Tumor Necrosis Factor-α Inhibits Insulin-Induced Increase in Endothelial Nitric Oxide Synthase and Reduces Insulin Receptor Content and Phosphorylation in Human Aortic Endothelial Cells

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Insulin exerts a vasodilatory effect through the release of nitric oxide (NO) from the endothelium. We have recently demonstrated that insulin also inhibits the expression of intracellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1), 2 major proinflammatory mediators, by human aortic endothelial cells (HAEC) and the proinflammatory mediator, nuclear factor (NF-κB), in the nucleus in parallel with an increase in endothelial nitric oxide synthase (e-NOS) expression. The inhibition of ICAM-1 by insulin is NO dependent. Because tumor necrosis factor-α (TNF-a) is proinflammatory and may thus inhibit the action of insulin at the endothelial cell level, we have now investigated whether TNF-a affects (1) insulin receptor content; (2) insulin receptor (IR) autophosphorylation induced by insulin, and (3) e-NOS expression by the endothelial cells. TNF-α (1 to 5 ng/mL) caused e-NOS inhibition in a dose-dependent fashion as measured by Western blotting. This inhibition was reduced with insulin addition. TNF-α also inhibited tyrosine autophosphorylation of the IR in HAEC induced by insulin and reduced IR β-subunit protein expression in HAEC. These effects of insulin and TNF-α were independent of cell proliferation, as cell counts did not change with insulin or TNF-α. Our data demonstrate that TNF-α may exert its effect by inhibiting IR autophosphorylation in HAEC and also by reducing IR protein (IRP) expression. Although the inhibition of IR autophosphorylation by TNF-α is known to occur at the adipocyte level, the data on the inhibitory effect of TNF-α on insulin-induced e-NOS expression and IRP contents are novel.

INSULIN HAS BEEN shown to have a potent vasodilatory effect in vivo on the arterial and the venous system. This effect is inhibited by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOS), and methylene blue, a NOS and guanylate cyclose inhibitor.[1-3] Thus, the vasodilatory effect of insulin appears to be mediated by the NOS-cyclic guanosine monophosphate (cGMP) pathway. Indeed, it has been shown that insulin induces an acute release of nitric oxide (NO) by human umbilical vein endothelial cells (HUVEC).[4,5] We have recently shown that insulin at physiologic and pharmacologic concentrations increases endothelial NOS (e-NOS) expression in human aortic endothelias cells (HAEC),[6] and that insulin also exerts an anti-inflammatory effect at the endothelial cell level by reducing the expression of intracellular adhesion molecule -1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), and the pro-inflammatory transcription factor, nuclear factor-κB (NF-κB).[7,8] Insulin-induced vasodilatation is impaired in patients with type 2 diabetes and/or obesity, 2 clinical states characterized by insulin resistance.[9-11] It is not clear why this resistance to the vasodilatory effect of insulin occurs in these patients.

It has recently been shown that adipose tissue expresses the cytokine TNF-α constitutively,[12] and that this expression is enhanced in the animal models of obesity, such as the OB/OB mouse, the db/db mouse, and the fa/fa Zucker rat.[13] The neutralization of TNF-α by soluble receptor to TNF-α (TNF-R), restores the normalization of insulin sensitivity in these insulin-resistant models.[12] Thus, metabolic insulin resistance in obesity may be modulated by TNF-α. An increased expression of TNF-α has also been demonstrated in adipose tissue of obese humans; this expression decreases with weight loss.[14,15] We have recently demonstrated that serum TNF-α concentrations are elevated in obese patients, and that the concentrations decrease with weight loss, with a concomitant decrease in serum insulin concentrations.[16] Thus, it is possible that TNF-α may be a mediator of insulin resistance in obesity. In addition, Yoshizumi et al[17] have demonstrated that treatment of bovine aortic endothelial cells with TNF-α downregulates e-NOS expression at the mRNA level by shortening its half-life. These facts raise the possibility that TNF-α may also contribute to the impaired vasodilatory response to insulin in obesity and type 2 diabetes. Therefore, we considered the possibility that TNF-α may also antagonize the effect of insulin at the endothelial cell level and thus create a state of insulin resistance, as in insulin-induced vascular responses. Furthermore, TNF-α is proinflammatory and is known to increase intranuclear NF-κB and to induce adhesion molecules and chemokines.

This report describes the effect of TNF-α on the expression of e-NOS by HAEC and the interaction of this cytokine with insulin in terms of insulin-induced e-NOS expression, insulin receptor (IR) autophosphorylation, and IR content.

MATERIALS AND METHODS

Cell Isolation and Harvesting

Human endothelial cells were harvested from human aortae and arterial vessels obtained from a 10-year-old Caucasian boy and a 27-year-old Caucasian male using the method described by Gospodarowicz et al.[18] The dissected aortae and arteries were placed in phosphate-buffered saline (PBS) supplemented with antibiotics. The vessels were incubated with 0.1% collagenase/dispase solution (Boehringer Mannheim, Indianapolis, IN) for 20 minutes. This suspension was spun and the cells collected and harvested in flasks coated with fetal bovine serum (FBS). The identity of the cells was confirmed by immunohis-

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tochemical staining, which was positive for factor VIII, Ulex europaeous, EN4, CD31, and negative for α-actin. All experiments were performed using cultures at a passage of 5 to 6. HAEC were allowed to reach 90% confluence, and cells were then taken to phenol-free endothelial cell growth media (Clonetics, Walkersville, MD) containing 2% charcoal/dextran stripped FBS (Hyclone, Logan, UT) for 24 hours. On the second day, the cells were induced with insulin (0, 100, and 1,000 μU/mL) and TNF-α (1 and 5 ng/mL). The medium was changed every 2 days for 6 days. After induction, the cells were collected and homogenized for Western blotting. For IR autophosphorylation, HAEC were incubated with TNF-α for 24 hours then induced with insulin for 10 minutes. The Institutional Review Board of State University of New York at Buffalo based at Millard Fillmore Hospitals approved the study.

Cell Proliferation Assay

The CellTiter 96 Aqueous nonradioactive proliferation assay (Promega, Madison, WI) was used to determine the number of viable cells. A total of 5,000 cells were cultured in a 100-μL volume/well in 96-well plates. Media was changed on a daily basis. Samples were run in triplicate. The absorbance was recorded at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Immunoprecipitation

IR was immunoprecipitated from HAEC by the method described by Springer.[19] Cell lysate was prepared by incubating harvested and washed cells in 1X lysis buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 50 mmol/L sodium fluoride, 1 mmol/L orthovanadate, 25 mmol/L beta glycerol phosphate, 100 mmol/L sodium pyrophosphate, 1.5 mg/mL benzimidine, 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 10 μg/mL pepstatin A) for 1 hour on ice. Lysate was centrifuged at 10,000 $\times$ g for 30 minutes in a microcentrifuge. Clarified supernatant was collected, and total protein concentration was determined using BCA protein assay (Pierce, Rockland, IL). A total of 2 to 3 mg of HAEC clarified cell lysate was precleared by the addition of 100 μL protein A sepharose slurry (50%) with mixing for 2 hours at room temperature. Lysate was centrifuged for 1 minute at 200 $\times$ g, and the supernatant was collected. Precleared supernatant was then incubated with 8 μg of polyclonal antibody against IR β-subunit (Transduction Labs, Lexington, KY) for 8 hours at 4°C with agitation. The immune complex was precipitated by the addition of 100 μL protein A sepharose slurry with gentle mixing for 6 hours at 4°C followed by centrifugation for 1 minute at 200 $\times$ g. The pellet was washed twice with 1 mL of wash buffer 1 (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1% Triton X-100, 0.025% sodium azide). The pellet was then washed by wash buffer 2 (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.025% sodium azide) followed by wash buffer 3 (50 mmol/L Tris-HCl, pH 6.8). A total of 40 μL of 2X gel loading buffer (100 mmol/L Tris-Cl, pH 6.8, 20 mmol/L DTT, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, and 20% glycerol) was added to the pellet, and the sample was boiled for 10 minutes followed by a 5-minute centrifugation at 10,000 $\times$ g, and the supernatant was collected and loaded into 6% polyacrylamide gels (SDS-polyacrylamide gel electrophoresis [PAGE]).

Western Blotting

Total cell lysate was prepared by washing the adherent cells with PBS followed by 1 mL boiling lysis buffer (1% SDS, 1 mmol/L sodium ortho-vanadate, 10 mmol/L Tris pH 7.4). Twenty micrograms of total cell lysate was electrophoresed on 6% gels (SDS- PAGE). The proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked and incubated with a monoclonal antibody against e-NOS (Transduction Labs). A monoclonal antibody against IR β-subunit (Transduction Labs) was used to detect IR quantity and an antiphosphotyrosine antibody (clone 4G10; Upstate Biotechnology, Lake Placid, NY) was used for tyrosine phosphorylation detection. Finally, the membrane was washed and developed using super signal chemiluminescence reagent (Pierce).

Statistical Analysis

The *t* test was used to for statistical analysis using SigmaStat software (Jandel Scientific, San Rafael, CA). The results are expressed as mean $\pm$ SE.

RESULTS

HAEC incubated with different concentrations of TNF-α (1 to 5 ng/mL) showed a dose-dependent inhibition of e-NOS, and only at high x-ray film exposure, e-NOS bands were detected on the 6th day (Fig 1). HAEC homogenates showed a dose-dependent increase in e-NOS following incubation with 0, 100, and 1,000 μU/mL of insulin for 6 days (Fig 2A). Insulin-induced e-NOS increase diminished significantly after the addition of 1 ng/mL TNF-α (Fig 2B). These effects of insulin and TNF-α were independent of cell proliferation, because cell counts increased in cultures with and without insulin and TNF-α at various concentrations to a similar extent. IR tyrosine autophosphorylation induced with insulin was inhibited by TNF-α ($P < .001$; Fig 3). In addition, IR protein (IRP) content also decreased significantly after TNF-α addition (Fig 4, $P < .05$). There was no dose-related difference in the extent of TNF-α–induced suppression of IR content. Normalization of IR tyrosine phosphorylation (IRTP) to IRP content showed that

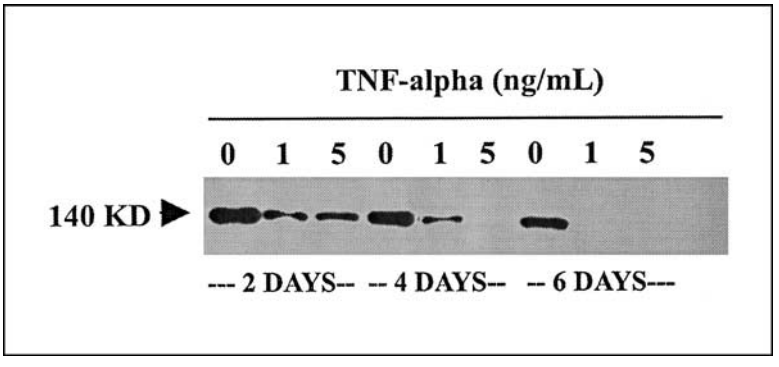


Fig 1. Western blot showing the inhibition of e-NOS by TNF-α over a period of 6 days. Note that TNF-α inhibits e-NOS significantly after 2 days, and this inhibition increases after 4 and 6 days.

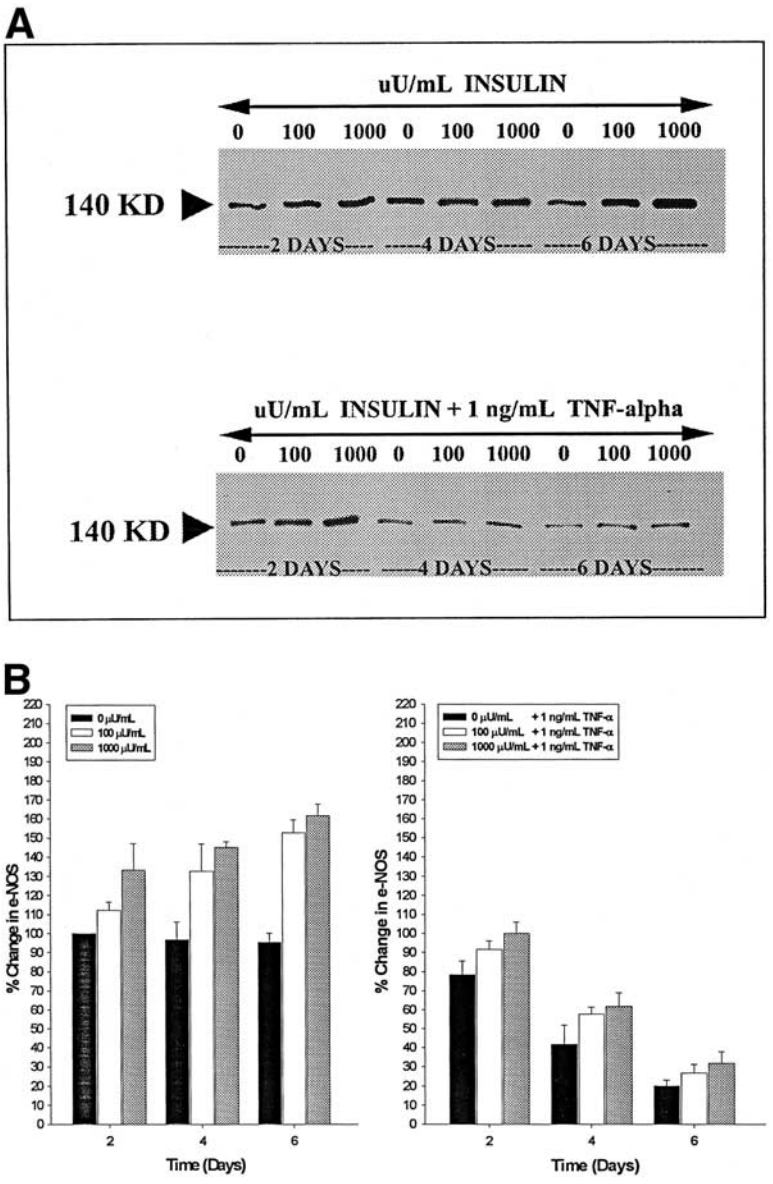


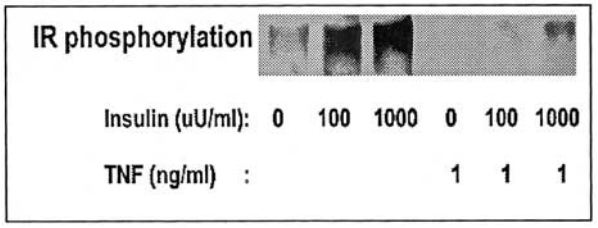
Fig 2. (A) A representative Western blot showing the effect of insulin and insulin and TNF-α on e-NOS over a period of 6 days. This blot is representative of 4 different experiments. (B) Densitometric analysis of e-NOS expression following incubation with insulin or insulin and TNF-α.

the reduction in IRTP was inhibited independently of the change in the IR content; the ratio of IR phosphorylation to IRP content diminished more markedly at 1,000 μU/mL insulin concentration than at 100 μU/mL without there being a significant difference in IRP content at the 2 concentrations (Fig 5).

DISCUSSION

TNF-α has been demonstrated to downregulate the expression of e-NOS mRNA in endothelial cells by a mechanism involving destabilization of e-NOS mRNA.[17] Our data demonstrate clearly for the first time that TNF-α inhibits insulin-induced increase in e-NOS expression and causes a decrease in IRP content and IR phosphorylation in HAEC. These effects are observed within 2 days of incubation. Longer periods of incubation with TNF-α cause a progressive marked inhibition of e-NOS, both with and without insulin coincubation. It is possible that TNF-α may contribute to the inability of insulin to cause vasodilatation in patients with obesity and type 2 diabetes with obesity through this mechanism, as both tissue expression[12-15] and plasma[16] content of TNF-α are elevated in the obese.

It has recently been shown that TNF-α induces a decrease in insulin-induced tyrosine phosphorylation of the IR and insulin receptor substrate-1 (IRS-1) in the adipocyte. This step is essential for the continuation/amplification of the insulin signal at the postreceptor level. Our data with endothelial cells are consistent with the observations on adipocytes. Our experiments show that TNF-α causes a reduction in insulin-induced tyrosine phosphorylation of the IR and also causes a 40% decrease of IRP concentrations within 24 hours of exposure. The concomitant decrease in e-NOS expression, IR content, and IR phosphorylation in HAEC following incubation with



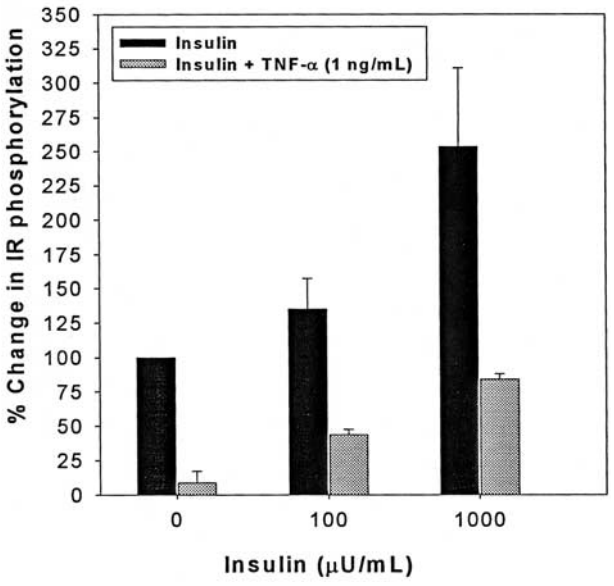
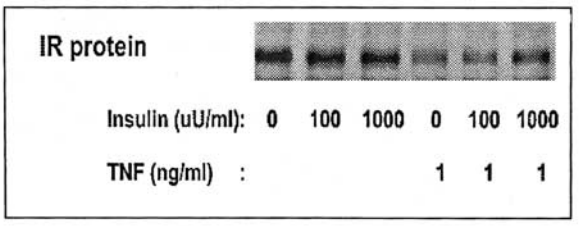


Fig 3. IRTP after a 24-hour incubation with or without 1 ng/mL TNF-α. HAEC were induced with insulin for 10 minutes. All values were normalized to 100%, and the following values were expressed as percent of basal level. The results are presented as mean ± SE.



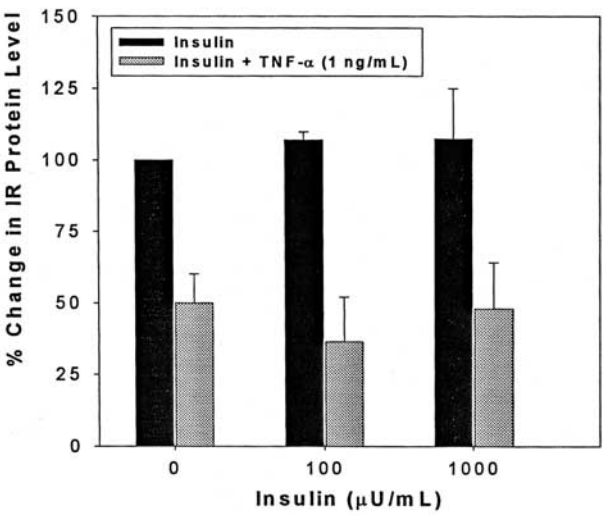


Fig 4. IRP content after a 24-hour incubation with or without 1 ng/mL TNF-α. HAEC were induced with insulin for 10 minutes. All values were normalized to 100%, and the following values were expressed as percent of basal level. The results are presented as mean ± SE. Note that insulin did not alter IRP, while TNF-α suppressed IRP consistently at all concentrations of insulin.

TNF-α suggest that the reduction in e-NOS may be mediated by the reduction in IR and IRTP. However, further investigation would be required to establish a definite causal/mechanistic relationship.

Whereas a diminution in insulin-induced IR phosphorylation by TNF-α has been demonstrated in adipocytes, there is no previous demonstration of TNF-α–induced reduction in IRP content in the adipocyte. Thus, TNF-α may alter postreceptor events by a reduction in IR autophosphorylation. Hotamisligil et al[20] have also demonstrated that TNF-α causes serine phosphorylation of IRS-1, and that this serine phosphorylated derivative of IRS-1 actually mediates the inhibition of insulin-induced tyrosine phosphorylation of the IR in adipocytes. Experiments to establish a possible similar role for serine phosphorylation of IRS-1 in the inhibition of IRTP in the endothelial cell would be of considerable interest. In view of the inhibitory effect of TNF-α on insulin-induced e-NOS expression, it would be of interest to investigate whether TNF-α also inhibits the acute effect of insulin on NO release by endothelial cells.

We have recently demonstrated that insulin reduces the expression of ICAM-1 in HAEC, and that this inhibition is

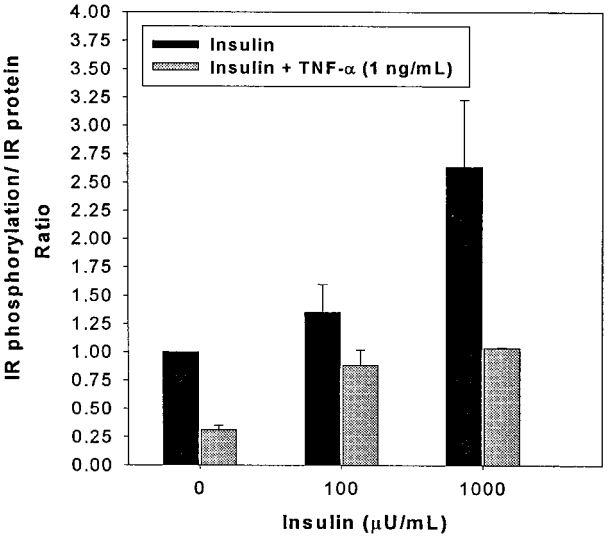


Fig 5. IRTP/IRP content ratio. Following incubation with insulin IRTP/IRP ratio increases, as expected. IRTP/IRP consistently decreases following incubation with TNF-α. Thus, TNF-α causes both a decrease in IRP and IRTP independently. The results are presented as mean ± SE.

mediated by NO, because the specific inhibition of e-NOS results in the neutralization of this effect.[7] Because TNF-α is known to increase the expression of ICAM-1,[21] it is possible that it induces this increase through the inhibition of insulin action and e-NOS in endothelial cells. The expression of ICAM-1 by the endothelium is important in the pathogenesis of inflammation and atherosclerosis. Its concentrations in plasma have been shown to be predictive of clinical events in coronary heart disease and cerebrovascular disease.[22] Our recent work also shows that insulin inhibits the intranuclear content of NF-κB and the expression of MCP-1 in endothelial cells.[8] Insulin has now been shown to exert an NF-κB suppressive effect in vivo and thus may be anti-inflammatory.[23] Furthermore, troglitazone, an insulin sensitizer and a peroxisome proliferator-activated receptorγ (PPARγ) agonist, also has potent anti-inflammatory effects, including the suppression of TNF-α[24]; troglitazone also restores vascular reactivity to normal following a short period of treatment.[25] It is known that TNF-α, a proinflammatory cytokine, stimulates intranuclear NF-κB and the expression of proinflammatory chemokines.

In conclusion, TNF-α inhibits insulin-induced enhancement of e-NOS expression, IRP content and tyrosine phosphorylation of IR in HAEC. Thus, increased expression and serum concentrations of TNF-α in the obese may contribute to the impairment of the vasodilatory effect of insulin in obesity and other insulin-resistant states, such as type 2 diabetes. These effects may be relevant to the pathogenesis of macrovascular disease in these states.

REFERENCES

1. Steinberg HO, Brechtel G, Johnson A, et al: Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. J Clin Invest 94:1172-1179, 1994
2. Scherrer U, Randin D, Vollenweider P, et al: Nitric oxide release accounts for insulin's vascular effects in humans. J Clin Invest 94: 2511-2515, 1994
3. Grover A, Padginton C, Wilson MF, et al: Insulin attenuates norepinephrine-induced venoconstriction. An ultrasonographic study. Hypertension 25:779-784, 1995
4. Zeng G, Quon MJ: Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. J Clin Invest 98:894-898, 1996
5. Guo JP, Murohara T, Buerke M, et al: Direct measurement of nitric oxide release from vascular endothelial cells. J Appl Physiol 81:774-779, 1996
6. Aljada A, Dandona P: Effect of insulin on human aortic endothelial nitric oxide synthase. Metabolism 49:147-150, 2000
7. Aljada A, Saadeh R, Assian E, et al: Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothelial cells through stimulation of nitric oxide. J Clin Endocrinol Metab 85:2572-2575, 2000
8. Aljada A, Ghanim H, Saadeh R, et al: Insulin inhibits NFκB and MCP-1 expression in human aortic endothelial cells. J Clin Endocrinol Metab 86:450-453, 2001
9. Baron AD, Brechtel G, Johnson A, et al: Interactions between insulin and norepinephrine on blood pressure and insulin sensitivity. Studies in lean and obese men. J Clin Invest 93:2453-2462, 1994
10. Vollenweider P, Randin D, Tappy L, et al: Impaired insulin-induced sympathetic neural activation and vasodilation in skeletal muscle in obese humans. J Clin Invest 93:2365-2371, 1994
11. McVeigh GE, Brennan GM, Johnston GD, et al: Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 35:771-776, 1992
12. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. Science 259:87-91, 1993
13. Yamakawa T, Tanaka S, Yamakawa Y, et al: Augmented production of tumor necrosis factor-alpha in obese mice. Clin Immunol Immunopathol 75:51-56, 1995
14. Kern PA, Saghizadeh M, Ong JM, et al: The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest 95:2111-2119, 1995
15. Hotamisligil GS, Arner P, Caro JF, et al: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 95:2409-2415, 1995
16. Dandona P, Weinstock R, Thusu K, et al: Tumor necrosis factor-alpha in sera of obese patients: Fall with weight loss. J Clin Endocrinol Metab 83:2907-2910, 1998
17. Yoshizumi M, Perrella MA, Burnett JC Jr, et al: Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res 73:205-209, 1993
18. Gospodarowicz D, Moran J, Braun D, et al: Clonal growth of bovine vascular endothelial cells: Fibroblast growth factor as a survival agent. Proc Natl Acad Sci USA 73:4120-4124, 1976
19. Stacker SA, Springer TA: Leukocyte integrin P150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. J Immunol 146:648-655, 1991
20. Hotamisligil GS, Peraldi P, Budavari A, et al: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α and obesity-induced insulin resistance. Science 271:665-668, 1996
21. Voisard R, Osswald M, Baur R, et al: Expression of intercellular adhesion molecule-1 in human coronary endothelial and smooth muscle cells after stimulation with tumor necrosis factor-alpha. Coron Artery Dis 9:737-745, 1998
22. Ridker PM, Hennekens CH, Roitman-Johnson B, et al: Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 351: 88-92, 1998
23. Dandona P, Aljada A, Mohanty P, et al: Insulin inhibits intranuclear nuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: Evidence for an anti-inflammatory effect? J Clin Endocrinol Metab 86:3257-3265, 2001
24. Ghanim H, Garg R, Aljada A, et al: Suppression of nuclear factor-κB and stimulation of inhibitor κB by troglitazone: Evidence for an anti-inflammatory effect and a potential antiatherosclerotic effect in the obese. J Clin Endocrinol Metab 86:1306-1312, 2001
25. Garg R, Kumbkarni Y, Aljada A, et al: Troglitazone reduces reactive oxygen species generation by leukocytes and lipid peroxidation and improves flow-mediated vasodilatation in obese subjects. Hypertension 36:430-435, 2000