Diabetes-Induced Oxidative Stress and Low-Grade Inflammation in Porcine Coronary Arteries

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Background—Multiple pathways contribute to accelerated coronary atherosclerosis in diabetics, including increased oxidative stress and inflammatory burden. Accordingly, the mechanisms of abnormal formation of reactive oxygen species and the changes in inflammatory gene expression were examined in diabetic coronary arteries.

Methods and Results—In pigs with streptozotocin-induced diabetes, superoxide formation was augmented in coronary media and adventitia because of increased NAD(P)H oxidase activity (3 months) accompanied by upregulated expression of its cytosolic subunit, p22phox. Diabetes-induced oxidative stress resulted in the inflammatory response in the adventitia (increased expression of interleukin-6, tumor necrosis factor-α, monocyte chemotactic protein-1, vascular cell adhesion molecule-1 [VCAM-1]) and in the media (VCAM-1). To examine the mechanisms of these changes, studies with isolated coronary fibroblasts were undertaken. Advanced glycation end products (AGEs), rather than glucose itself, upregulated expression of interleukin-6, VCAM-1, and monocyte chemotactic protein-1 mRNAs. These results were paralleled by increased interleukin-6 secretion (P<0.01) and augmented leukocyte adhesion to AGE-stimulated coronary cells (P<0.001). AGEs increased expression of phosphorylated forms of mitogen-activated protein kinases in coronary cells (ERK1/2 and JNK) and resulted in redox-sensitive expression of inflammatory genes that was inhibited by several inhibitors of oxidative pathways [NAD(P)H oxidase inhibitors, N-acetylcysteine, and pyrrolidine dithiocarbamate].

Conclusions—Diabetes increased NAD(P)H oxidase activity and oxidative stress, producing inflammatory responses in porcine coronary media and adventitia. AGEs activated ERK1/2 and JNK signaling pathways and induced the expression of several inflammatory genes in coronary cells in a redox-sensitive manner. These results suggest the involvement of AGEs in the development of accelerated coronary atherosclerosis in diabetes. (Circulation. 2003;108:472-478.)

Key Words: diabetes mellitus ■ inflammation ■ atherosclerosis

The rising prevalence of diabetes mellitus and its adverse impact on cardiovascular mortality and morbidity represents a major challenge in contemporary medicine1-2 (reviewed in Beckman et al2). Despite declining overall mortality from coronary heart disease in the United States, diabetics exhibited a smaller survival benefit, with diabetic women even experiencing increased mortality.3 Not only is diabetes associated with increased mortality in those without cardiovascular symptoms.4-6 Several characteristics of type 2 diabetes, including hypertension and central obesity, may promote macrovascular complications of this disease.7 On the cellular level, hyperglycemia, hyperinsulinemia, and proatherogenic dyslipidemia (marked by elevated levels of small, dense LDL particles and low HDL cholesterol) all contribute to complex vascular interactions.8-10

The presence of impaired glucose tolerance and a cluster of several risk factors confers higher cardiovascular risk long before diabetes is recognized clinically. Nevertheless, the role of tight glycemic control in the prevention of macrovascular complications of disease remains the subject of debate.11 These seemingly contradictory findings underscore the complexity of biochemical changes in diabetes. They include irreversible formation of advanced glycation end products (AGEs), which involves the most essential elements of the arterial wall (proteins, lipids, and nucleic acids).12 Increased AGEs and activation of the specific receptors (RAGE) on endothelial cells increases oxidative stress, reduces availability of nitric oxide, and activates protein kinase C, eventually
disrupting cellular functions. The AGE-RAGE dyad contributes to the process of endothelial activation and redox-sensitive expression of adhesive molecules, resulting in blood-borne inflammatory cells homing in disease-prone segments of the vasculature.\textsuperscript{13,14} Less is known concerning the impact of early diabetes-related changes on nonendothelial constituents of the arterial wall. Several studies have shown that poorly differentiated fibroblasts of the adventitia regulate the vascular redox state in response to several stimuli, thus contributing to cellular cross talk and altered vascular homeostasis.\textsuperscript{15–17} Because both increased oxidative stress and low-grade inflammation are hallmarks of diabetes, we have examined these responses in a porcine model of diabetes.\textsuperscript{18,19} The results show enhanced activity of NAD(P)H oxidase activity in coronary media and adventitia, which was associated with increased inflammatory gene expression. We also provide evidence that AGES are responsible for the activation of a broad panel of inflammatory mediators in a redox-sensitive manner in coronary cells. These results provide additional rationale for novel therapeutic strategies to address the deleterious effects of AGES in the coronary vasculature.

Methods

Animal Model

Domestic crossbred female pigs were used (weight 20 to 25 kg). Diabetes was induced by intravenous injection of streptozotocin (Biomol Research Laboratories, 150 mg/kg), whereas age-matched nondiabetic animals served as controls. Plasma glucose levels were measured before and 16 hours after drug administration and monitored every 2 weeks thereafter. Animals that failed to raise plasma glucose >250 mg/dL were excluded from the study. If the plasma glucose level was >500 mg/dL, insulin (4 to 8 U) was given subcutaneously each day to maintain glucose levels <500 mg/dL.

Superoxide Production and NAD(P)H Oxidase Activity

Superoxide anion (·O\textsubscript{2}\textsuperscript{-}) in coronary tissues was measured at 3 months after induction of diabetes by superoxide dismutase (SOD)-inhibitable conversion of nitroblue tetrazolium (NBT) to formazan.\textsuperscript{19} SOD-inhibitable NBT reduction was calculated as a measure of ·O\textsubscript{2}\textsuperscript{-} production (pmol · min\textsuperscript{-1} · mg wet weight\textsuperscript{-1}). To determine the enzymatic source of ·O\textsubscript{2}\textsuperscript{-} production, vascular tissues were preincubated with different inhibitors of oxidant enzymes for 30 minutes, followed by addition of NBT. The n value represents the number of vascular segments.

NAD(P)H oxidase activity in coronary tissues was measured by SOD-inhibitable cytochrome C reduction with NADH or NADPH as substrates.\textsuperscript{19} The activity of NAD(P)H oxidase was calculated as SOD-inhibitable cytochrome C reduction and expressed in nanomoles of ·O\textsubscript{2}\textsuperscript{-} per milligram per minute. The n value represents number of vessels.

Real-Time Reverse Transcription–Polymerase Chain Reaction

Gene expression was assessed by real-time reverse transcription–polymerase chain reaction (RT-PCR) at 3 and 6 months after induction of diabetes. Total RNA was isolated from tissues or cells with TRI reagent (Molecular Research Center). Gene transcripts were amplified in a separate tube to normalize variances in input RNA. The level of target mRNA in various samples was estimated by the relative standard method with a series of dilutions of RNA from porcine vascular cells or leukocytes.

Cell Isolation and Culture

Fibroblasts were isolated from coronary adventitia as described previously.\textsuperscript{20} The cells were grown in DMEM with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and glutamine 2 mmol · L\textsuperscript{-1} · mL\textsuperscript{-1} at 37°C in a humidified incubator with 5% CO\textsubscript{2}.

Adhesion Assay

The adhesion of freshly isolated porcine leukocytes onto the surface of coronary fibroblasts was examined. Porcine coronary fibroblasts (passages 3 to 8) were grown on a 96-well plate until ~90% confluence. Cells were then arrested in 0.5% FBS for 48 hours, followed by stimulation with glycated bovine albumin (AGE-BSA, AGE content 348.8 U/mg; Sigma) for 6 hours. Nonglycated bovine albumin (Con-BSA, AGE content 0.133 U/mg) was used as a control. After removal of AGE-BSA or Con-BSA, porcine leukocytes labeled with calcine-AM (Molecular Laboratory) were added to each well for 45 minutes (2·10\textsuperscript{5}/well). Nonadherent cells were

![Figure 1.](http://circ.ahajournals.org/)

**Figure 1.** Oxidative stress in control and diabetic coronary arteries. Superoxide production was measured in coronary media and adventitia by SOD-inhibitable NBT reduction at 3 months after induction of diabetes. Nondiabetic animals matched for age and sex were used as controls (n=3). DM indicates diabetes mellitus.

![Figure 2.](http://circ.ahajournals.org/)

**Figure 2.** Expression of NAD(P)H oxidase subunits in control and diabetic coronary arteries. Coronary media and adventitia were isolated at 3 months after induction of diabetes. Transcripts of NAD(P)H oxidase subunits were evaluated by real-time RT-PCR with primers and probes recognizing porcine sequences. Amounts of gene transcript were normalized by GAPDH mRNA (n=5 to 9 per bar). DM indicates diabetes mellitus.
removed by washing with PBS. Fluorescence intensity was measured before and after washing in a fluorescence multiwell plate reader (Cytofluor II, PerSeptive Biosystem).

Western Blot
AGE-stimulated coronary fibroblasts were washed with ice-cold phosphate buffer and lysed in cell lysis buffer (20 mmol/L MOPS [pH 7.0], 2 mmol/L EGTA, 5 mmol/L EDTA, 30 mmol/L sodium fluoride, 40 mmol/L β-glycerophosphate [pH 7.2], 10 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate, 5 mmol/L benzamidine, 5 μmol/L pepstatin A, 10 μmol/L leupeptin, and 0.5% Nonidet P-40). After being boiled for 5 minutes, equal amounts of protein (30 μg) were run on 10% SDS-PAGE. Proteins were transferred to a PVDF membrane. After being blotted with 5% fat-free milk in TBS-T, the blots were incubated overnight with primary antibodies (1:200; Cell Signaling Technology). After being washed, the blots were incubated with biotin-labeled secondary antibody, followed by incubation with avidin–horseradish peroxidase conjugate (Bio-Rad) for 30 minutes. An enhanced chemiluminescence kit (Perkin-Elmer) was used for detection.

Statistical Analyses
Data are expressed as mean±SEM. Student’s t test was used for 2-group comparisons. Statistical significance regarding multigroup comparisons was determined by ANOVA with Bonferroni correction. A value of P<0.05 was considered significant.

Results
Superoxide, NAD(P)H Oxidase, and Inflammation in Diabetic Coronary Arteries
Oxidative stress was measured by SOD-inhibitable NBT reduction at 3 months after induction of diabetes. Although normal coronary adventitia of control animals exhibited higher basal levels of -O2· than did media, both coronary layers of diabetic pigs showed significant increases in -O2· production in diabetic pigs (Figure 1). An inhibitor of NAD(P)H oxidase, diphenyliodonium (0.1 mmol/L), abolished -O2· generation in the media and adventitia, whereas inhibitors of xanthine oxidase (oxypurinol) and mitochondrial dehydrogenase (rotenone) showed no effects (data not shown). Consistent with the above results, significantly higher levels of NAD(P)H oxidase activity were observed in diabetic coronary arteries than in nondiabetic vessels (33.1±5.7 versus 9.2±3.8 pmol · mg⁻¹ · min⁻¹; n=3; P<0.05).

To further elucidate the contribution of NAD(P)H oxidase, its subunit expression was examined in control and diabetic animals. As shown in Figure 2, p22(phox) mRNA was upregulated in both media and adventitia, whereas the changes in gp91(phox) and p47(phox) were insignificant at 3 months of diabetes (n=5 to 9). Because reduced levels of endogenous antioxidants may contribute to increased oxidative stress, the activities of SOD and catalase were also determined. No significant changes in SOD or catalase activity were seen in diabetic coronary arteries (data not shown), which suggests that depletion of endogenous antioxidants did not contribute to the observed changes in oxidative stress.

To examine the downstream effects of increased oxidative stress, the expression of several inflammatory mediators was examined in diabetic coronary arteries at 3 and 6 months. As shown in the Table, diabetes significantly upregulated interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and vascular cell adhesion molecule-1 (VCAM-1) mRNAs in coronary adventitia (n=5 to 9; P<0.05 versus nondiabetic controls), whereas coronary media showed significant changes only in VCAM-1 expression.

![Figure 3](http://circ.ahajournals.org/lookup/suppl/doi:10.1161/01.CIR.0000134955.08761.C7/-/media/doi/01.CIR.0000134955.08761.C7/FIG3.JPG)

**Figure 3.** Effects of AGEs on inflammatory responses in coronary cells. Subconfluent coronary fibroblasts were arrested for 48 hours, followed by incubation with AGE-BSA or BSA for 4 hours. Transcripts of inflammatory mediators were measured by real-time RT-PCR and normalized by GAPDH mRNA(n=4/condition). Similar results were obtained in 3 separate experiments with different AGE-BSA and respective BSA. Control indicates BSA; AGE, AGE-BSA.
Effects of AGEs on Inflammatory Responses of Coronary Cells

To discern the mechanisms responsible for the upregulation of inflammatory responses in coronary cells, glucose and AGE-BSA were used. Incubation of coronary fibroblasts with glucose (25 to 50 mmol/L) for up to 7 days produced no increase in inflammatory mediators (data not shown). In contrast, AGE-BSA significantly augmented expression of IL-6, VCAM-1, and MCP-1 mRNAs (Figure 3; n=4/condition). Similar results were obtained in 3 separate experiments with AGE-BSA from 2 different sources (Sigma and Dr Vlassara). No increase was seen in TNF-α transcripts (data not shown). To further confirm that AGEs augment inflammatory mediators in coronary cells, the release of IL-6 protein from AGE-stimulated cells was measured with a porcine ELISA kit (R&D Systems). As expected, stimulation of coronary fibroblasts with AGE-BSA (400 μg/mL) increased IL-6 in conditioned medium compared with BSA-treated cells (38.12±4.07 versus 21.53±0.57 ng/10^5 cells; n=5/group; P<0.01). In addition, we have reasoned that increased VCAM-1 expression should result in enhanced adhesion of leukocytes to AGE-stimulated coronary fibroblasts. Incubation of coronary fibroblasts with AGEs (400 μg/mL) for 6 hours significantly enhanced leukocyte adhesion compared with cells incubated with BSA (11.4±1% versus 6.8±1%; n=21/group; P<0.001).

The activation of intracellular signal transduction pathways is necessary for mounting the observed inflammatory responses. Accordingly, coronary fibroblasts were stimulated with AGEs, and the expression of ERK1/2, JNK, and p38 mitogen-activated protein kinases was examined by Western blot. As shown in Figure 4, there was a rapid increase in phosphorylated forms of ERK1/2 and JNK, whereas p38 mitogen-activated protein kinase showed no change. The inhibitors of JNK, but not ERK, and the inhibitor of the downstream transcriptional factor, nuclear factor-κB (NF-κB), significantly attenuated AGE-induced changes in IL-6, MCP-1, and VCAM-1 (Figure 5). These results indicate that AGE-stimulated inflammatory gene expression involves functional activation of the JNK signaling pathway and the transcriptional factor NF-κB in coronary fibroblasts.

Redox-Sensitive Inflammatory Gene Expression in AGE-Stimulated Coronary Cells

To establish a causal relationship between AGE-induced oxidative stress and the above inflammatory responses, we examined whether antioxidants could interfere with inflam-
matory gene expression. Accordingly, cells were pretreated with various antioxidants for 1 hour, followed by stimulation with AGEs for additional 3 hours. The expression of inflammatory mediators was measured, and the inhibitory effects of antioxidants are shown in Figure 6. NAD(P)H oxidase inhibitors (diphenyliodonium and apocynin) and antioxidants (pyrroline dithiocarbamate and N-acetylcycteine) significantly attenuated AGE-induced expression of inflammatory mediators. In contrast, SOD, catalase, and N\textsuperscript{2}-nitro-L-arginine methyl ester showed no inhibitory effects (data not shown). These results indicate that AGEs induce redox-dependent changes in inflammatory gene expression in coronary vascular cells.

Discussion

The major findings of this study are as follows: (1) Diabetes increases oxidative stress in porcine coronary arteries because of augmented activity of NAD(P)H oxidase (increased enzymatic activity and expression of p22\textsuperscript{phox}); (2) An enhanced redox state is accompanied by the upregulation of inflammatory cytokines (IL-6 and TNF-\(\alpha\)), chemokines (MCP-1), and adhesive molecules (VCAM-1); (3) Studies in isolated coronary cells suggest that AGEs, rather than hyperglycemia per se, are responsible for redox-sensitive inflammatory responses in coronary adventitial cells.

Role of AGEs in Regulation of Vascular Homeostasis

Among multiple pathways that link oxidative stress and inflammation, AGE and RAGE interactions have been particularly noteworthy because they accumulate in diabetic vasculature and may contribute to accelerated atherosclerosis.\textsuperscript{21,22} In addition, oxidative stress itself contributes to the formation of AGEs that, on interacting with their receptor, produce further upregulation of free radicals and result in altered gene expression.\textsuperscript{3,4} The increased NAD(P)H oxidase activity and vascular inflammation observed in the present study raised the question as to the mechanisms and localization of altered gene expression in diabetic coronary arteries.\textsuperscript{25} To eliminate the possibility that the influx of blood-borne inflammatory cells into diabetic vessels augmented the redox state, we examined isolated coronary fibroblasts in vitro. AGEs induced phosphorylation of 2 signal transduction kinases (ERK1/2 and JNK) and produced inflammatory responses in adventitial cells that were attenuated by inhibitors of NAD(P)H oxidase or other antioxidants. These findings support the possibility of vascular protection with interventions that interfere with AGE signaling (eg, proximal or distal to its receptor). In fact, soluble RAGE, which prevents AGE-mediated signaling, has been shown to inhibit vascular inflammation and lesion formation in diabetic apolipoprotein E-deficient mice.\textsuperscript{26,27}

Adventitia and Vascular Inflammation

Although the induction of oxidative stress and inflammatory responses were seen in both coronary media and adventitia, the outer layer was more prone to diabetes-induced inflammatory responses (Table). These observations are consistent with the phenotypic plasticity of adventitial fibroblasts.\textsuperscript{28} Advanced differentiation and high levels of homeostatic gene expression of coronary smooth muscle cells may account for lower inflammatory responses under diabetic conditions.\textsuperscript{20,29} Adventitial release of cytokines and chemokines may result in cellular cross talk, as evidenced by intimal lesion formation after perivascular application of IL-\(1\beta\) or MCP-1.\textsuperscript{30,31} In human atherosclerosis, adhesive molecules (VCAM-1 and intercellular adhesion molecule) are highly expressed around adventitial vasa vasorum, which suggests additional vascular portals for inflammatory cells.\textsuperscript{32} In fact, several inflammatory cells (lymphocytes and mast cells) have been noted in the adventitia after fatal acute coronary syndromes or intractable vasospasm.\textsuperscript{33,35} Thus, the observed adventitial inflammation (including expression of chemokine and adhesive molecules) produced by AGE-activated resident fibroblasts may contribute to the retention of
blood-borne cells, resulting in “pan-arteritis” and increased susceptibility of diabetes to atherosclerosis.

**Study Limitations and Clinical Implications**

The major limitation of this study is that a porcine model of diabetes does not capture a full spectrum of the metabolic abnormalities seen in metabolic syndrome and type 2 diabetes that are major contributors to cardiovascular morbidity and mortality. Nonetheless, it is noteworthy that the metabolic syndrome and diabetes are accompanied by increased circulating levels of inflammatory cytokines and C-reactive protein.19,36 Because circulating measures of inflammatory burden (eg, C-reactive protein) are predictive of cardiovascular risk, whereas treatments that attenuate inflammation reduce cardiovascular events (eg, statins), the hypothesis that prevention of AGE signaling could decrease cardiovascular risk is quite attractive.37,38 In addition, treatments that reduce arterial accumulation of AGEs may improve vascular compliance, which is also linked to cardiovascular prognosis. Nevertheless, the results of the present experimental study should be interpreted in the context of several proinflammatory pathways leading to macrovascular disease in diabetes.7 In addition to AGE effects, other mechanisms include the proinflammatory role of adipose tissue or the direct effects of elevated VLDLs that activate NF-κB and mount inflammatory responses in vascular cells. Furthermore, the role of the JNK signaling pathway in mediating AGE-induced inflammatory responses in coronary cells awaits validation in vivo.

**Conclusions**

The present study demonstrated increased oxidative stress and augmented expression of inflammatory mediators in coronary arteries in a porcine model of diabetes. Results suggested the upregulation of NAD(P)H oxidase activity and p22phox subunit expression. Furthermore, AGEs induced several inflammatory mediators in coronary cells in a redox-sensitive manner with the involvement of the JNK signaling pathway. These results underscore the potential role of AGEs in the development of accelerated coronary atherosclerosis.

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**References**


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