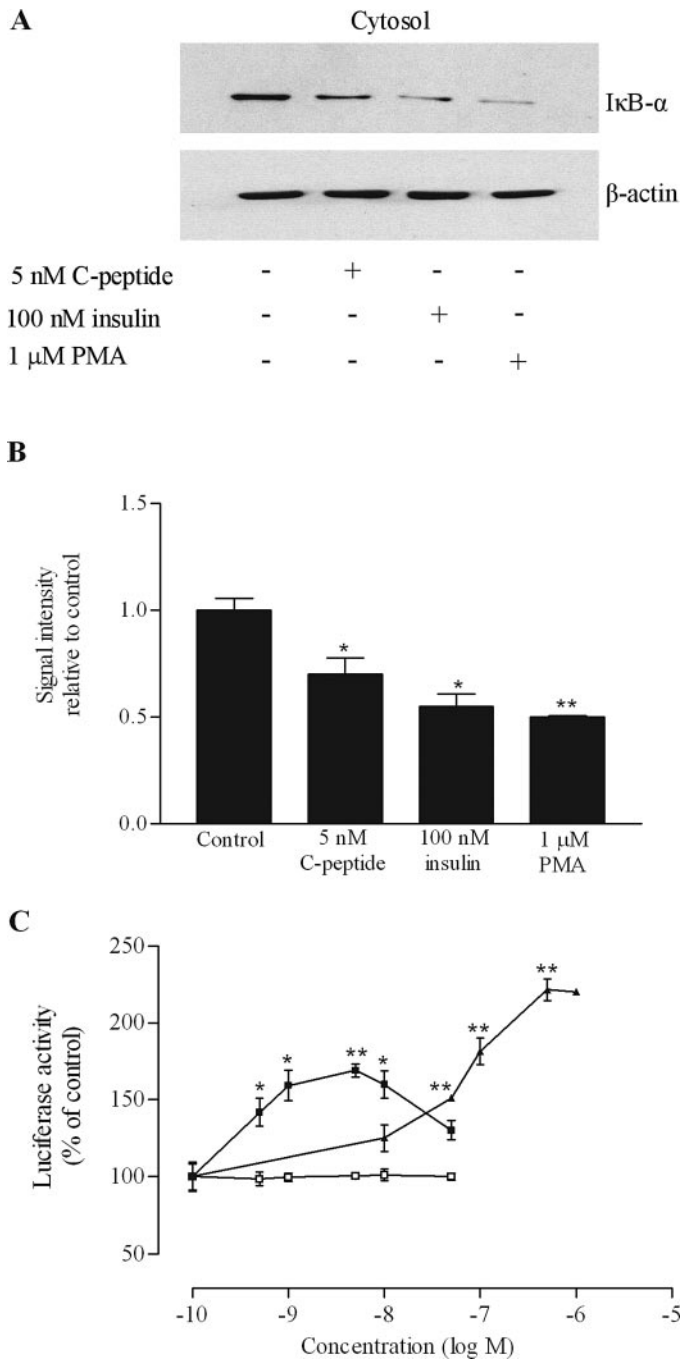


Figure 4. C-peptide and insulin activate NF-κB. (A) C-peptide and insulin enhance the cytosolic degradation of IκB-α as detected by Western blotting. (B) Densitometry and quantification of immunoblots; *n* = 4; **P* < 0.05, ***P* < 0.01 relative to control. (C) NF-κB activity in cells that were treated with C-peptide (■), scrambled C-peptide (□), or insulin (▲); *n* = 4 independent experiments with each condition performed in triplicate; **P* < 0.05, ***P* < 0.01 relative to negative control (designated 10⁻¹⁰ M).

specific anti-Gα_i antisera that recognized all three members of the Gα_i family. As shown in Figure 5B, specific anti-Gα_i were able to detect Gα_i proteins in OK cells. C-peptide caused a time-dependent increase in [³⁵S]GTPγS binding to

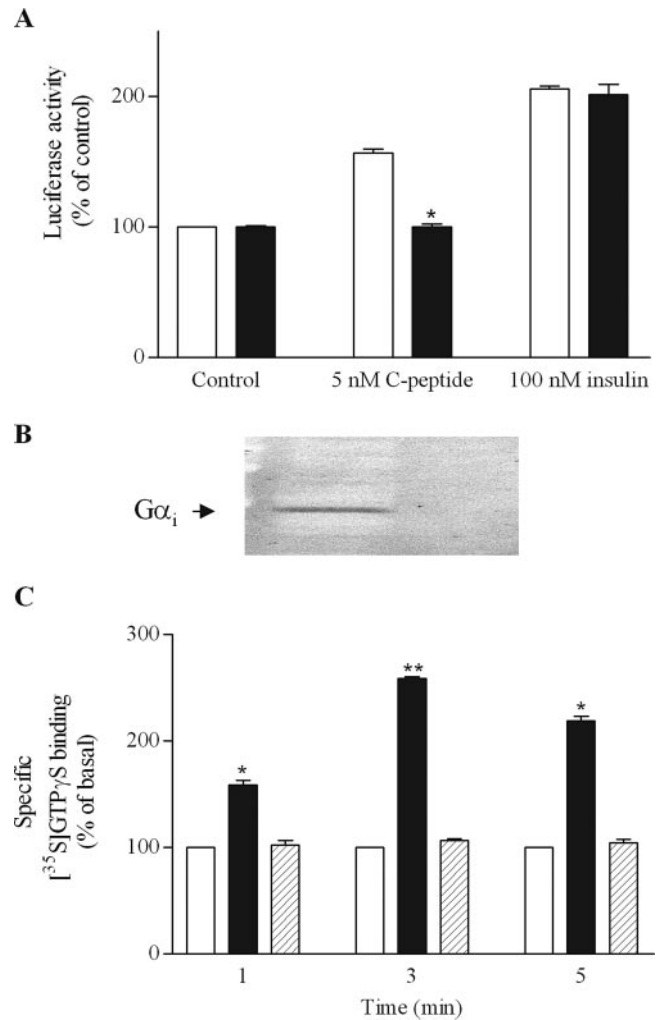


Figure 5. Activation of NF-κB by C-peptide is pertussis toxin (PTX) sensitive and therefore involves Gα_i. (A) NF-κB activity in cells that were treated with (■) or without (□) 100 ng/ml PTX before stimulation with C-peptide or insulin; *n* = 3; **P* < 0.01 versus non-PTX-treated, C-peptide-stimulated cells. NF-κB activity in cells that were not treated with PTX, C-peptide, or insulin was normalized to 100%, and all other conditions were compared with this. (B) Detection of Gα_i by immunoblotting. (C) C-peptide-stimulated [³⁵S]guanosine 5' triphosphate-γS (GTPγS) binding to Gα_i in cell membranes. Membranes were incubated in the absence (□; basal condition) or the presence (■) of 5 nM C-peptide or 5 nM sC-peptide (▨); *n* = 3 independent experiments with each condition performed in triplicate; **P* < 0.01, ***P* < 0.001 versus basal for relevant time point.

Gα_i in OK cell membranes, indicative of activation. Gα_i activation occurred rapidly, being detectable in 1 min and maximal 3 min after stimulation. Indeed, 5 nM C-peptide exposure resulted in [³⁵S]GTPγS binding of 258.3 ± 6.2% compared with basal levels, which were normalized to 100%. Basal [³⁵S] counts were approximately 350 to 500 cpm, and nonspecific binding accounted for ≤20% of total counts. In contrast, sC-peptide did not activate [³⁵S]GTPγS binding to any significant level (Figure 5C).

C-Peptide- and Insulin-Stimulated NF- κ B Activity Is Mediated by PI3-K but not Extracellular Signal Regulated Mitogen-Activated Protein Kinase

Mechanisms by which C-peptide mediated activation of NF κ B were examined according to the previously reported signaling effects of C-peptide in OK cells (11). Pretreatment of cells with 10 μ M PD98059, an inhibitor of the upstream extracellular signal regulated mitogen-activated protein kinase kinase 1, for 30 min before stimulation with 5 nM C-peptide or 100 nM insulin failed to attenuate the effects of C-peptide or insulin on NF- κ B activity. Conversely, pretreatment of the cells with 100 nM wortmannin, a PI3-K inhibitor, for 30 min significantly attenuated the effects of C-peptide and insulin on NF- κ B activity (Figure 6).

C-Peptide and Insulin Increase TRAF2 Protein Expression in OK Cells

To examine the role of NF- κ B-regulated genes in TNF- α -induced apoptosis of OK cells, we chose to study expression levels of TRAF2, a previously described NF- κ B-dependent survival gene (28). Treatment of OK cells with 300 ng/ml TNF- α resulted in a reduction of TRAF2 protein to 0.28 ± 0.04 -fold relative to basal levels. This reduction was completely inhibited by both C-peptide and insulin (Figure 7A). Indeed, in TNF- α -stimulated cells, C-peptide and insulin seemed to raise TRAF2 levels slightly above basal (Figure 7A). In non-TNF- α -stimulated cells, C-peptide and insulin significantly elevated TRAF2 protein 1.7 ± 0.03 - and 1.8 ± 0.05 -fold relative to basal, respectively (Figure 7B).

We next examined the ability of TNF- α to activate NF- κ B in our system (Figure 8). At a low concentration of 10 ng/ml, TNF- α was able to stimulate NF- κ B ($140 \pm 3.2\%$ compared with control), but 300 ng/ml TNF- α , a concentration that is capable of inducing toxicity, was unable to activate NF- κ B. Both C-peptide and insulin retained their capacity to activate NF- κ B in the presence of 300 ng/ml TNF- α . In addition, the combination

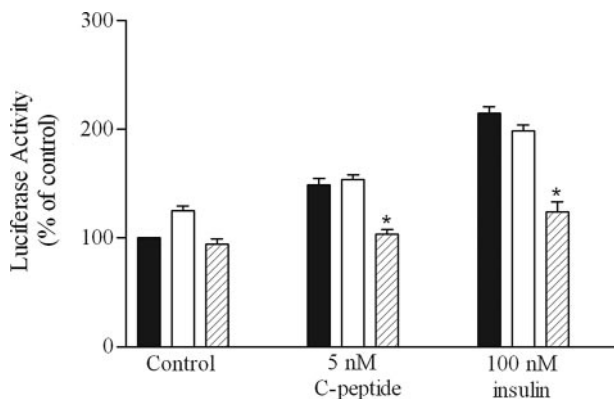


Figure 6. Phosphatidylinositol 3-kinase (PI3-K) involvement in activation of NF- κ B by C-peptide and insulin. NF- κ B activity in cells that were pretreated with wortmannin (▨) or PD98059 (□) or without inhibitor (■) before stimulation with C-peptide or insulin; $n = 3$ independent experiments with each condition performed in triplicate; * $P < 0.05$ relative to non-inhibitor-treated, C-peptide-, or insulin-stimulated cells.

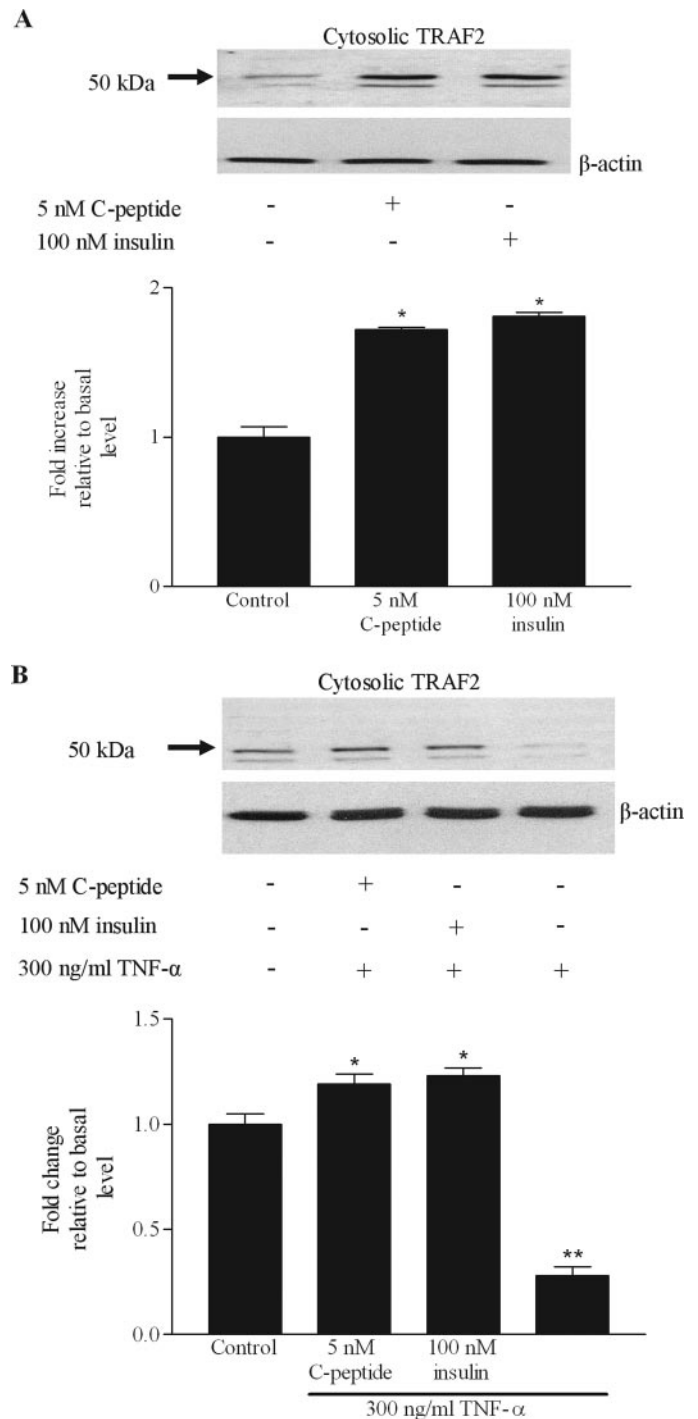


Figure 7. C-peptide and insulin increase the expression of TNF receptor-associated factor 2 (TRAF2). (A) C-peptide and insulin upregulate TRAF2. Shown are immunoblots for TRAF2 and the same blots reprobed for β -actin. Densitometric quantification of TRAF2 is shown in bottom panel; $n = 5$; * $P < 0.05$ versus control nonstimulated cells. (B) C-peptide and insulin antagonize TNF- α -induced reduction of TRAF2 protein. Cells that were preincubated with or without C-peptide or insulin then were stimulated by 300 ng/ml TNF- α . Densitometric quantification of TRAF2 is shown in the bottom panel; $n = 4$ blots from independent experiments; * $P < 0.05$, ** $P < 0.001$ relative to control cells that were treated with TNF- α alone.

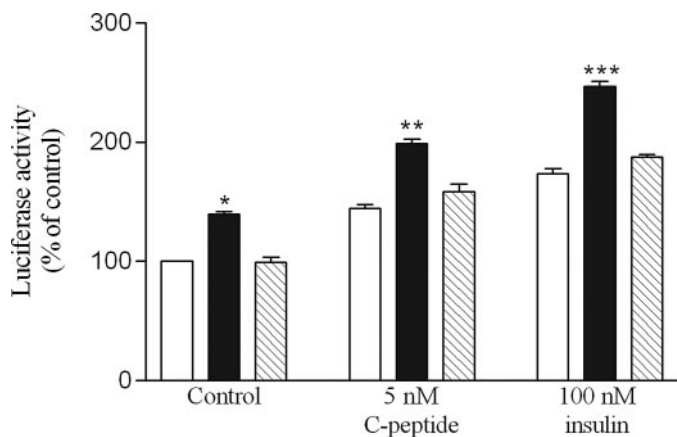


Figure 8. TNF- α at low concentrations activates NF- κ B. NF- κ B activity in cells that were preincubated with or without C-peptide or insulin then stimulated by 10 ng/ml TNF- α (■) or 300 ng/ml TNF- α (▨) or without TNF- α (□). Results are of $n = 3$ independent experiments with each condition performed in triplicate; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus non-TNF- α -stimulated.

of C-peptide or insulin with 10 ng/ml TNF- α resulted in an augmented NF- κ B response (198.7 ± 3.9 and $246.9 \pm 3.4\%$ compared with control, respectively).

Discussion

Development of diabetic nephropathy has been attributed to release of vasoactive hormones, growth factors, and inflammatory cytokines (29). Data from animal models of diabetes and from clinical studies in patients with diabetes have positioned TNF- α as a key mediator of nephropathy because urinary and renal interstitial levels are elevated in diabetic rats before the development of albuminuria (20), circulating TNF- α levels are elevated in patients with diabetic nephropathy (30,31), and TNF- α is an independent predictor of urinary albumin excretion in individuals with diabetes. Moreover TNF- α is known to be cytotoxic to kidney cells (32,33) and to compromise directly glomerular permeability, leading to proteinuria and therefore potentially to secondary proteinuric nephropathy as a result of increased protein delivery into the proximal tubule from any cause (34).

Kidney cell death by apoptosis has been documented widely in the course of renal injury, and proximal tubular cell loss through apoptosis contributes significantly to the tubular atrophy that invariably is observed in progressive renal failure (35). In diabetic nephropathy, TNF- α is an attractive candidate proapoptotic mediator. However, its effects on cell survival and death are complex in that the sensitivity of cells to TNF- α -induced cell death varies with the microenvironment. Therefore, the absence of survival factors and/or the presence of other lethal factors may promote apoptosis induced by TNF- α . We hypothesized that in type 1 diabetes, deficiency of insulin and/or C-peptide may play a role in the pathogenesis of diabetic nephropathy by rendering proximal tubular cells more

susceptible to the deleterious effects of cytokines such as TNF- α .

Our initial experiments indicated that OK cells were relatively resistant, in terms of cytotoxicity, to TNF- α when applied to the apical surfaces of nearly confluent monolayers. However, prevailing TNF- α levels in the kidney are elevated in diabetes, although the precise proximal tubular concentration is unknown. Furthermore, it is likely that the human TNF- α used in these studies differs in sequence from the corresponding opossum cytokine, therefore leading to reduced potency in this system.

More important, however, C-peptide at physiologically relevant concentrations confers protection against apoptosis that is induced by both TNF- α and serum removal in OK cells, a property that is shared by insulin. This protective effect is specific to some extent for TNF- α , because neither C-peptide nor insulin significantly inhibited cell death that was induced by the antitumor agent etoposide. Overall, these findings are consistent with our earlier observations that OK cells proliferate in the presence of C-peptide (11) and suggest that in kidney proximal tubular cells, both peptides that are released from pancreatic β cells should be regarded as growth and survival factors.

Other workers established that activation of NF- κ B may result in protection against TNF- α -induced cell killing (36–38). We postulated that C-peptide and insulin may inhibit TNF- α -mediated apoptosis *via* stimulation of NF- κ B activity. Our results support this hypothesis because (1) both insulin and C-peptide activate NF- κ B in OK cells, (2) the NF- κ B inhibitor MG-132 abolished the antiapoptotic effects of insulin and C-peptide, and (3) TNF- α failed to activate NF- κ B at concentrations that are required for the induction of apoptosis. Inhibition of NF- κ B with MG-132 only very slightly augmented apoptosis in OK cells that were treated with TNF- α alone, suggesting that basal cellular activity of NF- κ B is insufficient to control TNF- α -induced cell injury.

Insulin possesses antiapoptotic properties (39) that depend on activation of NF- κ B in mammalian cells (40,41). Until now, an NF- κ B stimulatory, antiapoptotic role of C-peptide has never been demonstrated. Data from Li *et al.* (39) showed that insulin protected SH-SY5Y cells against glucose-induced apoptosis in association with nuclear translocation and presumed activation of NF- κ B. Although in their studies addition of C-peptide seemed to enhance the effects of insulin, Li *et al.* were unable to demonstrate NF- κ B stimulatory, antiapoptotic properties role of C-peptide alone, concluding that C-peptide effects on neuronal signaling were mediated *via* enhanced insulin signaling.

No additive effects of C-peptide with insulin were observed in this study, and we believe that the cellular effects of C-peptide are a result of bioactivity of C-peptide and signaling in its own right. We previously demonstrated activation of PI3-K and ERK by C-peptide and insulin in OK cells (11), and we now present evidence that NF- κ B activation by these two agents, being sensitive to wortmannin but not to PD98059, depends on PI3-K activity but not that of ERK. Consistent with numerous reports of a crucial cell survival role for PI3-K (42–44), our data

suggest that insulin and C-peptide both initiate NF- κ B–dependent cell survival pathways downstream of PI3-K activation.

Clear differences exist between the modes of action of insulin and C-peptide. First, their NF- κ B activation dose-response curves are different. Insulin displays a typical sigmoidal dose-response curve of NF- κ B stimulation but is less potent than C-peptide. However, low insulin potency is often observed *in vitro*, where much higher than physiologic insulin concentrations generally are required to elicit signaling effects. The “bell-shaped” activation curve of NF- κ B activation by C-peptide is completely different from that of insulin but identical to that seen for activation of other signaling pathways by C-peptide (11,45,46). Second, PTX completely blocked NF- κ B activity that was evoked by C-peptide but had no effect on activity that was evoked by insulin. Together with similar observations by others (2,11,45), this finding indicates that C-peptide signals its effects *via* $G\alpha_{i/o}$, possibly *via* a G-protein–coupled receptor in contrast to receptor tyrosine kinase signaling of insulin. Using [³⁵S]GTP γ S binding, we now for the first time unequivocally demonstrate that $G\alpha_i$ proteins are activated by C-peptide binding to a G-protein–coupled receptor.

Although degradation of I κ B- α and activation of the NF- κ B–luciferase reporter suggested strongly that C-peptide and insulin had the capability to regulate gene transcription in OK cells, we wished to document definitively at the protein level that NF- κ B–regulated genes were altered under these conditions. We chose to investigate TRAF2, a key effector mediating TNF- α responses (47) and a critical regulator of TNF- α signaling to NF- κ B activation (48,49). TRAF2 gene transcription is NF- κ B regulated and may be increased by insulin (41). In resting OK cells, TRAF2 protein was increased in response to C-peptide or insulin in association with NF- κ B activation. This is the first evidence that C-peptide stimulates the expression of an adaptor protein that is recruited by activated TNF receptors. Conversely, TNF- α treatment was followed by a reduction in TRAF2 protein, in agreement with previous observations of ubiquitination and degradation of TRAF2 in response to TNF- α (50). However, both insulin and C-peptide were able to counteract the reduction of TRAF2 that resulted from higher concentration TNF- α treatment. These findings suggest that at higher concentrations in OK cells, TNF- α causes degradation of TRAF2, thereby directing cells toward apoptosis. By activating NF- κ B, however, both insulin and C-peptide enable cells to maintain healthy levels of TRAF2, thereby inhibiting proapoptotic signaling by TNF- α . It is interesting that in our studies in OK cells, TNF- α at 10 ng/ml was nontoxic and activated NF- κ B, a finding that is consistent with the report of Papakonstantini and Stourmaras (51), who described survival factor–like effects of 10 ng/ml TNF- α in OK cells.

Our study provides evidence for the ability of C-peptide, acting *via* $G\alpha_i$, to protect against TNF- α –induced apoptosis in kidney proximal tubular cells. C-peptide seems to share many properties with insulin but possesses its own signaling capabilities, and effort now is required to identify the C-peptide receptor. Despite being ignored for many years, it now is clear that C-peptide possesses important biologic properties and potentially may protect against diabetic complications. We believe

that the gradual accretion of data in the field of C-peptide function over recent years argues strongly for the urgent consideration of clinical trials of C-peptide in diabetic nephropathy.

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