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The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance

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Introduction

Insulin receptors (IRs) have been demonstrated on endothelial cells of both large and small blood vessels (1). Insulin binding to its receptor activates both PI3K/AKT and the Ras-MAP kinase pathway. In endothelial cells, the PI3K/AKT pathway mediates an antiapoptotic effect and also results in an increase in gene expression and activation of eNOS (2–4). These effects are enhanced by VEGF and fluid shear stress (5–8).

Multiple hypotheses have been put forward regarding the role of insulin action on vascular tissues as related to glucose homeostasis, control of blood pressure and blood flow, and development of vascular complications. Insulin’s effects with regard to vascular complications of diabetes can be considered to be both protective and deleterious. Protective effects of insulin include its ability to protect against apoptosis and to stimulate production of NO (4, 7), which results in relaxation of VSMCs and inhibits some of the process-
es associated with atherosclerosis. Deleterious effects of insulin are explained by its ability to activate several vascular mediators, such as VEGF, a strong angiogenesis factor, and endothelin-1 (ET-1), a known vasoconstrictor. Hypertension, especially salt-sensitive hypertension, is also associated with insulin resistance (9).

In addition, IR action on endothelium has been suggested to play a role in the control of glucose homeostasis in three ways. First, transendothelial transport of insulin is believed to be a rate-limiting step in insulin action on glucose disposal (10). While the exact mechanism(s) of the transendothelial transport in vivo remain debated (11), in endothelial cells in culture insulin transport across the endothelial barrier appears to be mediated by IR (12). Secondly, insulin has shown to act as vasodilator via activation of eNOS in skeletal muscle, and the resultant increase in blood flow has been suggested to contribute significantly to the increase in glucose uptake by skeletal muscle (13). Although the importance of this vasodilatory effect of insulin on whole-body glucose disposal remains controversial, this effect is impaired in individuals with insulin resistance (14–17). Finally, insulin has been suggested to act on endothelium to produce potential mediators of hormone action, such as NO and cGMP (18, 19). These substances can then diffuse to muscle and fat and act to increase glucose uptake.

To address to what extent insulin action in endothelial cells plays a role in glucose homeostasis and vascular tone, we have generated mice with a specific vascular endothelial cell insulin receptor knockout (VENIRKO). Here, we report the characterization of vascular development, function, and insulin action in
these animals and explore the role of the insulin receptor in retinal neovascularization.

Methods
Generation of mice and genotyping. VENIRKO mice were generated using the Cre-loxP system. Mice carrying an IR gene in which exon 4 is flanked by lox sites (20) were bred to generate using the Cre recombinase under control of the Tie2 promoter-enhancer. Previous studies have shown that Tie2 expression is limited to vascular endothelial cells and the endocardial cushion (21). As a result of the complex breeding, all the mice in these experiments have a mixed genetic background, including contributions from 129Sv, C57Bl/6, SJL, FVB, and DBA strains. To minimize the difference of genetic background, we used littermates from same breeding pairs as controls. Genotyping of the mice for the Cre transgene and the floxed IR alleles was performed by genomic PCR, using specific primers that allow identification of the presence or absence of the Tie2-Cre transgene and heterozygosity or homozygosity of the IR gene for the floxed or wild-type allele (22). Animals were housed on a 12-h light/dark cycle and were fed a standard rodent chow. All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with NIH guidelines. All experiments were carried out in male mice, except as otherwise noted.

Endothelial culture and evaluation of the Cre-mediated recombination of IR in vascular endothelial cells. Two approaches were used to derive microvascular endothelium for primary culture. Cerebrovascular microvessels were isolated by filtration of brain homogenates and digestion with collagenase (23). The digested vessels were filtered sequentially through nylon meshes of three different pore sizes 210, 88, and 55 µm, respectively. The microvessels retained on 88- and 55-µm filters were plated on 30-mm fibronectin-coated dishes and incubated in DMEM medium containing 10% FCS and 2% bovine brain EGF for 7–10 days. Nonendothelial cells were scraped off using a cotton bar under microscopic visualization. Cultures that were considered more than 95% pure based on morphological examination were used to prepare genomic DNA. Cre-mediated recombination of IR gene was analyzed by PCR using primers surrounding exon 4, as described previously (22).

To obtain enough endothelial cells to prepare RNA for real-time quantitative RT-PCR, primary cultures of endothelial cells were established using cells immunoselected from lungs (24). Briefly, a cell suspension was prepared from lungs by digestion with collagenase (1 mg/ml) for 45 min, followed by filtration using 40-µm nylon filters. Endothelial cells were then selected using a rat Ab to mouse CD-31 (Research Diagnostics Inc., Flanders, New Jersey, USA) coupled to magnetic beads (Dynabeads; Dynal Inc., Lake Success, New York, USA) and coated with sheep anti–rat IgG. Cells were plated on 1% gelatin-coated 30-mm dishes and grown for 6–7 days in DMEM containing 20% FCS, 2 mM l-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 µg/ml streptomycin, 100 U/ml penicillin, 100 µg/ml heparin, and 100 µg/ml endothelial cell growth supplement (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were then trypsinized and subjected to a second round of selection. For RNA from analysis, approximately 10⁶ endothelial cells in each genotype were harvested, and RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, California, USA) as described by the manufacturer.

Real-time quantitative RT-PCR. Primers and probes for real-time quantitative RT-PCR were designed using Primer Express 1.5a software (ABI Prism; Perkin-Elmer Applied Biosystems, Foster City, California, USA) and were as follows: for IR, 5′ primer, 5′-CAG AAT GTG ACA GAG TTT GAT GGG-3′; 3′ primer, 5′-CGG GTC AAT ATC CAC CAC AGT-3′; and probe: 6FAM (carboxyfluorescein)-CAG GAT TGT GTC TCC AAC AGC TG-TAMRA (N,N,N,N-tetramethyl-6-carboxyrhodamine). For VEGF, 5′ primer, 5′-GGA GTA CCC CGA GTA AGA GTA-3′; 3′ primer, 5′-AGC CTG CAC AGC GCA TC-3′; and probe, 6FAM-ATC TAC ACG GCC TCC TGT GTG CAG-TAMRA. For eNOS, 5′ primer, 5′-CCG CAA CGT CGA GAC CAG TAC-3′; 3′ primer, 5′-ATC AAA GCG GCC ATT TTC T-3′; and probe, 6FAM-AGC TCT GTC AAT ATG GGT GTA CCC-TAMRA. For ET-1, 5′ primer, 5′-TGG ACA TCA TCT GGG TCA ACA-3′; 3′ primer, 5′-GAC CTG GAA GAA CCT CCC AAT C-3′; and probe, 6FAM-TCC CGA GCG CGT CCT AGT-GTA-TAMRA. Real-time quantitative RT-PCR was done with the TaqMan One-Step RT-PCR Master Mix Reagents kit (Perkin-Elmer Applied Biosystems) as described by the manufacturer. The RT-PCR mixture (50 µl) contained 12.5 U of Multiscribe reverse transcriptase, 20 U of RNase inhibitor, IR primers and probe or VEGF primers and probe, eNOS primers and probe, or ET-1 primers and probe, at concentrations of 90–100 nM and 100 nM, for each set of primers and probe, respectively, and 5 ng of total RNA template from primary culture of lung endothelial cells. Amplification and detection of specific products was performed with the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems) with the following cycle profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. As an internal control, rodent GAPDH probe and primers (Perkin-Elmer Applied Biosystems) were used for RNA template normalization.

RNA isolation and Northern blot analysis. RNA from heart and aorta of control and VENIRKO mice was isolated using TRI Reagent ( Molecular Research Center Inc., Cincinnati, Ohio, USA), and 20 µg was subjected to Northern blot analysis using a 650-bp cDNA fragment of rat eNOS (kindly provided by Mark A. Perella and Arthur M.E. Lee, Harvard School of Public Health, Boston, Massachusetts, USA). Quantitation of eNOS mRNA was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA) and the levels normalized to those of the 36B4 mRNA.

Metabolic studies. Glucose-tolerance tests (GTTs) were performed in animals that had been fasted overnight for
16 h, using an intraperitoneal injection of a 2% glucose at a dose of 2 g/kg body weight. Insulin-tolerance tests (ITTs) were performed in the early afternoon in random-fed animals by intraperitoneal injection of 1.5 U/kg body weight of regular human insulin. Blood glucose levels were determined from whole venous blood obtained from the tail using an automatic glucose monitor (One Touch II; Lifescan, Milpitas, California, USA). Insulin concentrations in serum were determined with an ELISA assay using mouse insulin as standard (Crystal Chem Inc. Chicago, Illinois, USA). Serum-free fatty acid and triglyceride concentrations were analyzed immediately after separation of serum by colorimetric enzyme assays, using the NEFA C kit (Wako Chemicals USA Inc., Richmond, California, USA) and the GPO-Trinder assay (Sigma-Aldrich), respectively.

**Glycemic clamps to assess the kinetics of insulin action.** Euglycemic-hyperinsulinemic clamps were performed to evaluate differences in the time course of insulin action. Two intrajugular in-dwelling catheters were placed under anesthesia 4–6 days in advance of the study. The clamps were performed in awake, unanaesthetized mice after a 6-h fast. After basal tail vein blood glucose sampling at time –20, –10, and 0 min, insulin was administered intravenously at time 0 as a bolus (40 mU/kg) and constant infusion (4 mU/kg/min). This high dose of insulin was chosen to both suppress endogenous glucose production and stimulate peripheral glucose use. Blood glucose concentrations were measured at 2.5, 5, 7.5, 10, 15, 20, 25, 30, 40, 50, and 60 min. The intravenous infusion of dextrose (50%) was adjusted after each blood glucose determination in order to maintain euglycemia (25).

**Morphological analysis of retinal microvasculature.** Mice (17 days old) were sacrificed with an overdose of sodium pentobarbital and perfused through the heart with 50 ng of FITC-dextran (Sigma-Chemical) dissolved in 1 ml of 10% buffered formalin (Fisher Scientific Co., Fair Lawn, New Jersey, USA). The eyes were enucleated and fixed in 10% formalin for 12 h. The retinas were dissected and flat mounted with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, California, USA). Preparations were observed by fluorescence microscopy and photographed using a digital system.

**In vivo measurement of blood flow.** Mice were anesthetized, and jugular catheters were implanted for fluorescein infusion. The eyes were dilated (1% tropicamide, Mydriacyl; Alcon Laboratories Inc., Fort Worth, Texas, USA), and a Hamilton syringe containing 10% sodium fluorescein was connected to the externalized jugular vein catheter. Retinal blood flow was measured using a video fluorescein angiography methodology that has been described in detail previously (26–29).

**Blood pressure.** Blood pressure was measured in the morning by tail cuff photoelectric plethysmography in restrained, unanesthetized mice using an UR-5000 UEDA system (UEDA Electronic Works Ltd., Tokyo, Japan). Mice were trained to accept the measurement by conducting three mock blood pressure measurements sessions before the actual measurements. Statistical analysis of data. Data are expressed as means plus or minus SEM. Comparisons of the means were made using the unpaired Student t test. P values smaller than 0.05 were considered statistically significant.

**Results**

**VENIRKO mice lack IR and insulin action in endothelium.** Endothelial cell–specific inactivation of the IR was achieved by breeding mice carrying an IR gene in which exon 4 was flanked by lox sequences with Tie2 Cre transgenic mice. Intercrossing the Cre-positive and Cre-negative IR-lox heterozygous mice resulted in offspring of six genotypes: wild-type, IR-lox/+, and the IR-lox/lox, each
with or without the Cre transgene. During the characterization, no significant phenotypic differences were found among the three genotypes of mice negative for Cre and mice carrying the transgene only, with no IR-lox allele. Therefore, these four genotypes were combined to a single control group (control), which could be compared with mice positive for the Cre transgene and carrying one (heterozygous) or two lox-IR alleles (VENIRKO).

To assess inactivation of the IR gene, primary cultures of endothelial cells were prepared from brain or lung selected using anti–CD31 Ab, which recognize PECAM-1, a membrane protein specific for endothelial cells, monocytes, leukocytes, and platelets (24). Cre-mediated recombination of the IR gene in DNA from the endothelial cells was assessed using PCR with three primers, two specific for the intronic sequences upstream and downstream of exon 4 and a third specific for exon 4 (22). A single band of 266 bp was amplified from control, as predicted for the intact IR allele. By contrast, a single band of 220 bp was obtained with cells of VENIRKO mice, indicating complete recombination of the IR-lox allele (Figure 1, a and b).

This was confirmed by real-time quantitative RT-PCR for RNA isolated from primary cultures of endothelial cells, which revealed a 95–97% reduction in IR mRNA in VENIRKO cells as compared with control (Figure 1c). Using Western blot analysis, there was also little or no detectable IR determined in immunoprecipitates from VENIRKO mice as compared with the wild-type mice (data not shown).

Insulin produces a wide variety of effects in endothelial cells, including stimulation of angiogenesis and vasoregulation. To explore the role of the IR on gene expression in vascular tissues such as heart and aorta, Northern blot analyses were performed using eNOS probes. These revealed a 38.7% ± 7.1% decrease of eNOS expression in the heart (P < 0.05) and 61.8% ± 9.1% reduction of eNOS mRNA in the aorta (P < 0.05) as compared with control (Figure 2a). For better quantitation, we isolated total RNA from cultured endothelial cells and carried out real-time quantitative RT-PCR for VEGF as an angiogenic factor, eNOS as a vasodilator, and ET-1 as a vasoconstrictor. Two independent sets of endothelial cells were prepared from control and two from VENIRKO mice. Each RNA sample was analyzed by real-time quantitative RT-PCR on three independent experiments. VEGF expression in VENIRKO endothelial cells was comparable to that in control. On the other hand, both eNOS and ET-1 expression in VENIRKO were significantly reduced 30% ± 8% (P < 0.05) and 42% ± 8% (P < 0.05), respectively (Figure 2b).

**Structural integrity of vascular tissues and in vivo blood flow.**

Insulin signaling in endothelial cells activates AKT, and thus insulin could play a role in vascular development, angiogenesis, and endothelial cell apoptosis (4). Inactivation of the IR in endothelial cells, however, did not appear to compromise blood vessel development or
integrity. VENIRKO mice were born at the expected Mendelian frequency, were fertile, showed no defects in growth nor any gross morphological or functional abnormality. Survival up to 24 months of age was not different in VENIRKO mice as compared with control. Furthermore, no change in capillary count or structural alterations were observed in vascular structures of the brain, aorta, and kidney (data not shown). Integrity of capillary bed and small arteries was further examined using flat mount retinas (Figure 3). No abnormal architecture or evidence of compromised capillary integrity could be detected.

Role of IR in kinetics of insulin action in vivo. IRs have been shown to participate in transendothelial transport of insulin in endothelial cells in culture (30); however, whether transendothelial transport of insulin is rate limiting for insulin action in vivo remains controversial (10, 11). To address this issue, we used a modified frequently sampled euglycemic-hyperinsulinemic clamp to assess insulin action. Glucose infusion rates (GIRs) required to maintain euglycemia reflect the kinetic onset and maximal insulin stimulation of glucose turnover in vivo (Figure 4). Fitting GIR to a single two-parameter exponential rise to a maximum curve revealed no significant difference in the half-time for the onset of maximal insulin action (6.1 ± 1.4 vs. 4.4 ± 1.3 min, P = NS) nor maximal GIR (37.7 ± 2.5 versus 36.0 ± 2.6 mg/kg/min; P = NS). Thus, under this condition, endothelial cell IRs do not appear to play a major rate-limiting role in the access of insulin to metabolically active tissues.

Physiologic effects of IR inactivation in endothelial cells. At 2 months of age, fed and fasting glucose levels, fasting free fatty acid levels, and blood insulin concentrations were comparable in VENIRKO and control mice (Table 1). GTTs and ITTs were also indistinguishable (Figure 5). On the other hand, fasting triglyceride concentrations were reduced in VENIRKO mice approximately 20% (P < 0.01) (Table 1) and still slightly lower at 6 months. ITTs at 6 months of age showed that both control and VENIRKO mice had developed mild insulin resistance with a decrease in the response to insulin at 30 and 60 min. Nevertheless, VENIRKO mice remained more glucose tolerant during the GTT (Figure 5).

Effect of knockout of the IR on BP control. Previous studies have demonstrated that insulin has an acute vasodilatory effect (13) and have suggested that insulin resistance at the vascular level may play a role in hypertension. To assess this mechanism, BP and HR were measured at 16 months of age (Figure 6a). The HR in female VENIRKO mice was significantly slower than in control. SBP, MBP, and DBP in male VENIRKO mice were also slightly, but not significantly, lower as compared with control mice on a random diet.

To further explore vascular function and its consequences on insulin sensitivity, mice were placed on low-salt (0.05% NaCl) and high-salt (8.0% NaCl) diets for 2 weeks and then reevaluated for BP, HR, and insulin sensitivity. Consistent with the studies on the random diet, SBP, MBP, and DBP in male VENIRKO mice were also slightly, but not significantly, lower as compared with control mice on a random diet.

Table 1
Metabolic parameters at 2 and 6 months of age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 months</th>
<th>6 months</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Het VENIRKO</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>25.9 ± 0.4</td>
<td>26.3 ± 0.5</td>
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<tr>
<td>Fasting glucose (mg/dl)</td>
<td>59.3 ± 1.9</td>
<td>62.7 ± 3.2</td>
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<tr>
<td>Random fed glucose (mg/dl)</td>
<td>120 ± 3</td>
<td>123 ± 4</td>
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<tr>
<td>Free fatty acids (mEq/l)</td>
<td>1.39 ± 0.03</td>
<td>1.30 ± 0.07</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>160 ± 8</td>
<td>128 ± 9.8</td>
</tr>
<tr>
<td>Insulin (pg/ml)</td>
<td>317 ± 35</td>
<td>294 ± 48</td>
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The number of mice in each group for 2-month-old mice were: controls = 41–49; heterozygous (het) = 28–30; VENIRKO = 29–30. The number of mice in each group for 6-month-old mice were: controls = 45–57, het = 29–30, VENIRKO = 26–28. *P < 0.05 between control and VENIRKO; **P < 0.05 between heterozygous and VENIRKO.
The effect of changes in these vasoactive mediators is complex (34-36). A reduction in eNOS expression would be expected to be associated with an increase in vascular tone and BP. Indeed, a small increase in BP is observed in the eNOS knockout mouse (37). Decreased expression of ET-1, on the other hand, would be expected to increase relaxation of vascular tone and leads to a decrease in BP. Heterozygous inactivation of the ET-1 gene, however, results in hypertension in mice (38), due to an attenuation of parasympathetic activity. Thus, a disruption of the equilibrium between ET-1 and eNOS could lead to either hypertension or hypotension (36). VENIRKO mice have slightly lower BP than control mice and respond normally to high- and low-salt diets, probably as a result of the change in balance of eNOS and ET-1 expression that is critical for maintenance of BP in basal state. To further assess the microvascular function in VENIRKO mice, the response to the iontophoresis of acetylcholine (endothelium dependent) and sodium nitroprusside (endothelium independent) could be done.

Another proposed function of the endothelial IR is facilitation of transport of insulin (12). Our results reveal no alteration in the half-time for insulin stimulation of glucose turnover in VENIRKO mice. Many (10, 39, 40), but not all (25, 41), studies suggest the signal to suppress hepatic glucose production is generated, at least in part, indirectly by insulin action in the periphery, supporting the hypothesis that insulin transport across peripheral endothelium is a critical element determining the kinetics of insulin action in all major insulin-sensitive tissues (39). Moreover, the fact that GIR achieved in this study markedly exceeds basal glucose turnover (41) indicated that insulin crossed capillary endothelium and stimulated glucose uptake and use in peripheral tissues. Thus, in agreement with modeling studies (11), our results do not support a rate-limiting role for the IR in vivo in the transport of insulin across the endothelial barrier in stimulating glucose turnover. Our findings do not, however, exclude the possibility that the IR plays a role in transport of insulin at low insulin concentration or that the IR is a component of insulin transport through the endothelial cells mediating rate-limiting actions into other selected tissues, such as crossing the blood-brain barrier (42).

Despite the absence of IR on endothelial cells, glucose metabolism is comparable in control and VENIRKO mice. While this result would also suggest that insulin action is not critical for endothelial transport in muscle and fat, it is important to recall that glucose toler-
ance in the muscle- or fat-specific IR knockout mice is normal (20, 43). Thus a selective impairment of insulin action at these tissues may not be sufficient to cause diabetes. On the other hand, significant alterations in glucose tolerance are observed in mice with a liver-specific IR knockout (25) or a knockout of Glut4 in muscle (44) or fat (45). The difference in severity of glucose intolerance between the muscle IR and muscle Glut4 knockouts could also be consistent with the suggestion that insulin increases glucose uptake in muscle indirectly by producing vasodilatation (13) or by generating NO and cGMP that can stimulate glucose uptake by the myocytes (18, 19). Again, however, the finding of normal glucose tolerance in the VENIRKO mice indicates that these are not major mechanisms in control of blood glucose in vivo.

Serum triglyceride levels in VENIRKO mice are lower than control at 2 months of age and remain stable at 6 months, while control mice show a decrease in triglyceride concentration between 2 and 6 months. Free fatty acid concentrations in VENIRKO mice also tend to be reduced at 2 and 6 months, although this is not statistically significant. Clearance of triglyceride from the blood stream requires the action of lipoprotein lipase (LPL) on chylomicrons and very low-density lipoproteins at the luminal surface of the vascular endothelium. The expression of LPL in myocytes and its translocation to the luminal surface of the endothelial cell are both insulin-related processes (46). In this context, VENIRKO mice should have shown reduced LPL activity in endothelium and have higher triglyceride levels. Since they did not, it is possible that the expression of LPL in adipocytes or myocytes was enhanced and compensated for the LPL reduction in endothelial cells, that there is another LPL activation pathway in endothelial cells in an insulin-independent manner, or that endogenous production of triglyceride from liver and adipocyte might be reduced. Further investigation is necessary to address these issues.

To further explore the relationship between insulin sensitivity and vascular function, we challenged the mice with low- and high-salt diets. High-salt diets have been shown to produce insulin resistance, especially in those who respond with a marked increase in arterial pressure. As expected, insulin sensitivity in control mice tends to deteriorate from the low- to the high-salt diet. In contrast, VENIRKO mice show insulin resistance on a low-salt diet that remains unchanged when mice are shifted to a high-salt diet, suggesting that insulin signaling in endothelial cells may interact with regulatory mechanisms activated under low-salt diet conditions, such as the renin-angiotensin system, and regulate whole-body insulin sensitivity (47–49).

In conclusion, the endothelial cell IR knockout mouse shows normal glucose homeostasis and normal vascular development, but altered ET-1 and eNOS expression and modest alteration in regulation of BP and insulin sensitivity on a low-salt diet. The existence of counter-regulatory mechanisms may compensate for the deficiency of insulin effect, or insulin's role may be dispensable in metabolism and cardiovascular function.
Acknowledgments

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20. Bruning, J.C., et al. 1998. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without alter-