

Hyperinsulinemia Enhances Transcriptional Activity of Nuclear Factor- κ B Induced by Angiotensin II, Hyperglycemia, and Advanced Glycosylation End Products in Vascular Smooth Muscle Cells

Inga Golovchenko, Marc L. Goalstone, Peter Watson, Michael Brownlee, Boris Draznin

Abstract—Pathogenesis of macrovascular complications of diabetes may involve an activation of the transcription factor nuclear factor- κ B (NF- κ B) by hyperglycemia and advanced glycosylation end products (AGEs). Activation of NF- κ B is believed to be dependent on activation of the Rho family of GTPases. Although the precise mechanism of the Rho-mediated action is not completely understood, posttranslational modification of the Rho proteins by geranylgeranylation is required for their subsequent activation. We observed that in cultured vascular smooth muscle cells (VSMCs), insulin stimulated the activity of geranylgeranyltransferase (GGTase) I and increased the amounts of geranylgeranylated Rho-A from 47% to 60% ($P < 0.05$). GGTI-286, an inhibitor of GGTase I, blocked both effects of insulin. Increased availability of prenylated Rho-A significantly augmented the abilities of angiotensin II (Ang II), hyperglycemia, and AGEs to activate NF- κ B, as measured by NF- κ B response-element luciferase reporter activity. Preincubations of VSMCs with insulin for 24 hours doubled NF- κ B transactivation by Ang II, hyperglycemia, and AGEs. This priming effect of insulin was completely inhibited by GGTI-286. We demonstrate for the first time, to our knowledge, that insulin potentiates NF- κ B-dependent transcriptional activity induced by hyperglycemia, AGEs, and Ang II in VSMCs by increasing the activity of GGTase I and the availability of geranylgeranylated Rho-A. (*Circ Res.* 2000;87:746–752.)

Key Words hyperinsulinemia ■ muscle, smooth, vascular ■ angiotensin II ■ nuclear factor- κ B ■ hyperglycemia

Macrovascular disease has emerged as the major cause of morbidity and mortality in patients with type 1 and type 2 diabetes.^{1,2} Despite numerous clinical and epidemiological studies, the molecular nature of the pathogenesis of accelerated atherosclerosis in these patients remains incompletely understood.

Two landmark studies (the Diabetes Control and Complications Trial³ and United Kingdom Prospective Diabetes Study⁴) indicate that hyperglycemia plays a major role in the development of microvascular complications of diabetes and, possibly, macrovascular complications. Recently, two potential mechanistic explanations have emerged: (1) hyperglycemia's indirect influence on multiple steps in the pathogenesis of atherosclerosis via the glycation of proteins and lipoproteins, with subsequent formation of advanced glycosylation end products (AGEs) and (2) glycosylated low-density lipoprotein (LDL) particles,^{2,5–7} which are a direct influence of hyperglycemia (including an induction of oxidative stress) on certain early events in the pathogenesis of atherosclerosis, such as the activation of the transcription factor nuclear factor- κ B (NF- κ B).^{8–11} AGEs, modified LDLs, and hyper-

glycemia, along with angiotensin II (Ang II) (a contributing factor in the pathogenesis of hypertension), have been shown to activate the transcription factor NF- κ B.¹²

Even though hyperinsulinemia is a hallmark of insulin resistance, its role in the pathogenesis of macrovascular complications remains controversial.^{13–15} The suggestion that insulin may be involved in the process of atherogenesis was first proposed in 1969 by Stout and Vallance-Owen.¹⁶ Many epidemiological and clinical data have supported this suggestion,^{17–20} but direct biochemical and molecular evidence has been lacking in spite of the fact that insulin has been clearly shown to act on both vascular smooth muscle cells (VSMCs) and endothelial cells in vitro and in vivo.^{21–25}

Recently, we demonstrated a novel aspect of insulin action: its ability to activate the prenyl transferases farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) I and GGTase II,^{26,27} which posttranslationally modify Ras, Rho, and Rab proteins, respectively. Increased prenyl transferase activity augments the cellular amounts of prenylated small molecular weight GTPases of the Ras family.²⁸ Because only prenylated GTPases are activated by growth factors, insulin-

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induced increases in the availability of prenylated p21Ras result in the augmentation of the mitogenic responses of the cells exposed to hyperinsulinemia and other growth-promoting agents.^{29,30} We found that hyperinsulinemia significantly increased the amount of prenylated p21Ras in VSMCs and dramatically enhanced the responsiveness of these cells to platelet-derived growth factor (PDGF) with regard to DNA synthesis and amounts of vascular endothelial growth factor mRNA.³⁰ An inhibitor of FTase blocked the ability of insulin to increase the amount of farnesylated p21Ras and potentiate the action of other growth factors.^{26–30}

Because transactivation of NF- κ B seems to be Rho-A-dependent,^{31,32} we postulated that hyperinsulinemia (which does not have an independent effect on NF- κ B) augments the action of Ang II, AGEs, and hyperglycemia on NF- κ B transcriptional activity in VSMCs by increasing GGTase I activity and subsequent increased availability of geranylgeranylated Rho-A. This study was designed to experimentally explore this hypothesis.

Materials and Methods

Materials

Cell culture media and supplies were from Life Technologies, Gemini Bio-Products Inc, Promega, Calbiochem, and Sigma. Radioisotopes were from DuPont New England Nuclear. Insulin was from Eli Lilly and Company. Ras-CVLL and GGTI-286 were from Calbiochem; NF- κ B response-element luciferase (Luc) promoter-reporter construct containing several consensus NF- κ B response elements sequenced into the PGL₂-basic vector (Promega) was a gift from Dr Ken Tyler (University of Colorado Health Sciences Center, Denver, Colo); RSV- β -galactosidase construct was a gift from Dr Arthur Gutterrez-Hartmann (University of Colorado Health Sciences Center, Denver, Colo); AGEs were developed in the laboratory of Dr Michael Brownlee (Albert Einstein College of Medicine, Bronx, NY) by incubating bovine serum albumin with 500 mmol/L glucose for 12 weeks; Clostridium botulinum C3 transferase was from Calbiochem; and the reporter lysis buffer was from Promega. All reagents used and prepared for experiments were free of detectable or relevant levels of LPS.

Isolation, Characterization, and Growth of VSMCs

Bovine aortic smooth muscle cells are routinely isolated and grown from explants of small pieces of aortic tissue from adult animals. Cells are passaged from explant cultures into 75-cm² tissue culture flasks after trypsinization. Cells are then cultured in growth medium, consisting of minimal essential medium (Sigma) and containing 1 \times nonessential amino acids, 0.4 mmol/L glutamine, penicillin/streptomycin, and 10% FBS (Gemini Bio-Products Inc). Passages 1 through 4 were used in these experiments.

In Vitro GGTase I Activity Assay

VSMCs were challenged with insulin (100 nmol/L) in the absence or presence of the GGTase I inhibitor GGTI-286 (3 μ mol/L) for 30 and 60 minutes and 24 hours and then lysed in buffer (150 mmol/L NaCl, 5 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L sodium phosphate, 1% Triton X-100, 0.05% SDS, 1.5 μ mol/L aprotinin, 22 μ mol/L leupeptin, 50 mmol/L HEPES, pH 7.5). GGTase I activity was assayed in vitro using a modified version of a method by Moores et al.³³ Briefly, lysates containing endogenous GGTase I from control and insulin-treated VSMCs were incubated with 100 nmol/L Ras-CVLL protein and 100 nmol/L tritiated geranylgeranyl pyrophosphate (³H]GGPP) (15 mCi/mmol at 37°C for 30 minutes). The assay was stopped with 1 mL of 1 mol/L HCl in ethanol, filtered through Whatman GF/C glass-fiber filters, and air dried. Labeled protein was quantified by liquid scintillation spectrometry.

Separation of Prenylated and Unprenylated Rho-A
VSMCs were incubated with or without insulin (100 nmol/L) and in the absence or presence of GGTI-286 (3 μ mol/L) for 24 hours and lysed. Lysates were mixed with an equal volume of Triton X-114 and incubated at 37°C for 3 minutes, and aqueous and detergent phases were allowed to separate at room temperature, as previously described.^{26–30} The amount of prenylated Rho-A protein was expressed as a percentage of total cellular Rho-A immunoprecipitated from both phases.

³²P-Phosphorylation of α Subunit of GGTase I

VSMCs were incubated for 6 hours at 37°C in serum- and phosphate-free medium and then preincubated overnight with 250 μ Ci [³²P]orthophosphate (10 mCi/mmol). Cells were then incubated for 60 minutes with or without insulin (100 nmol/L), as previously described.^{26,27,29,30} The GGTase I α subunit was immunoprecipitated with antiserum to the α subunit and analyzed by 12% SDS-PAGE. Amounts of phosphorylation were visualized by autoradiography, and amounts of protein were determined by Western blotting.

VSMC Transfection

Early passage cultures (P1-P4) of VSMCs were plated in 6-well culture dishes at a density of 1.4 \times 10³ cells/mm² and maintained in growth medium for 18 hours. Transfection was performed in serum-free medium (OptiMEM and GIBCO/BRL) using Lipofectamine Plus Transfection Reagent (GIBCO/BRL), as described by the manufacturer. A chimeric promoter-reporter plasmid construct, which contained the gene encoding the firefly Luc gene under the control of several consensus NF- κ B transcription elements, was added to the VSMC transfection mix at a concentration of 0.3 μ g of DNA per well. In addition, VSMCs were cotransfected with a constitutively expressed β -galactosidase reporter plasmid construct (RSV- β gal, 0.3 μ g per well). The cells were subsequently serum-starved in 1 \times minimal essential medium (MEM), containing 1 \times nonessential amino acids, 0.4 mmol/L glutamine, and Pen/Strep, for 24 hours. Agonist treatment was performed in 1 \times MEM for durations of 4 to 24 hours. Cells were incubated for 24 hours in the absence or presence of insulin (100 nmol/L) with or without Ang II (30 nmol/L), C-3 botulinum toxin (40 nmol/L), glucose (25 mmol/L), GGTI-286 (3 μ mol/L), or AGE (100 μ g/mL). VSMCs were subsequently extracted in 1 \times Reporter Lysis Buffer (Promega) for analysis of reporter gene expression. Luc reporter activity, the marker for specific NF- κ B-dependent promoter transactivation, was corrected for differences in transfection efficiency, cell number, and extract recovery using β -galactosidase activity determined in the same cellular extract.

Statistical Analysis

Data were analyzed using the Student's *t* test, with *P*<0.05 considered statistically significant.

Results

Effect of Insulin on GGTase I and Geranylgeranylated Rho-A

Enzymatic activity of GGTase I was assessed by the ability of cell lysates to catalyze the attachment of ³H-geranylgeranyl pyrophosphate to a recombinant Rho analog, Ras-CVLL. Insulin, in a dose-dependent manner, significantly increased GGTase I activity in VSMCs. A maximal activation of GGTase I was achieved with 100 nmol/L at 30 minutes (41% increase), 60 minutes (54% increase), and 24 hours (47% increase) of incubation (Figure 1). This stimulatory effect of insulin was blocked by the GGTase I inhibitor GGTI-286.

We have previously shown that the mechanism of insulin action on FTase and GGTase II involved the phosphorylation of the α subunits of these enzymes.^{29,30} In the present study,

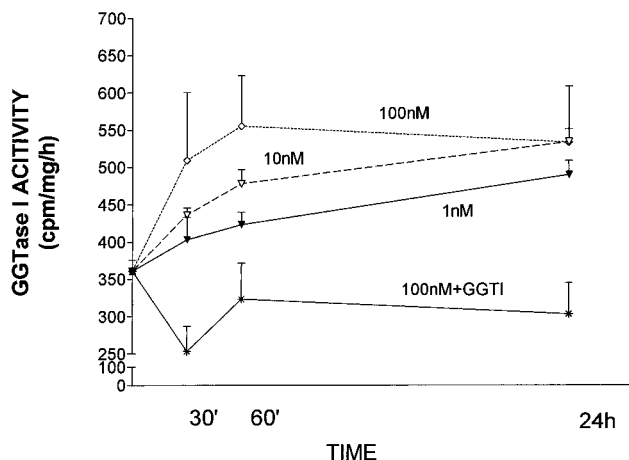


Figure 1. Effect of insulin on GGTase I activity in VSMCs. Cells were incubated with insulin (1, 10, or 100 nmol/L) for 30 or 60 minutes or 24 hours. In experiments with 100 nmol/L insulin, cells were also incubated in the absence or presence of the GGTase I inhibitor GGTI-286 (3 μ mol/L). Results represent the mean of 4 independent experiments. All values with 10 and 100 nmol/L insulin were significantly ($P<0.05$) different from time zero and from incubations with GGTI-286. Values with 1 nmol/L insulin were significant ($P<0.05$) at 60 minutes and 24 hours.

we demonstrate that incubation of VSMCs with insulin for 60 minutes results in a robust phosphorylation of the α subunit of GGTase I (Figure 2) without any change in the amount of protein. In concert with the previously reported specificity of insulin effect on FTase, the effect of insulin on GGTase I was not mimicked by insulin-like growth factor-1 (IGF-1) (Figure 2). Incubations of the cells with nanomolar concentrations of IGF-1 did not result in the phosphorylation of the α subunit of GGTase I.

Because increased activity of GGTase I was expected to augment the amounts of geranylgeranylated Rho-A in cells exposed to insulin, we used Triton X-114 extraction to measure the percentage of cellular Rho-A protein in the geranylgeranylated form. Only the prenylated forms of the small molecular weight GTPases were extracted into the detergent phase, whereas the unprocessed (unprenyated) GTPases remained in the aqueous phase.^{31,32} This technique has been characterized, standardized, and described in our previous publications.^{29–33} VSMCs incubated with insulin for 24 hours displayed increased amounts of geranylgeranylated Rho-A compared with control

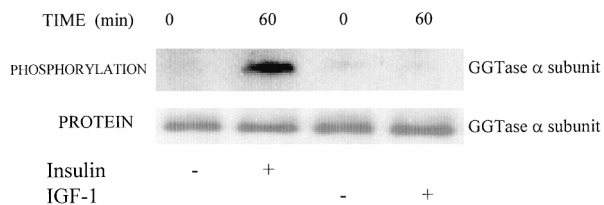


Figure 2. Effect of insulin on the phosphorylation of the α subunit of GGTase I in VSMCs. Cells were preincubated with [³²P]orthophosphate for 24 hours and then incubated with insulin (100 nmol/L) or IGF-1 (13 nmol/L) for 60 minutes. Increased amounts of phosphorylation of the α subunit were visualized by autoradiography (top), and amounts of protein were determined by Western blotting (bottom). A representative experiment of 3 identical runs is depicted.

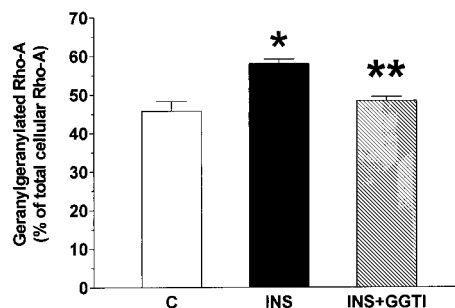


Figure 3. Effect of insulin on the amounts of geranylgeranylated Rho-A in VSMCs. Cells were incubated with insulin (INS) (100 nmol/L) for 24 hours in the absence or presence of GGTI-286 (INS+GGTI) (3 μ mol/L). Results are mean \pm SEM of 6 independent experiments. * $P<0.05$ vs control (C); ** $P<0.05$ vs insulin.

cells, whereas GGTI-286 completely blocked the effect of insulin (Figure 3). Furthermore, neither Ang II, hyperglycemia, nor AGEs had any effect on GGTase I or the amounts of prenylated Rho-A (not shown).

Insulin Potentiates Activation of NF- κ B by Ang II

Next we examined the pathophysiological consequences of insulin-induced increases in the availability of prenylated Rho-A in VSMCs. Because activation of NF- κ B has been shown to be Rho-A dependent,^{31,32} we used an NF- κ B response-element Luc promoter-reporter construct to assess the effect of insulin on the activation of NF- κ B by Ang II, hyperglycemia, and AGEs. We hypothesized that if insulin increases the availability of prenylated Rho-A, the Rho-A-dependent activation of NF- κ B by these agents will be increased in the presence of insulin. We have selected the NF-Luc reporter activity assay to quantitate the incremental influence of hyperinsulinemia on the activation of NF- κ B by Ang II, hyperglycemia, and AGEs.

Ang II-activated NF- κ B-dependent transcription increased 4-fold compared with control cells (Figure 4). Insulin had only a minor and insignificant influence on NF- κ B activation. However, preincubation of VSMCs with insulin (100 nmol/L) for 24 hours increased the effect of Ang II approximately 9-fold (Figure 4). Hyperinsulinemia did not affect the amounts of NF- κ B proteins (not shown). This priming effect of insulin was abrogated in the presence of GGTI-286, which blocked the ability of insulin to increase the availability of prenylated Rho-A for activation. The effect of GGTI-286 was very specific: the inhibitor did not affect the influence of insulin on Akt or the influence of Ang II on NF- κ B (not shown). Additionally, the GGTI-286 inhibitory effect on insulin-induced potentiation of Ang II action was not mimicked by an inhibitor of FTase. To confirm that the influence of Ang II on NF- κ B transactivation was Rho-A dependent, we performed similar experiments in the presence of Clostridium botulinum C3 transferase (C-3 toxin), which ADP-ribosylates and inactivates Rho-A.³⁴ C-3 toxin completely blocked the effect of Ang II on NF- κ B-mediated transcription in the absence (Figure 4) or presence (not shown) of insulin. Neither GGTI-286 nor C-3 toxin had any effect on the basal Luc activity in these or subsequent experiments.

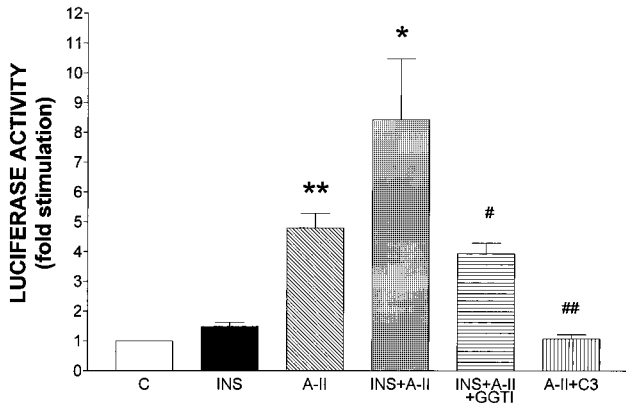


Figure 4. Potentiating effect of insulin on the ability of Ang II to activate NF- κ B response-element Luc construct in VSMCs. Cells transfected with NF- κ B response-element Luc (as described in Materials and Methods) were incubated with either insulin (INS) (100 nmol/L) or Ang II (30 nmol/L) or preincubated with insulin and then challenged with Ang II. Separate plates of cells were also treated with GGTI-286 (INS+Ang II+GGTI) (3 μ mol/L) or C-3 toxin (40 nmol/L). Results are mean \pm SEM of 4 to 8 independent experiments per group. ** P <0.01 vs control (C); * P <0.05 vs Ang II; # P <0.05 vs insulin+Ang II; ## P <0.001 vs insulin+Ang II. A-II indicates angiotensin II.

Insulin Potentiates Activation of NF- κ B by Hyperglycemia

Incubations of VSMCs with 25 mmol/L glucose for 12 hours resulted in a 1.6-fold increase in NF- κ B-dependent transcriptional activity compared with controls (Figure 5), confirming previous suggestions that hyperglycemia has an independent influence on NF- κ B.¹² Preincubations of these cells with 100 nmol/L insulin for 24 hours augmented NF- κ B-dependent transactivation by hyperglycemia from 2-fold (glucose alone) to approximately 7-fold. A combination of insulin with mannitol in similar concentrations was without effect, thus ruling out a nonspecific effect of hyperosmolarity. The priming effect of insulin on glucose was absent in the presence of GGTI-286 (Figure 5), indicating that insulin

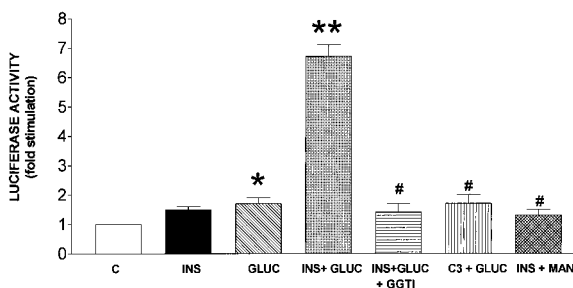


Figure 5. Potentiating effect of insulin on the ability of hyperglycemia to activate NF- κ B response-element Luc construct in VSMCs. Cells transfected with NF- κ B response-element Luc construct (as described in Materials and Methods) were incubated with either insulin (INS) (100 nmol/L) or glucose (GLUC) (25 mmol/L) or preincubated with insulin and then challenged with hyperglycemia (INS+GLUC). Separate plates of cells were also treated either GGTI-286 (INS+GLUC+GGTI) (3 μ mol/L), C-3 toxin (C3+GLUC) (40 nmol/L), or insulin (100 nmol/L) and mannitol (25 mmol/L) (INS+MAN). Results are mean \pm SEM of 4 to 6 independent experiments per group. * P <0.05 vs control (C); ** P <0.01 vs glucose; # P <0.01 vs insulin+glucose.

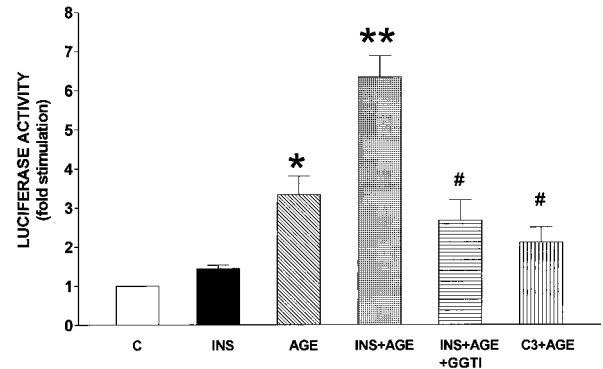


Figure 6. Potentiating effect of insulin on the ability of AGEs to activate NF- κ B response-element Luc in VSMCs. Cells transfected with NF- κ B response-element Luc construct (as described in Materials and Methods) were incubated with insulin and then challenged with AGE (100 μ g/mL). Separate plates of cells were also treated either with GGTI (INS+AGE+GGTI) or C-3 toxin (C3+AGE). Results are mean \pm SEM of 4 to 6 independent experiments. * P <0.05 vs control (C); ** P <0.05 vs AGE; # P <0.05 vs insulin+AGE.

potentiates NF- κ B-mediated transcription via the Rho pathway.

Insulin Potentiates Activation of NF- κ B by AGEs

Finally, we examined the influence of AGEs on the activation of NF- κ B in the absence or presence of insulin. In the absence of insulin, AGEs activated NF- κ B-dependent transcription >3-fold compared with controls (Figure 6), in agreement with previously published data.¹¹ We now demonstrate that the effect of AGE was significantly increased to >6-fold by the preincubation of VSMCs with 100 nmol/L insulin for 24 hours. C-3 toxin blocked the effect of AGEs on NF- κ B activation in the absence and presence (not shown) of insulin, indicating its dependence on Rho-A. GGTI-286 eliminated the priming influence of insulin on the ability of AGEs to activate NF- κ B.

Potentiating Effect of Insulin Is Dose-Dependent

To ascertain the pathophysiological significance of the priming effect of insulin, we performed several key experiments with physiological, high physiological, and pharmacological doses of insulin. Insulin concentrations of 0.1 nmol/L, 1 nmol/L, and 10 nmol/L are equivalent to 13.9 μ U/mL, 139 μ U/mL, and 1390 μ U/mL, respectively. The first two concentrations are commonly seen in fasting and postprandial states in individuals with insulin resistance. The potentiating influence of hyperinsulinemia was clearly dose-dependent (Figure 7). Although the dose-dependent effect of insulin was less apparent in experiments with Ang II (Figure 7A), the ability of increasing concentrations of insulin to augment the influence of hyperglycemia (Figure 7B) and AGEs (Figure 7C) was particularly evident. The maximal potentiating effect of insulin was observed at 10 nmol/L.

Discussion

Type 2 diabetes mellitus has been clearly shown to associate with an accelerated progression of atherosclerosis.^{1,2,13,22} The molecular mechanisms involving hyperglycemia and AGEs

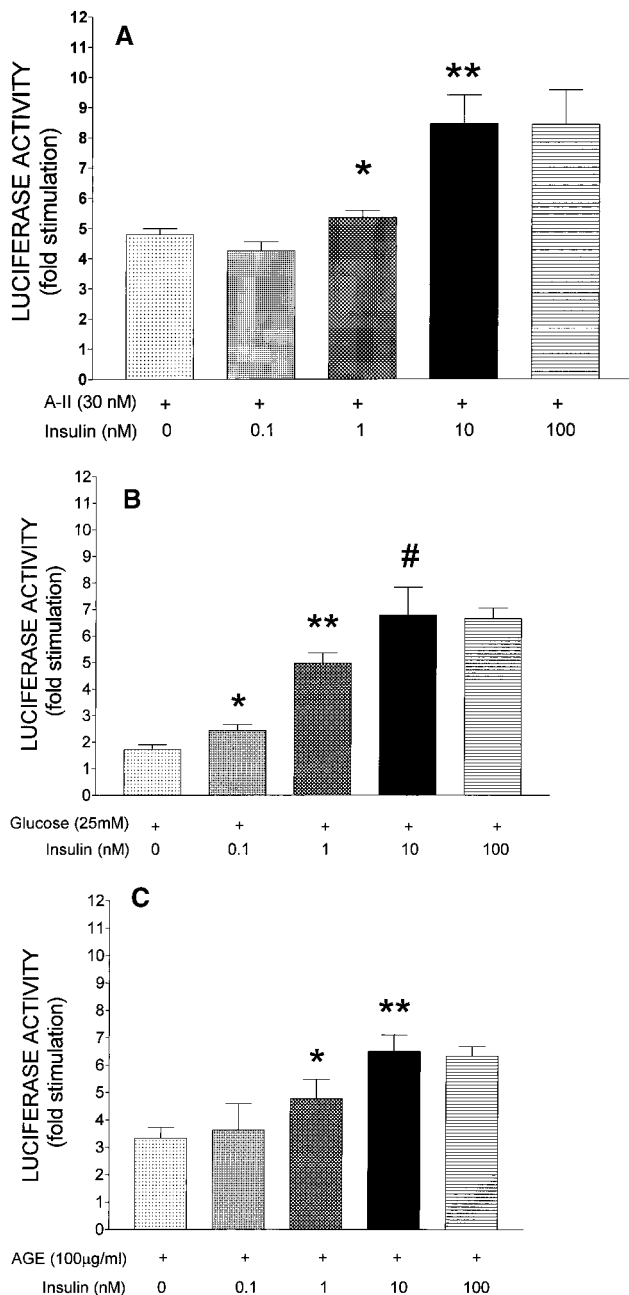


Figure 7. Dose-dependent influence of insulin on the ability of Ang II (A), hyperglycemia (B), and AGEs (C) to activate NF- κ B response-element Luc promoter construct in VSMCs. Cells were preincubated with 0.1, 1, 10, or 100 nmol/L insulin for 24 hours and then challenged with one of the agonists, as described in the legends to Figures 4 through 6. Control cells were exposed to agonist alone. Results are mean \pm SEM of 4 to 6 independent experiments per group. A, * P <0.05 vs control; ** P <0.05 vs 1 nmol/L. B, * P <0.05 vs control; ** P <0.01 vs 0.1 nmol/L; # P <0.05 vs 1 nmol/L. C, * P <0.05 vs control; ** P <0.05 vs 1 nmol/L. A-II indicates angiotensin II.

in the pathogenesis of atherosclerosis are still largely enigmatic. The latest evidence suggests that activation of the transcription factor NF- κ B by hyperglycemia and AGEs may be critically important in this process.^{10,12,35–37} In contrast, the contribution of hyperinsulinemia to the progression of atherosclerosis has not been established despite many epidemi-

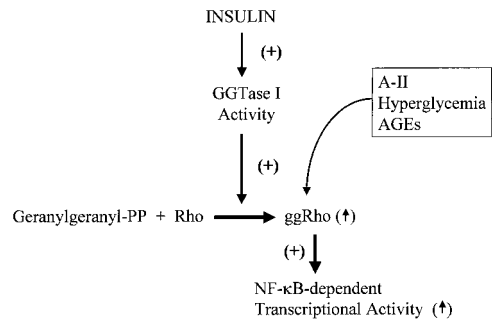


Figure 8. Mechanism of the priming effect of insulin on Ang II-stimulated, hyperglycemia-stimulated, and AGE-stimulated NF- κ B-dependent transcriptional activity. Insulin stimulates the phosphorylation and activation of GGTase I with resultant increases in the amounts of geranylgeranylated Rho (ggRho) made available for activation by Ang II, hyperglycemia, and AGEs. The greater availability of ggRho results in an enhanced NF- κ B-dependent transcriptional activity in response to these factors. A-II indicates angiotensin II.

ological studies, suggesting that hyperinsulinemia may be detrimental.^{16–20} For these reasons, we investigated the effects of hyperinsulinemia on the ability of hyperglycemia, AGEs, and Ang II to activate NF- κ B in VSMCs.

The salient feature of this study is the ability of hyperinsulinemia to potentiate the Rho-A-mediated effects of Ang II, hyperglycemia, and AGEs on NF- κ B activity. Insulin itself has only a minor and non-statistically significant influence on NF- κ B. However, in the presence of hyperinsulinemia, the effects of Ang II, hyperglycemia, and AGEs on NF- κ B-dependent transcription more than doubled (Figures 4 through 6). This potentiating effect of insulin is dose-dependent and clearly present at physiological and high physiological concentrations of insulin (Figure 7). Most of the results depicted in Figures 2 through 6 have also been observed with lower concentrations of insulin. Hyperinsulinemia stimulated GGTase I activity and increased the amounts of geranylgeranylated Rho-A in VSMCs (Figures 1 and 3). Increased availability of geranylgeranylated Rho-A provides a background for an exaggerated NF- κ B-dependent transcriptional response to Ang II, hyperglycemia, and AGEs (Figure 8). Moreover, in the presence of the GGTase I inhibitor GGTI-286, the ability of insulin to potentiate the effects of Ang II, hyperglycemia, and AGEs on NF- κ B was completely blocked, suggesting that hyperinsulinemia works via activation of GGTase I.

Rho-A is one of a dozen Rho GTPases that belongs to a large subgroup of the Ras superfamily of small molecular weight GTPases that regulate a wide spectrum of cellular functions.³⁸ Rho proteins function as molecular switches and exist in either an active GTP-bound conformation or an inactive GDP-bound state. Similar to all members of the Ras superfamily, the Rho GTPases are posttranslationally modified by prenylation before activation by GTP loading.³⁹ Most Rho proteins are geranylgeranylated by geranylgeranyl transferase I.³⁹ Thus, the magnitude of Rho-mediated cellular responses depends on the availability of geranylgeranylated Rho and the degree of its activation by GTP loading.

Recently, Rho proteins have been shown to be involved in the activation of NF- κ B.^{40,41} Evidence has been provided that

Rho GTPases stimulate the phosphorylation of the I- κ B, its subsequent degradation, and the release of NF dimers to the nucleus.^{12,35} It has also been suggested that the generation of reactive oxygen species by Rho GTPases might trigger NF- κ B activation.^{42,43} Additionally, NF- κ B can be activated via molecular pathways other than those related to Rho-A.

The transcription factor NF- κ B was first identified in mature B lymphocytes, where it interacted with the B site of the κ light chain gene enhancer.⁴⁴ It was soon found to be present in other cells as well, including VSMCs.^{10,45} NF- κ B is a heterodimer composed of a 50-kDa and 65-kDa subunit. When inactive, NF- κ B is sequestered in the cytoplasm with the inhibitor I- κ B.⁴⁴ Many cytokines, lysophosphatidic acid, Ang II, and other agents activate NF- κ B by stimulating the phosphorylation and degradation of I- κ B,^{12,44} allowing subsequent translocation of the released NF dimer to the nucleus.

Increasing evidence implicates NF- κ B in the pathogenesis of atherosclerosis.^{10,46,47} NF- κ B plays an important role in the regulation of a variety of genes that are induced in the atherosclerotic lesion, such as vascular cell adhesion molecule, intercellular adhesion molecule, and E-selectin.^{48–50} Conceivably, activation of NF- κ B by hyperglycemia, AGEs, oxidized LDL, and oxidant stress in patients with diabetes could contribute to early development of atherosclerosis and its rapid progression. This study suggests that ambient hyperinsulinemia could potentiate the influence of these factors on NF- κ B in VSMCs and thereby contribute to the accelerated development of atherosclerosis in patients with metabolic syndrome X.

We recently demonstrated that insulin stimulates prenylation of Ras, Rho, and Rab.^{26–30} In the presence of ambient hyperinsulinemia tissue amounts of farnesylated p21Ras and geranylgeranylated Rho-A, Rab-3 and Rab-4 were significantly increased.^{26–30} We also showed that hyperinsulinemia-induced increases in the amounts of farnesylated p21Ras augmented cellular responses to other growth factors, such as IGF-1, epidermal growth factor, and PDGF.^{29,30} For example, in rat and porcine VSMCs, hyperinsulinemia increased the ability of PDGF to stimulate DNA synthesis and the amounts of vascular endothelial growth factor mRNA.³⁰ This potentiating influence of hyperinsulinemia was abrogated in the presence of an inhibitor of FTase.³⁰

The mechanism of the insulin effect on FTase seems to include the phosphorylation of the α subunit of FTase.⁵¹ Insulin-stimulated phosphorylation of the α subunit was not mimicked by IGF-1, epidermal growth factor, or PDGF and required the presence of the intact insulin receptor.²⁹ Thus, even in high concentrations, the effects of insulin on the phosphorylation of the prenyl transferases and their subsequent activation are specific for this hormone and its cell-surface receptor.²⁹ Because the same α subunit also belongs to GGTase I,⁵² we examined the effect of insulin on GGTase I activity in various tissues. We found that insulin indeed stimulated GGTase I activity and increased the amounts of geranylgeranylated Rho-A in 3T3-L1 fibroblasts, MCF-7 breast cancer cells,⁵³ and VSMCs (Figures 1 and 3). This study also demonstrates that IGF-1 does not promote the phosphorylation of the α subunit of GGTase I (Figure 2). In VSMCs, increased availability of prenylated Rho-A aug-

ments the magnitude of the activation of NF- κ B by Ang II, hyperglycemia, and AGEs (Figure 7) and thereby may contribute significantly to the development and progression of atherosclerosis.

Our present data indicate that insulin significantly augments the activity of GGTase I, robustly increases the phosphorylation of the α subunit of GGTase I, and increases the amount of geranylgeranylated Rho-A from 40% to 45% to 66% to 65% of the total cellular Rho-A. Even though the increment is only 20% to 25%, cells do not have more than 70% to 75% of the total Rho protein in the prenylated form at any given moment. Furthermore, this increment in the amount of prenylated Rho-A results in approximately doubling of the magnitude of the nuclear effects of Ang II, hyperglycemia, and AGEs. Thus, the effect of hyperinsulinemia on the amounts of prenylated Rho-A seems to be consistent, significant, and pathophysiologically relevant.

In summary, these results support our hypothesis that hyperinsulinemia creates a state of increased mitogenic responsiveness to other growth-promoting agents by activating the prenyl transferases and augmenting the amounts of prenylated Ras and Rho GTPases available for activation (Figure 8). For the first time to our knowledge, these observations demonstrate the possible biochemical and molecular basis for the detrimental influence of hyperinsulinemia. Because ambient hyperinsulinemia may exaggerate the detrimental influence of poor metabolic control (ie, the influence of hyperglycemia and AGEs), therapeutic interventions directed at the control of glycemia in patients with insulin resistance should be accompanied by measures directed at the reduction of the levels of insulinemia as well.

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