Tumor Necrosis Factor-α Induces Endothelial Dysfunction in the Prediabetic Metabolic Syndrome

Andrea Picchi,* Xue Gao,* Souad Belmadani,* Barry J. Potter, Marta Focardi, William M. Chilian, Cuihua Zhang

Abstract—Inflammation is a condition that underscores many cardiovascular pathologies including endothelial dysfunction, but no link is yet established between the vascular pathology of the metabolic syndrome with a particular inflammatory cytokine. We hypothesized that impairments in coronary endothelial function in the obese condition the prediabetic metabolic syndrome is caused by TNF-α overexpression. To test this, we measured endothelium-dependent (acetylcholine) and -independent vasodilation (sodium nitroprusside) of isolated, pressurized coronary small arteries from lean control and Zucker obese fatty (ZOF, a model of prediabetic metabolic syndrome) rats. In ZOF rats, dilation to ACh was blunted compared with lean rats, but sodium nitroprusside–induced dilation was comparable. Superoxide (O$_2^-$) generation was elevated in vessels from ZOF rats compared with lean rats, and administration of the O$_2^-$ scavenger TEMPOL, NAD(P)H oxidase inhibitor (apocynin), or anti–TNF-α restored endothelium-dependent dilation in the ZOF rats. Real-time PCR and Western blotting revealed that mRNA and protein of TNF-α were higher in ZOF rats than that in lean rats, whereas eNOS protein levels were reduced in the ZOF versus lean rats. Immunostaining showed that TNF-α in ZOF rat heart is localized in endothelial cells and vascular smooth muscle cells. Expression of NAD(P)H subunits p22 and p40-phox were elevated in ZOF compared with lean animals. Administration of TNF-α more than 3 days also induced expression of these NAD(P)H subunits and abrogated endothelium-dependent dilation. In conclusion, the results demonstrate the endothelial dysfunction occurring in the metabolic syndrome is the result of effects of the inflammatory cytokine TNF-α and subsequent production of O$_2^-$.

Key Words: coronary microcirculation • endothelial dysfunction • inflammation • metabolic syndrome • superoxide
Determination of Plasma TNF-α
Plasma TNF-α was measured using BIO-Plex mouse 3-plex assay (Bio-Rad Laboratories, Calif). Conjugate beads were added to the microplate to assess the level of TNF-α in plasma. TNF-α concentrations (picograms per milliliter) were automatically calculated by BIO-Plex Manager software using a standard curve derived from a recombinant cytokine standard.

Treatment With TNF-α, Apocynin, and TNF-α Neutralization
We administered TNF-α (10 μg/kg, IP, R&D Systems) to lean rats for 3 days to determine whether TNF-α can inhibit coronary arterial dilations mediated by NO. We also tested whether TNF-α-affect ed mRNA expression of NAD(P)H in lean rats. We then administered NAD(P)H oxidase inhibitor apocynin (100 mg/kg, IP for 3 days) to lean rats for 3 days to determine whether apocynin can protect the vasodilation-induced in ZOF rats and test whether apocynin affected the protein expression of TNF-α and eNOS. Acetylcholine (ACh) was used as an activator of NO-mediated vasodilation.

The neutralizing antibody to TNF-α (anti-TNF-α) used in these studies was 2E2 monoclonal antibody (2E2 Mab. 94021402, NCI Biological Resources, At). At 12 to 16 weeks of age, all rats received the neutralizing anti-TNF-α (2E2 Mab. I.P., 0.625 mg/mL per kilogram per day, 3 days, IP). The rationale for this dose of antibody was based on our estimates of TNF-α expression (in the low nanogram or picogram range); the dose of antibody was able to neutralize 10- to 100-fold this amount of TNF-α.

Functional Assessment of Isolated Small Coronary Arteries
A branch of the septal coronary artery (40 to 100 μm in diameter, ∼0.5 mm in length) was carefully dissected for in vitro study, as described previously. Vessels were then cannulated with glass micropipettes, pressurized to 60 cm H2O intraluminal pressure, and bathed in physiological salt solution (PSS). The inner diameters of coronary arteries were measured using video microscopic techniques. After developing a stable basal tone (ie, spontaneous con traction to 60% to 70% of maximal diameter), the experimental interventions were performed. The concentration–diameter relationships for ACh (0.1 μmol/L to 10 μmol/L) and sodium nitroprusside (SNP) (1 nmol/L to 1 μmol/L) were then established. The contributions of the NO pathway in these vasodilations were examined by treating the vessels with the NOS inhibitor N-monomethyl-L-arginine (L-NMMA) (10 μmol/L, 30-minute incubation).

To determine whether TNF-α was a player in a role in endothelial injury in the metabolic syndrome, endothelial-dependent and -independent dilation was assessed in coronary arteries from anti TNF-α-treated rats. To determine the role of TNF-α and O2− anion in altered vasoreactive responses in the metabolic syndrome, the above vasodilatory functions were examined in the presence of the O2− scavenger (a membrane-permeable O2− dismutase mimetic) TEMPOL (1 mmol/L, 60-minute incubation). The contributions of NAD(P)H oxidase, xanthine oxidase, and mitochondrial respiratory chain in generating O2− were assessed by treating the vessels with the NAD(P)H oxidase inhibitor apocynin (10 μmol/L), the xanthine oxidase inhibitor allopurinol (10 μmol/L), or the mitochondrial respiratory chain inhibitor rotenone (1 μmol/L) for 60-minute incubation, separately. All drugs were administered extraluminally in these functional studies.

Measurement of NO Production
NO release was measured directly by using the APOLLO 4000 (World Precision Instruments), an optically integrated multichannel free radical analyzer. NO detection was based on an electrochemical (amperometric) principle that involves the oxidation of NO on the surface of an electrode and the measurement of the subsequent (redox) current generated. Isolated coronary arteries (3 to 4 vessels per sample) were initially incubated in PSS at 37°C for 30 minutes. After this equilibration period, ACh (1 μmol/L) was administered and NO release was detected. Vehicle control studies were run in parallel with the experimental groups for comparison.

mRNA Expression of TNF-α and NAD(P)H Oxidase Subunits by Real-Time PCR
Total RNA was extracted from left ventricular coronary arteries using TRIzol reagent (Life Technologies Inc) and was processed directly to cDNA synthesis using the SuperScript III Reverse Transcriptase (Life Technologies Inc). The primers of TNF-α and NAD(P)H oxidase subunits (p22-phox, p40-phox, p-47-phox, and gp-91-phox) were designed (primer 3 software) and synthesized (Qiagen). cDNA was amplified using qRT-PCR Kit with SYBR Green (Life Technologies Inc). Data were calculated by 2−ΔΔCT method and presented as fold change of transcripts for TNF-α gene and NAD(P)H oxidase gene in ZOF rats normalized to GAPDH, compared with lean control rats (defined as 1.0-fold).

Protein Expression of TNF-α and eNOS by Western Blot Analyses
For Western blot analysis, coronary arteries (4 to 6 vessels per sample) were separately homogenized and sonicated in lysis buffer (Cellytic MT Mammalian Tissue Lysis/Extraction Reagent, Sigma). Protein concentrations were assessed with the use of BCA Protein Assay Kit (Pierce), and equal amounts of protein (40 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham). TNF-α and eNOS protein expression were detected by Western blot analysis with the use of TNF-α primary antibodies (Santa Cruz Biotechnology) and eNOS primary antibody (Abcam) in 4 groups of lean rats, ZOF rats, ZOF rats treated with anti-TNF-α (0.625 mg/mL per kilogram per day, 3 days, IP), or ZOF rats treated with apocynin (100 mg/kg per day, 3 days, IP), Horseradish peroxidase–conjugated goat anti-mouse was used as the secondary antibody (Abcam). Signals were visualized by enhanced chemiluminescence (ECL) (Amersham) and quantified by Quantity One (Bio-Rad Versadoc imaging system).

Immunohistochemical Analyses
To identify and localize proteins in sections of arteries or myocardial tissue, we used immunohistochemistry. The slides from formalin-fixed hearts were incubated with blocking solution (BSA 3% in Tris buffer), then incubated with polyclonal goat antibody against TNF-α (R&D systems) and the endothelial cell marker von Willebrand factor (ab6994, Abcam) or smooth muscle α-actin (1A4, Calbiochem) or macrophages (mouse anti rat CD68, Serotec), and then incubated with a secondary fluorescent antibody (Alexa Fluor 488 and Alexa Fluor 568, Molecular probe). Sections were finally mounted in an antifading agent (Slowfade gold with DAPI, Molecular probe) and then the slides were observed and analyzed using a fluorescence microscope (Leica microscope with a ×63 objective). For every section, a negative control (without primary antibody) was performed.

Measurement of O2−
Dihydroethidium
The production of O2− was evaluated in coronary arteries with the oxidative fluorescent dye dihydroethidium (DHE). Isolated coronary arteries (40 to 100 μm in diameter) from lean and ZOF rats were incubated in PSS containing vehicle at 37°C for 60 minutes, followed by embedding the arteries in OCT compound (Tissue-Tek) for cryostat sections. DHE fluorescence for O2− affected both endothelial and smooth muscle layers of vessels was measured in lean and ZOF rats or ZOF rats with anti–TNF-α or ZOF rats with apocynin. Images were obtained using a Nikon fluorescence microscope (605-nm long-pass filter). Control and experimental tissues were placed on the same slide and processed under the same conditions.

Electron Paramagnetic Resonance Spectroscopy
The homogenate (4 to 6 isolated small coronary arteries) was prepared as described previously. Superoxide quantification from the
electron paramagnetic resonance (EPR) spectra was determined by double integration of the peaks, with reference to a standard curve from horseradish peroxidase generation of the anion from standard solutions of hydrogen peroxide, using p-acetamidophenol as the cosubstrate, then normalized by protein concentration.

**Data Analysis**

At the end of each experiment, the vessel was relaxed with 100 μmol/L SNP to obtain its maximal diameter at 60 cm H₂O intraluminal pressure. All diameter changes in response to agonists were normalized to the vasodilation in response to 100 μmol/L SNP and expressed as a percentage of maximal dilation. All data are presented as mean±SEM. Statistical comparisons of vasomotor responses under various treatments were performed with 2-way ANOVA, and intergroup differences were tested with Bonferroni inequality. Significance was accepted at *P*<0.05.

**Results**

**Plasma Concentration, mRNA, and Protein Expression of TNF-α**

Figure 1A shows elevations of plasma concentration of TNF-α. Figure 1B (real-time PCR) shows elevations of TNF-α expression. Figure 1C (Western blotting) shows the protein expression of TNF-α in isolated coronary small arteries of lean control rats, ZOF rats, ZOF rats treated with anti-TNF-α (0.625 mg/kg per day, 3 days, IP), or ZOF rats treated with apocynin (100 mg/kg per day, 3 days, IP). The results in Figure 1 corroborate the functional results shown below.

**Cellular Source of TNF-α Expression in the Metabolic Syndrome**

Markers for endothelial cells (ECs) (von Willebrand factor), vascular smooth muscle cells (VSMCs) (α-actin), or macrophages (mouse anti-rat CD68), along with TNF-α to establish the cell type expressing the TNF-α, showed that TNF-α in rat heart was localized in endothelial cells and vascular smooth muscle cells (Figure 2). Experiments were performed without the primary antibodies to test whether or not staining specificity was related to the nonspecific binding of the secondary antibodies, which showed no staining in heart sections, indicating that the signals were attributable to specific binding of the primary antibody.

**NO-Mediated Vasodilation to ACh**

To show NO dependency of ACh dependent dilation in lean control rats, we studied responses to the agonist before and after treatment with L-NMMA. Vasodilation to ACh was NO-mediated because it was blocked by L-NMMA.

**NO Production and Protein Expression of eNOS**

The real-time NO production from isolated vessels of lean Zucker (n=5) and ZOF (n=5) rats in response to ACh was measured by electrochemical detection (NO is proportional to current). In control vessels, ACh stimulated a large increase in NO production. In ZOF vessels, ACh-stimulated NO production was diminished. The concentrations of NO normalized to protein concentration (real-time NO production, in nmol/L per milligram) were 52.33±4.8 (n=5, lean rats) and 26.37±2.19 (n=5, ZOF rats, *P*<0.05 versus lean). Western blotting shows the protein expression of eNOS (Figure 4B) in isolated coronary arteries of lean control, ZOF rats, ZOF rats treated with anti-TNF-α (0.625 mg/kg per day, 3 days, IP), or ZOF rats treated with the NAD(P)H oxidase inhibitor apocynin (100 mg/kg per day, 3 days, IP).
endothelial dysfunction in the metabolic syndrome. We tested the dose-response curve to flow-dependent dilatation (range of ΔP [that is linearly related to flow] from 4 to 60 cm H2O), a response that is endothelial dependent but agonist independent. Figure 5C shows abrogated flow-dependent dilatation in ZOF rats compared with leans.

We investigated whether TNF-α treatment increased vascular NAD(P)H oxidase activation in lean rats by administering TNF-α (10 μg/kg per day) to lean rats for 3 days and evaluating the mRNA expression of NAD(P)H oxidase subunits p22-phox, p40-phox, p47-phox, p67-phox, and gp-91-phox. The results (Figure 6) indicate that TNF-α plays a critical role in the oxidase stress occurring during the metabolic syndrome by inducing expression of NAD(P)H oxidase.

Roles of Superoxide, Xanthine Oxidase, Mitochondrial Respiratory Chain, and NAD(P)H Oxidase in the Metabolic Syndrome–Induced Vascular Dysfunction

O2⁻⁻ production is postulated to be linked to TNF-α. We hypothesized that scavenging O2⁻⁻ with TEMPOL would restore vasodilation in ZOF rats. To establish the pathway for O2⁻⁻ production, we administered the NAD(P)H oxidase inhibitor apocynin (10 μmol/L), the xanthine oxidase inhibitor allopurinol (10 μmol/L), or the mitochondrial respiratory chain inhibitor rotenone (1 μmol/L) to determine whether vasodilation to ACh would be restored in ZOF rats (Figure 7).

The Metabolic Syndrome–Induced Superoxide Production in Small Coronary Arteries Isolated From ZOF Rats

Figure 8A shows DHE fluorescence imaging of O2⁻⁻ in small coronary arteries. Setting the scanning threshold to obtain a clear background image of the blood vessel allowed identification of the smooth muscle and endothelial layers. In control conditions (nondiabetic, ie, lean Zucker rats), DHE oxidative fluorescence revealed sparse levels of O2⁻⁻ throughout the vessel wall. Production of O2⁻⁻ was significantly elevated in both endothelial and smooth muscle layers of ZOF rats compared with control (lean Zucker). Neither apocynin nor neutralizing antibodies to TNF-α affected baseline levels of artery fluorescence (not shown), but these 2 agents markedly reduced the TNF-α–induced fluorescent signals for O2⁻⁻ separately. Figure 8B shows the results from EPR spectroscopy to quantify the production of O2⁻⁻.

Discussion

Our results suggest that overexpression of TNF-α induces activation of NAD(P)H oxidase and production of O2⁻⁻, leading to endothelial dysfunction in the metabolic syndrome. Importantly, our findings support the concept that TNF-α plays a pivotal role in endothelial dysfunction in the metabolic syndrome based on the following observations. Antibody neutralization of TNF-α prevented coronary endothelial dysfunction and reduced O2⁻⁻ generation and protein expression of eNOS in ZOF rats. Blockade of NAD(P)H oxidase mimicked the actions of anti–TNF-α on O2⁻⁻ production, protein expression of TNF-α and eNOS, and endothelial function in ZOF rats. Molecular evidence indicated that the

Role of TNF-α in the Metabolic Syndrome–Induced Vascular Dysfunction and the Expression of NAD(P)H Oxidase

We administered TNF-α (10 μg/kg per day, 3 days, IP) and assessed responses of microvessels to ACh to show the chronic administration of TNF-α in lean rats could mimic the responses of vessels from ZOF rats (Figure 5A). Administration of the inflammatory cytokine abrogated endothelium-dependent dilatation, which was reversed by apocynin. We also found that administration of anti–TNF-α (2E2 monoclonal antibody) restored endothelial function in ZOF rats (Figure 5B). This observation is the cornerstone of our hypothesis that overexpression of this inflammatory cytokine produces
expression of TNF-α (mRNA and protein) and mRNA expression of NAD(P)H oxidase subunits p22-phox and p40-phox were significantly increased, but protein expression of eNOS was diminished in ZOF rats. Our findings provide further understanding of the mechanism(s) underlying endothelial dysfunction in the metabolic syndrome and the development of type 2 diabetes.

**TNF-α and the Metabolic Syndrome**

A large body of evidence shows that the metabolic syndrome is associated with inflammation. TNF-α is overexpressed in obesity and likely mediates insulin resistance in the major animal models of obesity; neutralization of TNF-α with soluble TNF-α receptors results in the restoration of insulin sensitivity and thereby demonstrates that the proinflammatory cytokine TNF-α is the mediator of insulin resistance. Studies in human show increased TNF-α, interleukin-6 (IL-6), C reactive protein (CRP), and migration inhibitory factor (MIF) plasma levels, indicating metabolic syndrome is an inflammatory state. We found increased TNF-α mRNA expression (5-fold), plasma concentration of TNF-α (8-fold), and protein expression of TNF-α (more than 3-fold in small coronary arteries) in ZOF rats. If TNF-α is, as we and others suspect, 1 of the initiators of a cytokine cascade, then it may also be 1 of the genes that is induced very quickly following perturbation. Our immunostaining results show TNF-α in ZOF rat heart is localized in ECs and vascular smooth muscle cells. We believe O₂⁻ production in vascular
endothelial cells and smooth muscle cells by TNF-α would limit NO bioavailability and reduce NO-dependent dilation. Finally, TNF-α can be “stored” within cells in a “pre” form, and the enzyme TNF-α converting enzyme13 can rapidly increase levels of the active cytokine.

Endothelial Dysfunction in the Metabolic Syndrome

In the present study, endothelium-dependent vasodilation and NO production was attenuated in small coronary arteries from ZOF versus control lean rats. Although in the functional studies, basal tone tended to be lower in the vessels isolated from ZOF rats versus those from lean rats, it did not reach statistical significance. The present results provide the first direct evidence that the metabolic syndrome is associated with impaired agonist-induced NO production and NO-mediated dilation in the coronary microcirculation.

There is no previous report on the endogenous role of TNF-α in this respect, and it has been unclear whether TNF-α plays a direct role in endothelial dysfunction in the metabolic syndrome. Recent evidence supports the idea that the effect of TNF-α is NO dependent by producing a rapid inhibitory action on NO synthase (NOS) in the endothelium via activation of a sphingomyelinase/ceramide signaling pathway; this mechanism is supposed to mediate the action of TNF-α, thereby contributing to vascular endothelial dysfunction in coronary circulation under different pathological conditions with increased cytokines.5,10,14 In the present study, we documented that TNF-α is critical for the development of endothelial dysfunction in the model of the metabolic syndrome. TNF-α expression was significantly increased in ZOF rats; anti-TNF-α restored NO-mediated coronary arterial dilation in ZOF rats but did not affect the endothelium-

Figure 5. ACh-induced dilation was significantly blunted by 3 days treatment with TNF-α (10 μg/kg, n=5), but 3 days treatment of anti-TNF-α (0.625 mg/kg, n=6) protected endothelial dysfunction induced by TNF-α in lean control rats. Three days of treatment with the NAD(P)H oxidase inhibitor apocynin (100 mg/kg) restored the impaired endothelial function induced by TNF-α in vessels isolated from lean rats (n=5) (A). Anti-TNF-α restored NO-mediated coronary arterial dilation in ZOF rats but did not affect the vasodilation in lean control rats (B). Flow-dependent dilation in ZOF rats was also decreased relative to lean controls (C). *P<0.05, lean rats vs ZOF rats; #P<0.05, lean rats+TNF-α vs lean rats+TNF-α+anti-TNF-α; ##P<0.05, lean rats+TNF-α vs lean rats+TNF-α+apocynin.

Figure 6. Real-time PCR shows mRNA expression of NAD(P)H oxidase from left ventricular coronary arteries in lean control and ZOF rats. Bars represent the increased expression in ZOF rats as a fold change of lean control. mRNA expression for NAD(P)H oxidase p22-phox and p40-phox is higher in ZOF rats than in lean control rats, and treatment by TNF-α (10 μg/kg, 3 days, IP) significantly increased the mRNA expression of NAD(P)H oxidase subunits p22-phox and p40-phox in lean rats. Data are shown as mean±SD, n=4. *P<0.05 vs lean rats.
dependent vasodilation in lean controls. Furthermore, administration of anti–TNF-α protected endothelial dysfunction induced by TNF-α in isolated vessels from lean control rats, confirming that the monoclonal anti–TNF-α was specific.

Impaired endothelium-dependent vasodilation was restored in ZOF rats after the treatment with TNF-α antibodies for 3 days (IP), and 3 days treatment with TNF-α (IP) mimicked endothelial dysfunction in lean rats, both demonstrating the pivotal role of this inflammatory cytokine in the vascular pathology of the metabolic syndrome. This result is in agreement with those of previous studies showing that TNF-α can decrease the release of endothelial NO and induce impairment of endothelium-dependent relaxation in a variety of vascular beds.

Our results also provide insight into the basis for the endothelial dysfunction induced by the metabolic syndrome, namely oxidative stress. O$_2^-$ scavenging by TEMPOL improved endothelium-dependent vasodilation in ZOF rats. Anti–TNF-α or apocynin attenuated the production of O$_2^-$ in ZOF rats, thereby confirming the critical role of O$_2^-$ in the endothelial dysfunction occurring in the metabolic syndrome. These data are consistent with data from previous studies demonstrating that vessels from patients with diabetes generate significantly more O$_2^-$ by increasing the activity of NAD(P)H oxidase. Furthermore, the studies affirmed that the endothelium itself is a net contributor to total vascular O$_2^-$ release. The link between TNF-α overexpression and O$_2^-$ production has been investigated by Zhang et al., who found that tiron, a cell-permeable O$_2^-$ scavenger, and polyethylene glycol–conjugated superoxide dismutase (PEG-SOD) prevented TNF-α–induced impairment of endothelium-dependent vasorelaxation in coronary arterioles. Our results are consistent with these studies and support our hypotheses that TNF-α and O$_2^-$ are connected in the production of oxidative stress and endothelial dysfunction in the metabolic syndrome.

Figure 7. Incubation of coronary arteries with the O$_2^-$ scavenger TEMPOL (1 mmol/L, n=7) (A) or the NAD(P)H oxidase inhibitor apocynin (10 μmol/L, n=8) (B) restored vasodilation in ZOF rats, but the xanthine oxidase inhibitor allopurinol (10 μmol/L, n=5) or oxypurinol (100 μmol/L, n=4) or the mitochondrial respiratory chain inhibitor rotenone (1 μmol/L, n=4) did not (data not shown).

Furthermore, incubation with TEMPOL (A) (n=7), apocynin (B) (n=8), allopurinol (data not shown, n=4), oxypurinol (data not shown, n=4), or rotenone (data not shown, n=4) did not alter the vasodilatory responses to ACh in lean controls. *P<0.05 vs lean rats.

Figure 8. DHE fluorescence (A) was markedly elevated in both endothelial (arrow head) and vascular smooth muscle (arrow) cells of arterial sections in ZOF rats (n=4) compared with lean rats (control). Both anti–TNF-α and apocynin significantly reduced the fluorescent signals in ZOF rats. However, neither apocynin nor anti–TNF-α markedly reduced the fluorescent signals (n=4). Data shown are representative from 4 separate experiments. B shows the results from EPR spectroscopy to quantify the production of O$_2^-$.

O$_2^-$ production was higher in isolated coronary arteries from ZOF rats vs lean rats (P<0.05). Administration of anti–TNF-α or apocynin reduced the production of O$_2^-$ to the level observed in the lean controls. n=6 (B). *P<0.05 vs lean rats; #P<0.05 vs ZOF rats.
Effect of eNOS on TNF-α–Induced Endothelial Dysfunction in the Metabolic Syndrome

The vascular endothelium is a major target for the actions of TNF-α. TNF-α can decrease the release of endothelial NO and induce impairment of endothelium-dependent vasodilation in a variety of vascular beds; however, the signaling pathways that couple TNF-α stimulation to endothelial dysfunction in small coronary arteries remain to be understood. Zhang et al demonstrated that TNF-α inhibited NO-mediated, endothelium-dependent vasorelaxation in coronary arterioles by activating sphingomyelinase and enhancing O2·− generation in coronary endothelial cells. In the presence of L-NMMA, TNF-α had no further inhibitory effect on the responses to endothelium-dependent vasodilators, suggesting that the effect of TNF-α is NO dependent.

TNF-α is known to affect eNOS expression by affecting the half-life of its mRNA. We show ZOF rats generate little or no NO. The protein expression of eNOS is significantly attenuated in isolated arteries in ZOF rats compared with the lean animals. We know that TNF-α impairs endothelial function by reducing NO bioavailability, but little information is available in the literature on how TNF-α affects endothelial NOS (eNOS) activity in coronary arteries. Although there appears to be an inconsistency between NO-mediated dilation and eNOS protein, ie, restoration of dilation when eNOS protein is still decreased, we believe there are some factors that complicate this reasoning. Not all eNOS is active: some is bound to caveolin, and, secondly, some eNOS may be dysfunctional because of limitations in BH4 or other cofactors. The total amount of eNOS may not reflect the total production of NO and the ensuing dilation because there may not be a simple match of eNOS protein with NO-mediated dilation.

The Major Source of O2·− Production in the Metabolic Syndrome

Although there are multiple intracellular sources for formation of oxygen free radicals (eg, mitochondria, xanthine oxidase, NAD(P)H oxidase, etc), our results support the idea that the major enzyme activated by TNF-α in the metabolic syndrome is NAD(P)H oxidase. We can state with conviction that the major source of O2·− was NAD(P)H oxidase in the metabolic syndrome because NAD(P)H oxidase inhibitor apocynin significantly reduced O2·− production, as measured by DHE fluorescence and EPR. Moreover, the antagonism of NAD(P)H oxidase virtually normalized endothelium-dependent vasodilatation. Our results indicate that the pathway for TNF-α–induced endothelial dysfunction is mediated by activation of NAD(P)H oxidase and the subsequent production of O2·−. Guzik et al have also shown that activity and protein levels of the vascular protein NAD(P)H oxidase system are increased in diabetic vessels and that the significantly increased O2·− production in human blood vessels from patients with diabetes is mediated by upregulated NAD(P)H oxidase activity and by a striking increase in endothelial O2·− production mediated by eNOS uncoupling. To the contrary, we did not find any improvement in endothelial function after incubation with rotenone and allopurinol, suggesting that mitochondria and the xanthine oxidase system are not likely to be the source of O2·− production. Guzik et al measured O2·− production in diabetic and nondiabetic vessels in response to a range of potential oxidase inhibitors. Their results showed that oxypurinol and rotenone had minimal or modest effect on O2·− production, whereas diphenylene iodium, an inhibitor of flavin-containing oxidases such as NAD(P)H oxidases, abolished O2·− production. Our results demonstrate the production of TNF-α is essential in eliciting this oxidative stress.

Guzik et al reported that the protein expression of NAD(P)H oxidase subunits p22, p47, and p67-phox are significantly increased in diabetic human tissue versus normal control. Our real-time PCR results indicate that mRNA expression of NAD(P)H oxidase subunit p22-phox and p40-phox are increased ~2-fold in ZOF rats versus lean rats. This supports our functional results, in which the experiments with apocynin suggest that NAD(P)H oxidase might be a major enzymatic vascular source of reactive oxidative species (ROS) in ZOF rats. Most interestingly, the TNF-α treatment also increases the mRNA expression of vascular NAD(P)H oxidase subunits p22-phox and p40-phox in the control animals. Further studies are needed to determine the mechanism linking TNF-α receptor activation to NAD(P)H oxidase.

The present study indicates that the model of the metabolic syndrome increases TNF-α, which stimulates endothelial generation of O2·− through activation of NAD(P)H oxidase in the endothelium and contributes to the endothelial dysfunction. To our knowledge, this is the first functional study to link the mechanism(s) of the model of the metabolic syndrome—in terms of endothelial dysfunction—to TNF-α, the subsequent activation of NAD(P)H oxidase, and thus the production of O2·− in coronary artery endothelium. Taken together, these results further support our hypothesis that TNF-α and O2·− are connected in the production of oxidative stress and endothelial dysfunction in the metabolic syndrome. In conclusion, we found that TNF-α overexpression impairs endothelium-dependent vasodilation in coronary arterioles of rats affected by the model of the metabolic syndrome. The impaired endothelial function can be restored toward normal by administration of TNF-α antibodies. The mechanism by which TNF-α affects endothelial function seems to be an increased superoxide production by NAD(P)H oxidases, which, in turn, leads to a reduced NO bioactivity by direct scavenging. These results confirmed that inflammatory cytokines play a pivotal role in the vascular pathology of the metabolic syndrome and provide new findings into the understanding of interactions among inflammation, diabetes, and atherosclerosis. These findings may provide further insight into a novel therapeutic target for cardiovascular diseases associated with elevated levels of TNF-α.

Acknowledgments

We appreciate the support of Systems Biology & Translational Medicine at Texas A&M University after Hurricane Katrina, specifically, Drs Harris Granger, David Zawieja, and Lih Kuo, for unconditional help. Also, Drs Gregory J. Bagby, Johnny Porter, and Marvin K. Harris provided expert assistance.

Sources of Funding

This study was supported by grants from research funds established by Dr Harris Granger at Texas A&M University; American Heart

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Association Grant-in-Aid 0455435B; Atorvastatin Research award 2004-37; American Heart Association Scientist Development Grant 110350047A (to C.Z.); NIH Centers of Biomedical Research Excellence grant P20 RR18766 (to Dr Stephen Lanier); and NIH grants HL32788 and HL65203 (to W.M.C.).

Disclosures
None.

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Circ Res. published online June 1, 2006;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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