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Insulin exhibits short-term anti-inflammatory but long-term proinflammatory effects *in vitro*☆

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ABSTRACT

Although insulin is indispensable for maintaining glucose homeostasis, it is still controversial whether or not a high concentration of insulin is deleterious. We examined the effect of insulin on the transcriptional activity of NF-κB, which mediates the expression of a variety of inflammation/coagulation-related genes using hepatocyte cell lines *in vitro*. We found that insulin (1 nM) alone caused minimal increase in NF-κB-mediated transcription. On the other hand, when cells were simultaneously treated with proinflammatory cytokines such as TNFα, the following dual effect of insulin was observed: short-term (6 h) suppressive, and long-term (36 h or later) stimulatory effects. The former effect was transient and appears to be mediated by the phosphatidylinositol 3 kinase (PI_3K) signaling pathway. The latter effect, in contrast, was more pronounced, enhancing the TNFα-stimulated NF-κB-dependent transcription by more than sevenfold. This positive effect was NF-κB-specific, and was eliminated by mitogen-activated protein kinase (MAPK) inhibitors. Altogether, our data suggest that insulin has short-term anti-inflammatory but long-term proinflammatory effects. From a clinical standpoint, this implies that low basal and periodically high plasma insulin is beneficial, whereas a sustained rise in plasma insulin, as often seen in patients with obesity, may induce atherothrombotic disorders, because of the NF-κB-mediated overexpression of proinflammatory/procoagulant/antifibrinolytic proteins in the liver.

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1. Introduction

Insulin has two aspects of actions in the human: one as a metabolic hormone and the other as a growth factor (Kitamura et al., 2003). During development, insulin acts as a growth factor, playing an important role in the regulation of cell growth and differentiation. In contrast, insulin is primarily a metabolic hormone in the adult, and is indispensable for the regulation of glucose homeostasis: the lack or impaired action of insulin results in diabetes mellitus, characterized by high blood glucose. However, insulin still appears to keep the dual action after development. Recent advances in the investigation of the molecular mechanism of insulin have revealed the diversity of signaling pathways, with two major systems consisting of the phosphatidylinositol 3-kinase (PI_3K)-mediated and the mitogen-activated protein kinase (MAPK)-mediated pathways (Kanzaki and Pessin, 2001). The metabolic effect of insulin is mediated by the PI_3 kinase-mediated pathway, whereas that of growth factor is mediated by the MAPK pathway, although the physiological role of the latter in the adult is not completely understood.

Recently, a third aspect of insulin action has emerged: the anti-inflammatory and/or proinflammatory action. Dandona et al. (2001, 2002) proposed that insulin has an anti-inflammatory effect in such a way that the hormone exhibits an inhibitory effect on NF-κB-mediated transcription in mononuclear cells. Since NF-κB is a key transcription factor in a variety of inflammation-related genes, suppression of NF-κB-dependent gene expression would be anti-inflammatory. Interestingly, however, there is a long-lasting notion that a high concentration of insulin enhances vascular disorders such as atherosclerosis which is currently regarded as inflammatory in nature (Fontbonne, 1994; Stout, 1996; Ross, 1999; Libby, 2002), and, if this is the case, insulin might be a proinflammatory hormone. Thus, the effects of insulin on inflammation are complex and the precise mechanisms underlying the phenomena have not been entirely elucidated. Moreover, the signaling pathway(s) mediating the effects of insulin on inflammation, either proinflammatory or anti-inflammatory, remains unknown.

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In this study, we examined the effect of insulin on the transcriptional activity of NF-κB-dependent gene expression in hepatic cells. The liver is one of the major targets of insulin, and a variety of metabolic effects are caused by insulin. We obtained results suggesting that insulin has not only short-term anti-inflammatory but also long-term proinflammatory actions, by exerting inhibitory and stimulatory effects on NF-κB-mediated transcription, respectively.

2. Materials and methods

2.1. Reagents

Human insulin was obtained from Sigma (St. Louis, MO, USA). Human TNFα and IL-1β were from PeproTech (Rocky Hill, NJ, USA). PD98059, SB203580, wortmannin, and LY294002 were from Calbiochem (San Diego, CA, USA). pCRE (cAMP-response element)-Luc and pNF-κB-Luc reporter plasmids were purchased from Stratagene (La Jolla, CA, USA).

2.2. Plasmid construction

5′-promoter regions of the human fibrinogen-γ gene (≈1.0 kb), the human plasminogen activator inhibitor-1 (PAI-1) gene (≈0.7 kb), and the human C-reactive protein (CRP) gene (≈0.25 kb including a proximal promoter region) were cloned by PCR, and incorporated into a pA3Luc luciferase reporter plasmid. These constructs were designated as Fibrinogen-Luc, PAI1-Luc, and CRP-Luc.

2.3. Cell culture and transfection

HuH7, a human hepatoma cell line, was obtained from RIKEN cell bank (Saitama, Japan), and maintained in DMEM (high glucose) supplemented with 10% FBS. The cells were transfected stably with the pNF-κB-Luc reporter plasmid containing five tandem repeats of NF-κB binding sites and with the RSV-Neo plasmid, and a mixed polyclonal cell line after selection with G418 (Invitrogen, Carlsbad, CA, USA), designated HuH7NF, was used in most of the subsequent studies. In some experiments, HuH7 and HepG2 cells (obtained from Prof. Joseph A. Majzoub, Children's Hospital, Boston, USA) were transfected transiently with a variety of reporter plasmids using a lipofection method.

2.4. Experiments

Cells were plated and cultured with DMEM supplemented with 10% FBS in 24-well plates. Twenty-four hours after plating, the culture medium was changed to DMEM with 1% FBS, and the cells were cultured for an additional 24 h. In each experiment, test reagents, prepared in concentrations that were 1000-fold higher than the final ones, were added directly into the culture medium (reagent volume:medium volume = 1:1000), and the cells were incubated for the defined time interval. In the experiments using both TNFα and insulin, TNFα was present until the end of experiment, in combination with insulin. Inhibitors used were added 30 min before the addition of insulin. At the end of incubation, the culture medium was removed, and the cells were harvested for the determination of luciferase activity.

2.5. Luciferase assay

Luciferase assay was performed as previously described (Aoki et al., 1997), and light output was measured for 20 s at room temperature using a luminometer (Berthold Lumat LB9507, Bad Wildbad, Germany).

2.6. RT-PCR

Endogenous expression of the two major components of NF-κB (p50 and p65), IkappaB (IκB)-β, insulin receptor, and GAPDH mRNAs were examined by RT-PCR using Superscript III (Invitrogen) and Taq DNA polymerase (Takara, Kyoto, Japan). The primer sets used were as follows: sense, 5′-CACCTAGCTGCCAAAGAAGG-3′ and antisense, 5′-AGGCTCAAAGTTCTCCACCA-3′ for p50; sense, 5′-TCAATGGCTACACAGGACCA-3′ and antisense, 5′-CACTGTCACCTGGAAGCAGA-3′ for p65; sense, 5′-GCCGACGCAGATGAATGGTG-3′ and antisense, 5′-TCCGGTTTGTCAAGGTCAGC-3′ for IκBβ; sense, 5′-CCTTCAAGAGATGATTCAGATG-3′ and antisense, 5′-TGTTCATTAGACAGGCCTTGGT-3′ for insulin receptor; sense, 5′-GCACCGTCAAGGCTGAGAAC-3′ and antisense, 5′-GTGCTAAGCAGTTGGTGGTGC-3′ for GAPDH.

2.7. Western blotting

The HuH7NF cells were cultured in 3.5-cm-diameter dishes, treated with insulin (1 nM, 48 h) and TNFα (100 pM, 24 h), and cytosolic/nuclear proteins were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockfold, IL, USA). Then the extracts were applied for electrophoresis, and incubated with anti-ERK/pERK (Cell Signaling, Beverly, MA, USA), anti-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-phospho-NF-κB p65 (Ser276) (cell signaling) antibodies, and with horseradish peroxidase-conjugated anti-mouse (or rabbit) immunoglobulin G (IgG) (Santa Cruz). Finally, the bands were detected by chemiluminescence using ECL plus Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer's instructions.

2.8. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out using a commercially available non-RI EMSA kit (LightShift Chemiluminescent EMSA kit; Pierce). Briefly, the HuH7NF cells were treated with insulin (1 nM, 48 h) and TNFα (100 pM, 24 h), and nuclear extract was prepared using NE-PER (Pierce). The extract was then incubated for 6 h with the double-stranded, 3′-end-biotinylated oligonucleotide probe (50 fmol) encompassing the consensus NF-κB binding sequence (sense, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ biotin; antisense, 5′-GCCTGGGAAAGTCCCCTCAACT-3′ biotin), and the mixture was subjected to 4% nondenaturing polyacrylamide gel electrophoresis (160 V for 4 h). Finally, the biotinylated DNA was transferred to a nylon membrane, cross-linked, and then the biotin-labeled DNA was detected with digital imaging apparatus (LightCapture, ATTO, Tokyo, Japan). An NF-κB oligonucleotide probe without biotinylation (cold probe; 400×) was also used as a competitor, to confirm the specificity of the bands.

2.9. Data analysis

Samples in each group of the experiments were in triplicate or quadruplicate. All data were expressed as mean ± S.E.M. When the statistical analyses were performed, data were compared by one way analysis of variance with Fisher's multiple range test, and *P* values below 0.05 were considered significant.

3. Results

3.1. Expression of each NF-κB component and insulin receptor in HuH7NF and HepG2 cells

The presence of mRNAs for representative components of NF-κB (p50, p65, and IκBβ) and insulin receptor in HuH7NF and HepG2 cells was analyzed by RT-PCR. mRNA for GAPDH was used as a positive control. We found amplified products with the appropriate band size in all cases (Fig. 1), indicating the expression of endogenous mRNAs for all the proteins examined.

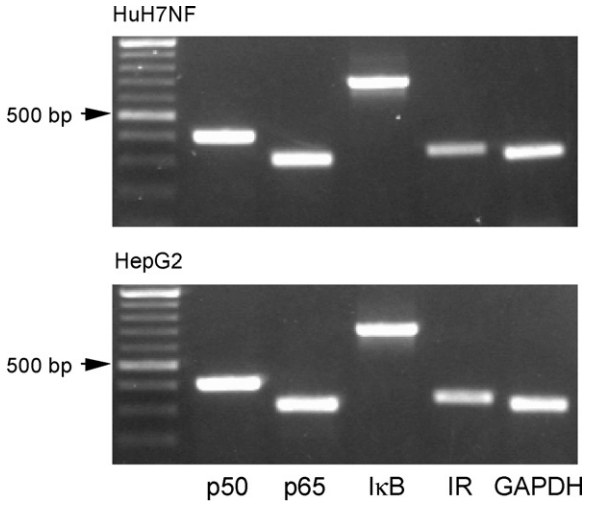


Fig. 1. RT-PCR analysis of the expression of NF-κB p50, p65, IκBβ (IκB), insulin receptor (IR) and GAPDH mRNAs in HuH7NF and HepG2 cells. The figures show photographs of the ethidium bromide-stained products using agarose gel electrophoresis. cDNA produced from an RT reaction using total RNA from each cell line was amplified by PCR with pairs of oligonucleotide primers specific for each mRNA. No band was amplified in the same reaction without reverse transcriptase (not shown).

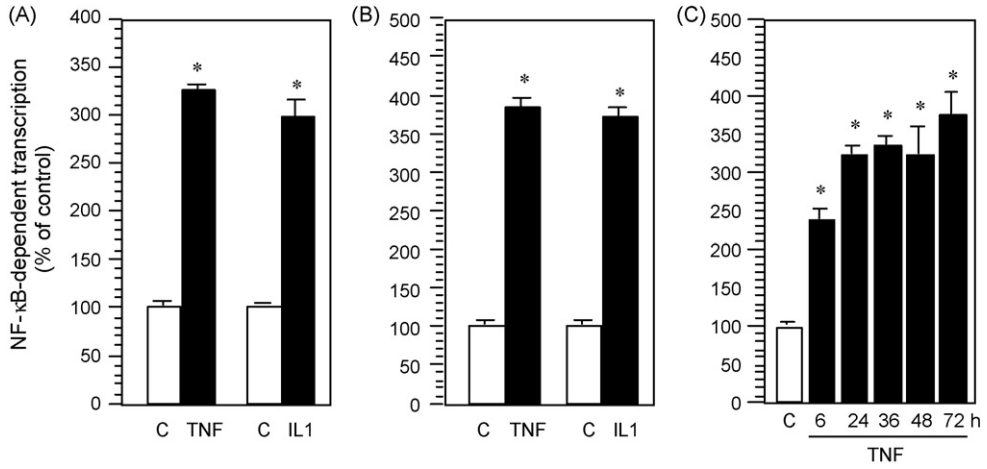


Fig. 2. Effects of TNFα and IL-1β on the NF-κB-dependent transcription in HuH7NF and HepG2 cells. (A) HuH7NF cells were treated with TNFα (TNF; 100 pM) or IL-1β (IL1; 100 pM) for 24 h. (B) A similar experiment was carried out using HepG2 cells transfected transiently with NF-κB-luciferase reporter plasmid. (C) HuH7NF cells were treated with TNFα (TNF; 100 pM) for 6–72 h. Each value is shown as a percentage of the control value. $^{*}P < 0.05$ vs. control (C).

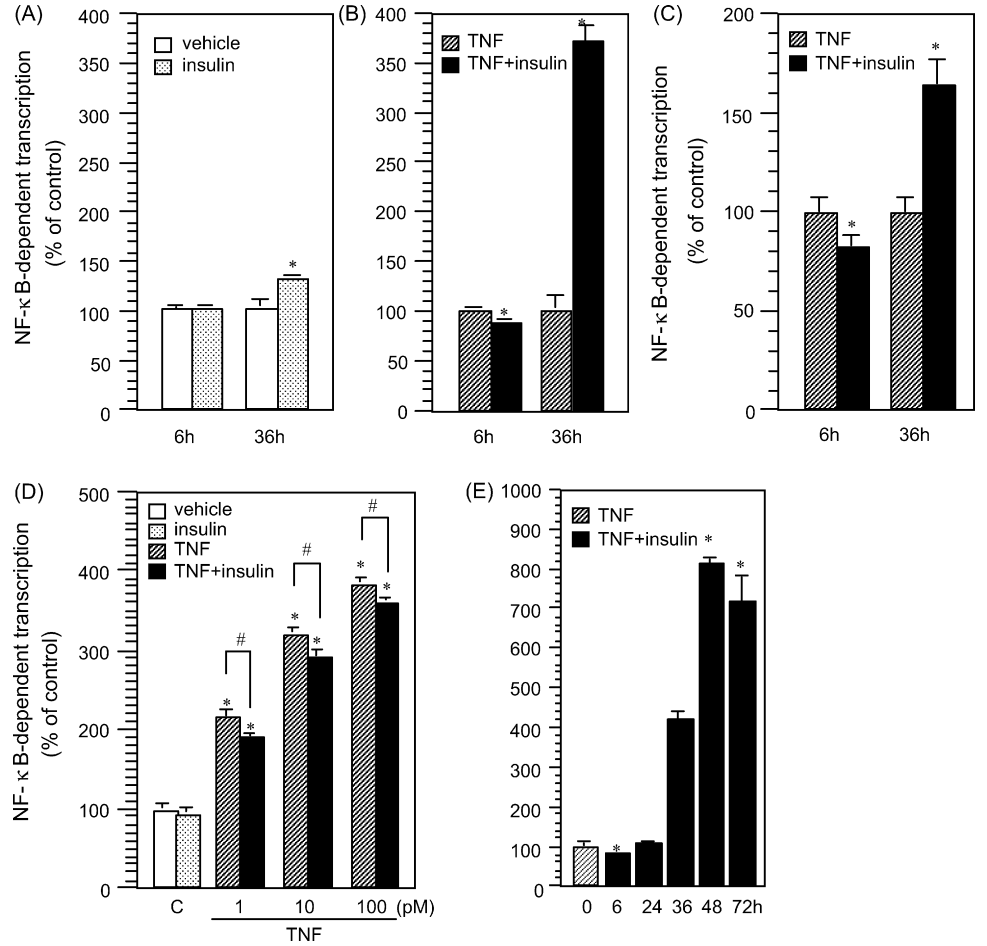


Fig. 3. Effects of insulin on basal or TNFα-stimulated NF-κB-dependent transcription in HuH7NF and HepG2 cells. (A) Effects of insulin alone. HuH7NF cells were treated with insulin (1 nM) for 6 or 36 h. (B and C) Combined effects of insulin and TNFα. HuH7NF cells were treated with TNFα (TNF; 100 pM) for 24 h, and also with insulin (1 nM) for 6 or 36 h (B). A similar experiment was carried out using HepG2 cells transfected transiently with NF-κB-luciferase reporter plasmid (C). (D) Dose–response effect of TNFα. HuH7NF cells were treated with or without various concentrations of TNFα (1–100 pM) for 24 h, and also with insulin (1 nM) for 6 h. E. Time-course of the combined effects of insulin and TNFα. HuH7NF cells were treated with TNFα (100 pM) for 24 h, and also with insulin for 6–72 h. Each value is shown as a percentage of the basal value [vehicle (A and D) or TNFα alone (B, C, and E)]. $^{*}P < 0.05$ vs. vehicle (A and D) or TNFα alone (B, C, and E). $^{\#}P < 0.05$ vs. TNFα alone (D).

3.2. Proinflammatory cytokines stimulate NF-κB-dependent transcription in HuH7NF and HepG2 cells

Using the HuH7NF and HepG2 cell lines, we first examined whether proinflammatory cytokines such as TNFα or IL-1β stimulate NF-κB-dependent transcription, as has been reported *in vivo*. As shown in Fig. 2, treatment of the cells with each cytokine potently stimulated the NF-κB-mediated transcription, indicating that the HuH7NF and HepG2 cells are cytokine-responsive.

3.3. Insulin shows dual effects on cytokine-stimulated NF-κB-dependent transcription in HuH7NF and HepG2 cells

We then examined the effect of insulin on NF-κB-dependent transcription. In HuH7NF cells, insulin (1 nM) had no effect at 6 h and a minimal stimulatory effect at 36 h on the basal activity of NF-κB-mediated transcription (Fig. 3A). Interestingly, however, the effect of insulin on cytokine-treated promoter activity was quite different: insulin showed a mild but significant suppressive effect on TNFα-stimulated NF-κB-mediated promoter activity by short-term (6 h) treatment, whereas it markedly enhanced the TNFα-stimulated promoter activity after long-term (36 h) treatment (Fig. 3B). Similar results were obtained in HepG2 cells transfected transiently with NF-κB-luciferase reporter plasmid (Fig. 3C). The short-term inhibitory effect of insulin on TNFα-induced NF-κB activation was observed regardless of the concentration of TNFα used (Fig. 3D). A more precise time-course experiment using HuH7NF cells showed that again insulin significantly suppressed TNFα-stimulated transcriptional activity at 6 h but then changed to potentiate the activity, with an up to eightfold amplification on TNFα-stimulated NF-κB-dependent transcription being observed (Fig. 3E).

3.4. Long-term insulin treatment enhances nuclear translocation and DNA binding of NF-κB protein

To see if long-term insulin enhances the nuclear translocation and DNA binding of NF-κB, Western blotting and EMSA analysis were carried out using anti-NF-κB p65 antibody and the canonical NF-κB probe, respectively. The results showed that treatment of the cells with TNFα increased the nuclear, but not cytosolic, amount of NF-κB p65 protein (Fig. 4A). The concomitant treatment with insulin did not influence the TNFα-induced translocation. Interestingly, however, insulin significantly enhanced the NF-κB protein binding stimulated by TNFα, when compared with TNFα alone (Fig. 4B).

3.5. Effects of signal transduction inhibitors on short-term inhibitory effect of insulin

To identify the signal transduction pathway(s) involved in the observed short-term suppressive effect of insulin on NF-κB-mediated transcription, we tested the effect of a variety of signal transduction inhibitors. Treatment of the cells with MAPK inhibitors PD98059 or SB203580 did not interrupt the suppressive effect of insulin, whereas the PI_3K inhibitor wortmannin completely eliminated the effect (Fig. 5A). Another experiment using a different PI_3K inhibitor LY294002 showed similar results (Fig. 5B). These results suggest that the short-term inhibitory effect of insulin is mediated by the PI_3K-mediated metabolic pathway.

3.6. Long-term insulin treatment enhances ERK phosphorylation

We then tried to clarify the signal transduction pathway(s) involved in the enhancing effect of insulin on NF-κB-mediated tran-

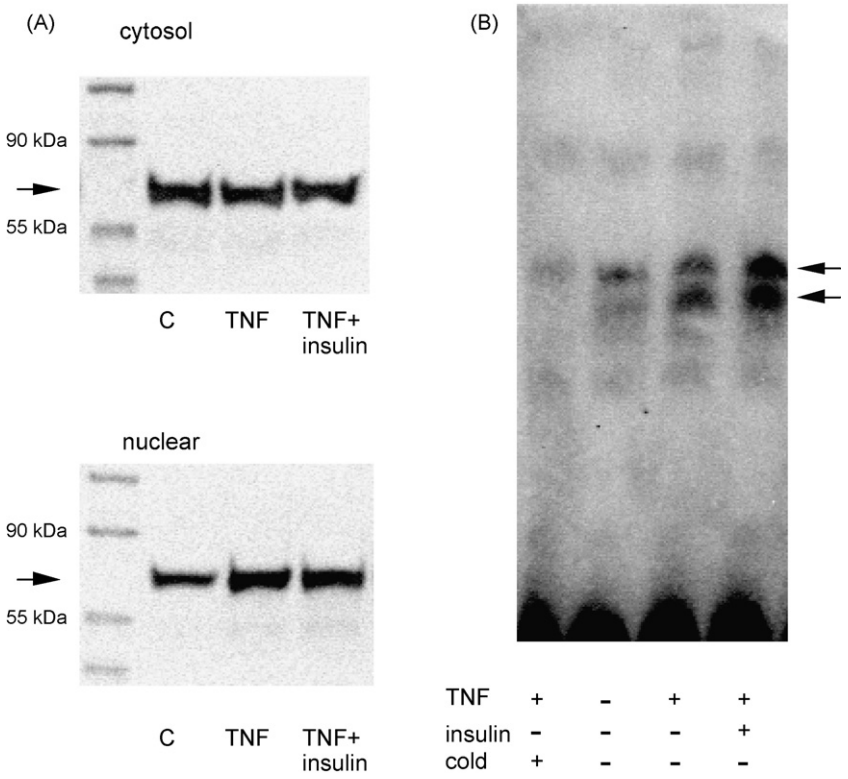


Fig. 4. Effects of TNFα and insulin on nuclear translocation and DNA binding of NF-κB in HuH7NF cells. Cells were treated with vehicle (C), TNFα alone (TNF; 100 pM, 24 h), or TNFα with insulin (1 nM, 48 h). The cells were then harvested, and the extracted cytosolic and nuclear proteins were applied for Western blotting using anti-NF-κB p65 antibody (A). The nuclear protein was also used for EMSA analysis (B). Cold, cold probe used as a competitor.

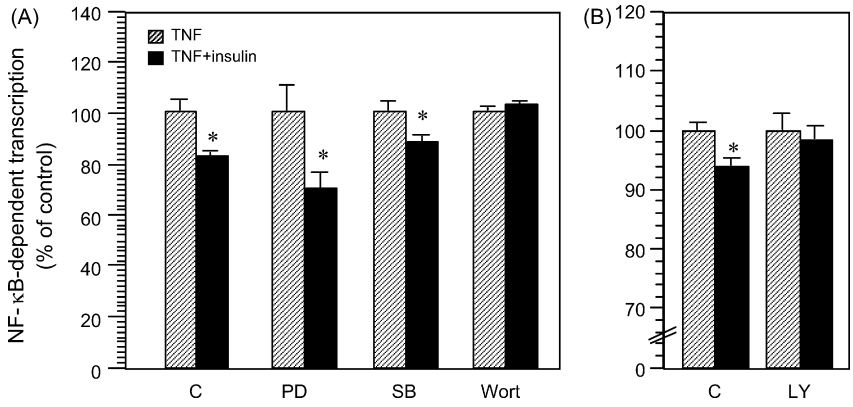


Fig. 5. Effects of kinase inhibitors on the short-term insulin suppression of TNFα-stimulated NF-κB-dependent transcription in HuH7NF cells. (A) Cells were treated for 24 h with TNFα (TNF; 100 pM), and with insulin (1 nM; closed bar) or vehicle (shaded bar) for 6 h. Cells were also treated with vehicle (C), PD98059 (PD; 20 μM), SB203580 (SB; 10 μM), or wortmannin (Wort; 1 μM) 30 min prior to the addition of insulin until the end of experiment. (B) A similar experiment as (A) was carried out using LY294002 (LY; 20 μM). *$P < 0.05$ vs. value of TNFα alone.

scription. Since the p42/44 MAPK (ERK) pathway is known to be the major signaling pathway mediating the growth effect of insulin, we examined the effect of long-term insulin treatment on ERK activation. The results showed that insulin significantly enhanced the TNFα-stimulated ERK phosphorylation (Fig. 6A). The amount of total ERK was not different among the groups.

3.7. Effects of signal transduction inhibitors on long-term potentiating effect of insulin

To confirm the involvement of MAPK pathway in the enhancing effect of insulin on the NF-κB-mediated transcription, we examined various kinase inhibitors. We found that treatment of the cells with the PI_3K inhibitor wortmannin modestly dampened but did not diminish the stimulatory effect of insulin (Fig. 6B). In contrast, MAPK inhibitors PD98059 and SB203580 both completely abolished the effect. These results strongly suggest that the long-term enhancing effect of insulin on TNFα-stimulated NF-κB-mediated transcription is mediated by the MAPK-related growth pathway of insulin signaling.

3.8. Long-term insulin treatment enhances NF-κB p65 phosphorylation

Since transcriptional activity of NF-κB is known to be stimulated not only by nuclear translocation but also by protein phosphorylation, we examined the effect of long-term insulin treatment on Ser276 phosphorylation of p65. The results showed that insulin significantly enhanced the phosphorylated form of p65 (Fig. 6C). The amount of total p65 was not different between the two groups.

3.9. Promoter specificity of the long-term potentiating effect of insulin

To confirm that the above observed positive effect of insulin is a promoter-specific event, we carried out transient transfection experiments in HuH7 cells using three different reporter constructs. Among the reporter plasmids tested, similar positive effects were observed only in the NF-κB-mediated transcription and not in the CRE- or RSV-mediated transcription (Fig. 7A). Finally, to see whether long-tem insulin treatment influences the expression of atherothrombosis-related genes, we carried out similar experiments using the reporter constructs containing the 5′-promoter region of human fibrinogen, PAI-1 and CRP genes, all of which are known to be NF-κB-responsive and clinically athero-/thrombogenic. We found that long-term insulin treatment significantly enhanced the TNFα-stimulated transcriptional activity of these genes (Fig. 7B), like what we observed in the NF-κB-luciferase reporter construct. Altogether, these results suggest that the potentiating effect of insulin on TNFα-stimulated promoter

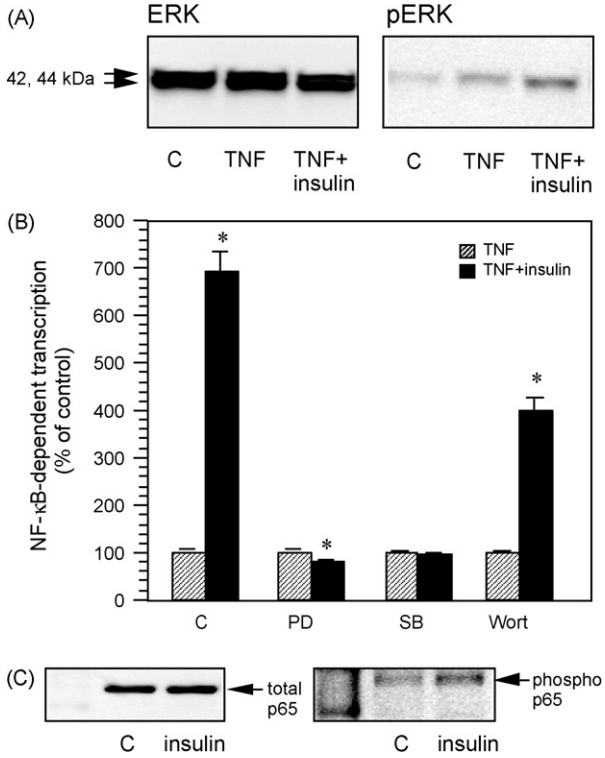


Fig. 6. (A) Effects of TNFα and long-term insulin treatment on ERK phosphorylation in HuH7NF cells. Cells were treated with TNFα (100 pM, 24 h), and with insulin (1 nM, 48 h) or vehicle (C). The cells were then harvested, and applied for Western blotting using total or phosphorylated ERK (pERK) antibodies. (B) Effects of kinase inhibitors on the long-term insulin augmentation of TNFα-stimulated NF-κB-dependent transcription in HuH7NF cells. Cells were incubated for 24 h with TNFα (TNF; 100 pM), and with insulin (1 nM; closed bar) or vehicle (shaded bar) for 48 h. Cells were also treated with vehicle (C), PD98059 (PD; 20 μM), SB203580 (SB; 10 μM), or wortmannin (Wort; 1 μM) 30 min prior to the addition of insulin until the end of experiment. Each value is shown as a percentage of the value of TNFα alone. *$P < 0.05$ vs. value of TNFαalone. (C) Effects of long-term insulin treatment on Ser276 phosphorylation of the NF-κB p65 in HuH7NF cells. Cells were treated with insulin (1 nM, 48 h) or vehicle (C). The cells were then harvested, and applied for Western blotting using the anti-total or Ser276-phosphorylated p65 antibodies.

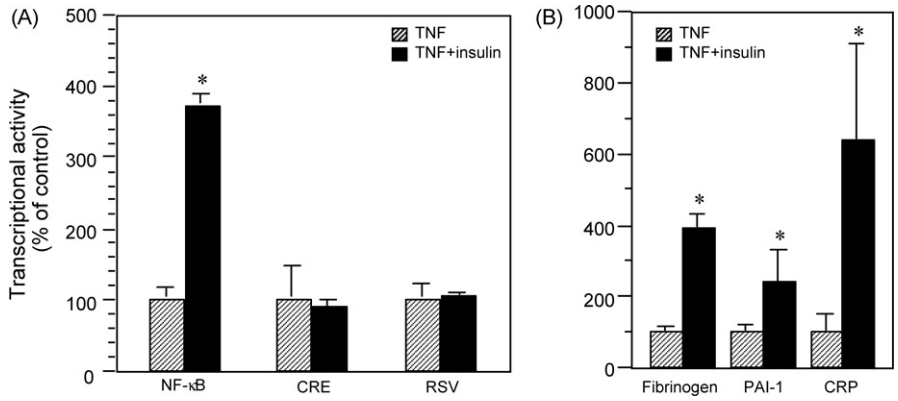


Fig. 7. Effects of long-term insulin treatment on various promoter-mediated transcriptions in HuH7 cells. (A) Cells were transfected transiently with pNF-κB-, pCRE-, or pRSV-luciferase reporter plasmids. Then the cells were treated for 24 h with TNFα (TNF; 100 pM), and also with insulin (1 nM; closed bar) or vehicle (shaded bar) for 48 h. Each value is shown as a percentage of the value of TNFα alone. (B) Cells were transfected transiently with either Fibrinogen-Luc, PAI1-Luc, or CRP-Luc plasmid, and then treated as (A). Each value is shown as a percentage of the value of TNFα alone. *$P < 0.05$ vs. value of TNFα alone.

activity is NF-κB-specific, and that similar effects occur in NF-κB-dependent, atherothrombosis-related genes such as fibrinogen, PAI-1 and CRP.

4. Discussion

In this study, we showed that insulin has a short-term inhibitory effect and a long-term stimulatory effect on NF-κB-mediated transcription in hepatic cells. The effect is promoter-specific, and is obvious when the transcription is simultaneously stimulated by proinflammatory cytokines such as TNFα. Furthermore, experiments with kinase inhibitors suggested that the short-term effect appears to be mediated by the metabolic pathway, whereas the long-term effect is mediated by the growth pathway of insulin signaling. Since NF-κB is a key transcription factor for a variety of inflammation-related genes, our present data raise a new concept that insulin has both anti-inflammatory and proinflammatory nature. This may partly explain the deleterious effect of sustained hyperinsulinemia frequently observed in patients with the metabolic syndrome and/or insulin resistance.

NF-κB is a ubiquitous transcription factor and exerts diverse effects in a variety of organs (Barnes and Karin, 1997). In the liver, NF-κB is known to play a central role in the transcription of inflammation- or acute phase protein-related genes such as fibrinogen-γ, PAI-1, CRP, serum amyloid A, and coagulation factor VIII (Cha-Molstad et al., 2000; Bing et al., 2000; Begbie et al., 2000; Fuller and Zhang, 2001; Ruan et al., 2001). Most of the protein products of these genes are proinflammatory, procoagulatory or antifibriolytic, and thus sustained expression is thrombotic and/or atherogenic, resulting in coronary and/or cerebrovascular events. Indeed, CRP is currently used as a marker of arteriosclerosis, which is now recognized as an inflammatory disease (Ross, 1999; Libby, 2002). Thus, our results in this study suggest that the short-term effect of insulin is protective, whereas the long-term effect is promotive in the progression of atherothrombotic disorders.

The inhibitory effect of insulin on NF-κB function has been recently reported. Dandona et al. (2001, 2002) clearly showed that 6-h treatment of insulin inhibits nuclear translocation of NF-κB *in vivo*; this fits well with our current data. Although the degree of suppression in our *in vitro* experimental model is less than that reported *in vivo*, we suppose that the apparently mild effect is due to the long intracellular half life of the reporter protein, which makes the inhibition less prominent. In this study, the short-term suppressive effect of insulin was eliminated by PI_3K inhibitors such as wortmannin and LY294002, suggesting the involvement of the metabolic pathway of insulin signal, although the precise molecular mechanism is not clear. Insulin is primarily a metabolic hormone in the human and plays an indispensable role in glucose homeostasis. In healthy adults, the basal plasma insulin level is low and a spiky increase is observed after each meal, and thus the anti-inflammatory effect of insulin is expected to be dominant. Indeed, a DCCT-EDIC study shows that patients intensely treated with the basal-bolus insulin regimen are protected from vascular events (Nathan et al., 2003). In some cases, the anti-inflammatory effects of insulin may last for a longer time than that observed *in vitro*, because the combined treatment of glucose, insulin, and potassium (GIK) is reported to suppress inflammatory parameters over 48 h in patients with acute myocardial infarction or who have undergone coronary surgery (Chaudhuri et al., 2004; Visser et al., 2005; Koskenkari et al., 2006), possibly due to the beneficial effect of GIK treatment on the ischemic organ and/or on whole body metabolism, with resultant reduction in inflammatory responses. In contrast, in patients with the metabolic syndrome with obesity and/or insulin resistance, chronic and sustained increase in plasma insulin concentration, both basal and post-prandial, is usually observed, and in this case, the proinflammatory effect of insulin may be dominant. Indeed, the plasma levels of fibrinogen and PAI-1 are shown to be elevated in patients with insulin resistance (Imperatore et al., 1998; Abbasi et al., 1999), and this may partly be explained by the long-term effect of insulin.

The proinflammatory effect of insulin was inhibited by MAPK inhibitors, suggesting the involvement of the growth pathway of insulin. We confirmed that long-term insulin treatment clearly enhanced ERK phosphorylation. Furthermore, activation of NF-κB by MAPK-mediated signal transduction has been well characterized previously (Schulze-Osthoff et al., 1997). In our experimental condition, the long-term effect of insulin was quite remarkable and more than an 8-fold potentiation on TNFα-stimulated NF-κB-mediated transcription was observed; since TNFα alone caused a 3-fold increase of basal NF-κB activity in this study, the net effect is more than a 24-fold increase. Interestingly, however, our Western blotting and EMSA data showed that the effect on NF-κB p65 nuclear translocation was stimulated by TNFα but not enhanced by insulin, although increase in NF-κB binding in EMSA was observed. Recent studies suggest that the transactivation capacity of NF-κB is determined not only by nuclear translocation (quantitative change) but also by phosphorylation of p65 (RelA) (qualitative change) (Vanden Berghe et al., 1998; Vermeulen et al., 2002, 2003), which is caused by a variety of signaling pathways including MAPKs. Larsen et al. (2005) showed Ser276 phosphorylation of p65 by ERK activa-

tion, and we also found the increase in the Ser276-phosphorylated form of p65 following insulin stimulation. Thus, we assume that the activation of MAPK by long-term insulin treatment leads to NF-κB-dependent transcription not by nuclear translocation but by phosphorylation of p65, with the resultant increase in DNA binding and/or recruitment of co-activators such as p300/CBP.

All of our experiments were carried out with culture medium containing high concentrations of glucose (≈30 mM). Glucose intolerance is frequently observed in the metabolic syndrome, and combination of long-term insulin effect with sustained and/or post-prandial hyperglycemia may also facilitate the activation of NF-κB, because we recently reported that high glucose itself activates NF-κB-dependent transcription using the same experimental model (Iwasaki et al., 2007). High glucose is also reported to blunt the short-term organoprotective (anti-apoptotic) effect of insulin in an animal model of myocardial ischemia/reperfusion (Zhang et al., 2006).

There has been a long debate whether insulin itself is an independent risk factor for the vascular disorders (Fontbonne, 1994; Stout, 1996). Animal studies provide controversial results (Sato et al., 1989; Shamir et al., 2003). The discrepancy of the results among the studies may partly be due to the concentration of insulin used, because high concentration of insulin may act through the IGF-I receptor in the vascular wall. Clinically, patients with insulinoma have high plasma insulin levels but an increase in cardiovascular events is not observed (Leonetti et al., 1993). In contrast, the incidence of macrovascular thrombotic events is reported to be higher in pre-diabetic patients with hyperinsulinemia (Standl, 1995). One clue to resolve the puzzle is that, in this study, the long-term proinflammatory effect of insulin was obvious when the NF-κB-mediated transcription was simultaneously stimulated by proinflammatory cytokines such as TNFα. In the metabolic syndrome, accumulation of multiple risk factors is thought to be responsible for the resultant atherosclerotic events (Reaven, 1995). As mentioned above, the combination of proinflammatory cytokine (TNFα) and sustained high insulin caused the dramatic (>24-fold) increase in NF-κB-mediated gene expression, and thus the observed synergism might be responsible for the molecular basis of the "multiple" risk factor syndrome. In other words, the deleterious effect of insulin may become overt when the patient accompanies obesity with high cytokine (including adipokine) levels. The UKPDS study supports this notion, showing that hyperinsulinemia alone is not, but in combination with other risk factors can be, a cardiovascular risk factor (Adler et al., 1999). Animal studies also show hyperinsulinemia as a causative risk factor when rats/mice are obese, insulin resistant, or treated with continuous insulin injection (Gruen et al., 2005; Sista et al., 2005).

We investigated the long-term effects of insulin using a hepatocyte cell line in this study, and thus our data may also be related to the pathogenesis of hepatic steatosis. NF-κB is known to be activated in an animal model of non-alcoholic steatohepatitis (Dela Peña et al., 2005), and a recent report shows that inhibition of NF-κB activation efficiently prevents high sucrose-induced liver steatosis and inflammation (Beraza et al., 2008). Since a variety of inflammation-related as well as coagulation/thrombosis-related gene expression is induced by NF-κB, its activation by the combination of proinflammatory cytokines and insulin might, at least partly, be responsible for the development of hyperinsulinemia-associated steatohepatitis frequently observed in patients with the metabolic syndrome.

Finally, our data provide some clinical implications on the molecular mechanism of insulin resistance in the metabolic syndrome. Insulin resistance is shown to occur through the metabolic pathway mediated by insulin receptor substrate (IRS)-PI_3K, whereas signal transduction mediated by the Src-Ras-MAPK pathway is reported to be intact (Nawano et al., 1999; Jiang et al., 1999; Yamaguchi et al., 2003; Wang et al., 2004). Because insulin is primarily a metabolic hormone to maintain glucose homeostasis in the human, insulin resistance in the metabolic pathway causes compensatory hyperinsulinemia. In this situation, overstimulation of the growth pathway may occur, with the resultant NF-κB activation as shown in this study. This concept is in agreement with the data obtained from IRS2 knockout mice presenting the accelerated neointima formation in the metabolic pathway-selective insulin-resistant state (Kubota et al., 2003). Since the short-term metabolic effects of insulin via PI_3K pathway is beneficial, our data suggest that elimination of insulin resistance with sustained hyperinsulinemia and restoration of the pulsatile secretory pattern of insulin is important to avoid the deleterious facet of insulin action.

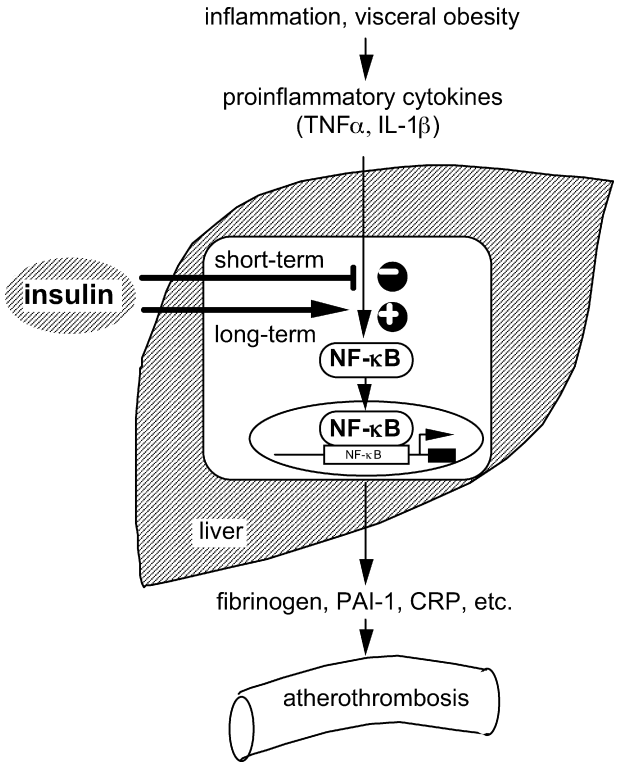


Fig. 8. Hypothetical representation of the pathophysiology of hyperinsulinemia. Insulin acutely suppresses but chronically amplifies the proinflammatory cytokines-mediated transcription of NF-κB-dependent genes. In a clinical situation, the resultant increase in the protein products such as fibrinogen, PAI-1, or CRP will be causative to thrombotic/atherosclerotic events.

In summary, our present data suggest that insulin has not only a short-term anti-inflammatory action but also a long-term proinflammatory action, by either inhibiting or amplifying the effect of proinflammatory cytokine-NF-κB axis (Fig. 8). Normalization of the insulin secretory pattern appears to be important to avoid the NF-κB activation with the resultant pro-atherothrombotic events, although further *in vivo* study is necessary to support this hypothesis.

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