Interaction of Diabetes and Hypertension on Determinants of Endothelial Adhesiveness

Philip S. Tsao, Josef Niebauer, Ricardo Buitrago, Patrick S. Lin, Bing-yin Wang, John P. Cooke, Y-d. Ida Chen, Gerald M. Reaven

Abstract—Epidemiological studies have established that diabetes mellitus and hypertension are independent risk factors for atherosclerosis. One of the earliest abnormalities seen in atherogenesis is enhanced monocyte adherence to the endothelium. The mechanisms by which diabetes mellitus or hypertension enhances monocyte–endothelial cell interactions are incompletely characterized. It is not known whether there are additive interactions between these risk factors on endothelial adhesiveness for monocytes. Male Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats were fed a normal or fructose-enriched diet. In some cases, animals were injected with streptozotocin (35 mg/kg body weight) to induce diabetes. After 2 weeks, plasma was drawn for biochemical measurements, and thoracic aortas were harvested, opened longitudinally, and exposed to fluorescently labeled mouse monocyte cells (WEHI 78/24, 2×10⁶/mL) for 30 minutes on a rocking platform. Adherent cells were counted by epifluorescence microscopy. WEHI 78/24 binding to aortic segments from SHR animals was elevated compared with segments from WKYs. Fructose feeding alone had no effect on endothelial adhesiveness. When WKYs were made hyperglycemic by STZ injection, monocyte binding was 160% of the control value. Elevated monocyte binding was also observed in aortas derived from SHR animals injected with STZ, indicating an additive effect of hypertension and hyperglycemia. To determine whether alterations in oxidative state played a role in the endothelial adhesiveness, aortic segments were exposed to lucigenin (250 μmol/L) for measurement of superoxide anion. Aortic segments from SHR elaborated 120% more superoxide anion than did controls. Elevated free-radical production was also observed in aortas from diabetic WKYs. Furthermore, thoracic aortas derived from diabetic SHR animals elaborated more superoxide anion than did any of the other groups (374%, P<0.05). Immunohistochemical staining for monocyte chemotactic protein-1 demonstrated increased expression in aortas isolated from diabetic WKY and SHR compared with control vessels. These studies demonstrate that both diabetes and hypertension lead to increased monocyte adherence to the endothelium. This abnormality is associated with increased vascular superoxide production and monocyte chemotactic protein-1 expression. Furthermore, there appears to be an additive interaction between hyperglycemia and hypertension in their effects on endothelial adhesiveness and its determinants. (Arterioscler Thromb Vasc Biol. 1998;18:947-953.)

Key Words: atherosclerosis ■ free radicals ■ glucose ■ chemokine
Mean arterial blood pressure was monitored by the tail-cuff method sensitive to the diabetogenic effects of STZ. To investigate the role of high-fructose chow and STZ injection (fructose 50%, fructose 11%, fat, and 22% protein; Teklad Labs, n=4), and high-fructose chow and STZ injection (fructose: STZ 35 mg/kg, n=5). The fructose-enriched diet leads to insulin resistance, hyperinsulinemia, and hypertriglyceridemia and renders the rats more sensitive to the diabetogenic effects of STZ. To investigate the role of a second risk factor, hypertension, we also studied SHRs (8 to 9 weeks of age at the beginning of the experiments) that were also assigned to one of the same three regimens (chow, n=6; fructose, n=4; and fructose+STZ, n=6). After 2 weeks, blood was drawn from the tail vein for determination of plasma glucose (in mg/dL), triglycerides (in mg/dL), and insulin (in μU/mL) concentrations. Mean arterial blood pressure was monitored by the tail-cuff method before and after the treatment regimen. The equipment used included magnetic animal holders connected to a manual scanner (model 59, HTC), and a dual-channel recorder (model 1202, Linear Instruments). These protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University. Aortic rings were removed and placed into ice-cold, oxygenated PBS. A 30-mm vian artery and cleaned of adventitial tissue. Aortic segments were then carefully opened longitudinally and placed into 35-mm culture dishes containing 2 mL HBSS. Aortic strips were fixed to the culture dishes carefully underlaid with a layer of fetal calf serum and then centrifuged at 400 g for 15 minutes. The cell suspension was separated labeled cells from the remaining dye.

Probes) for 15 minutes at room temperature. The cell suspension was then carefully underlaid with a layer of fetal calf serum and then centrifuged at 400g to separate labeled cells from the remaining dye. Cells were washed in complete medium and resuspended in HBSS containing 1 mmol/L Ca2+1, 1 mmol/L Mg2+, and 2 mmol/L HEPES for binding studies.

Binding Studies
Monocyte binding studies were performed as previously described. In brief, the animals were killed by decapitation and thoracic aortas were removed and placed into ice-cold, oxygenated PBS. A 30-mm segment of aorta was excised immediately distal to the left subclavian artery and cleaned of adventitial tissue. Aortic segments were then carefully opened longitudinally and placed into 35-mm culture dishes containing 2 mL HBSS. Aortic strips were fixed to the culture dish with the use of 25-gauge needles and placed on a rocking platform at room temperature. After 10 minutes HBSS was replaced by 2 mL HBSS containing fluorescently labeled WEHI 78/24 cells (2×10^6/mL) for 30 minutes; the dishes were rotated 120° every 10 minutes. Nonadherent monocytes were then washed off, and adherent cells were counted by epifluorescence microscopy from at least 20 different sites. Data are expressed as a percentage of adherent cells on the thoracic aorta from a control animal studied in parallel.

Superoxide Anion Measurements
To determine whether changes in endothelial adhesiveness were associated with alterations in vascular superoxide anion generation, the following studies were performed. Superoxide anion production by aortic segments was monitored by lucigenin chemiluminescence and a modification of the method previously reported. Aortic rings (2 cm) were resuspended in HBSS containing lucigenin (bis-N-methylacridinium nitrate, 250 μmol/L). Superoxide anion generation was monitored in a Turner Designs luminometer for 1 minute with a 30-second delay. The relative specificity of lucigenin-induced chemiluminescence by superoxide anion is demonstrated by the potent effect of Tiron (an intracellular scavenger of superoxide anion) to block the signal and the lack of effect of H2O2 scavengers.

NOx Measurements
In some experiments, the aortic segments were prepared as described above and incubated with 1 mL HBSS medium containing calcium ionophore (1 μmol/L, Sigma) and l-arginine (100 μmol/L, Sigma) at 37°C to induce NO production. After 120 minutes, samples of the medium (100 μL) were collected for measurement of NOx (NO and one-electron oxidation products of NO). NOx levels were measured by using a commercially available chemiluminescence apparatus (Dasibi model 2108) after reduction of the samples in boiling acidic vanadium(III) at 98°C. Boiling acidic vanadium quantitatively reduces NO3− and NO2− to NO, which is quantified by the chemiluminescence detector after reaction with ozone. Signals from the detector were analyzed by a computerized integrator and recorded as areas under the curve. Standard curves for NOx/NO3 were linear over the range of 100 pmol to 5 nmol.

Immunohistochemistry
Segments of freshly isolated thoracic aortas were rinsed in cold PBS and fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Immunohistochemical analysis was performed by using primary antibodies raised against rat MCP-1 (a generous gift from Dr T. Yoshimura, National Cancer Institute, Frederick, Md) or AGEs (Wako). Sections were incubated with primary antibody or unrelated IgG for 1 hour at room temperature. After the sections were washed three times in PBS for 15 minutes, secondary antibody (FITC-labeled goat anti-mouse IgG, Zymed Laboratories) was applied for 25 minutes at room temperature. Balloon-injured rat carotid arteries and glycosylated albumin (kindly provided by M. Ohno, University of Tokyo) were used as positive controls for AGE staining.

Data Analysis
All values in text are expressed as mean±SEM of n independent experiments. Differences between specific means were tested by ANOVA with post hoc analysis using Fisher’s protected least significant difference test. A value of P<0.05 was accepted as statistically significant.

Results
Metabolic Studies
Table 1 summarizes the biochemical data. Although plasma glucose and TG concentrations were similar in chow-fed SHRs and WKYs, SHRs had significant increases in both plasma insulin concentrations and blood pressure. Plasma glucose concentrations increased modestly in both groups of rats in response to the fructose-enriched diet. Plasma insulin concentrations increased by ~20 μU/mL in both SHRs and WKYs in response to fructose, and the difference between the two groups of rats was maintained. Plasma TG concentrations also increased in both WKYs and SHRs in response to fructose. Blood pressure remained similar in fructose-fed WKYs but increased in SHRs, further exaggerating the difference between the two groups. Plasma insulin concentrations decreased after STZ in SHRs and WKYs and were associated with increases in both plasma glucose and TG concentrations. However, it is apparent from Table 1 that
these changes were much greater in magnitude in SHRs. Blood pressure did not change after STZ injection in WKY animals but did increase further in SHR animals.

Studies of EC Adhesiveness

To investigate whether these metabolic perturbations were associated with alterations in monocyte adhesiveness, thoracic aortas were isolated and functional binding assays performed. As shown in Figure 1, endothelial adhesiveness for monocytes was 178% higher in aortas from chow-fed SHRs than in WKYs (P < 0.05). These values did not change in either group in response to the fructose-enriched diet. STZ-induced diabetes was associated with significantly increased adhesiveness in thoracic aortas from both WKYs and SHRs (164% of control, P < 0.05) and SHRs (267% of control, P < 0.01). Moreover, the increase in STZ-treated SHR animals was significantly greater than in SHR animals (P < 0.05), indicating a positive interaction between hypertension and hyperglycemia with respect to their effect on endothelial adhesiveness.

Diet/Treatment Regimens

<table>
<thead>
<tr>
<th>Group/Animal</th>
<th>Glucose, mg/dL</th>
<th>Insulin, μU/dL</th>
<th>TG, mg/dL</th>
<th>MABP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>143±2.1</td>
<td>35±2.8</td>
<td>64±5.0</td>
<td>127±2</td>
</tr>
<tr>
<td>SHR</td>
<td>135±2.9</td>
<td>57±8.8*</td>
<td>67±5.6</td>
<td>165±2*</td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>151±3.8</td>
<td>56±8.8*</td>
<td>208±40*</td>
<td>125±2</td>
</tr>
<tr>
<td>SHR</td>
<td>153±2.7</td>
<td>76±14*</td>
<td>313±43*†</td>
<td>176±4†</td>
</tr>
<tr>
<td>STZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>202±26*</td>
<td>45±3.9</td>
<td>295±56*</td>
<td>135±2</td>
</tr>
<tr>
<td>SHR</td>
<td>444±34*†</td>
<td>30±5.9†</td>
<td>738±124*†</td>
<td>183±3*†</td>
</tr>
</tbody>
</table>

MABP indicates mean arterial blood pressure.

*P < 0.05 vs chow-fed WKYs.
†P < 0.05 vs chow-fed SHRs.

TABLE 1. Responses in WKYs and SHRs to Diet/Treatment Regimens

Figure 1. Effect of diabetes and hypertension on monocyte-EC interactions in WKYs and SHRs. Aortic endothelium from SHRs displayed increased adhesiveness for monocytes compared with WKY endothelium. Fructose feeding alone did not affect monocyte adhesion. Diabetes induced by STZ injection in combination with fructose feeding resulted in increased monocyte-EC interactions. Data are expressed as mean±SEM. *P < 0.05; †P < 0.01 vs chow-fed WKYs; and §P < 0.05 vs chow-fed SHRs.

Figure 2. Superoxide production in aortas from SHRs was elevated compared with WKYs. Diabetes increased superoxide generation over basal conditions in WKY animals. Moreover, diabetes had an additive effect with hypertension on superoxide production. Data are expressed as mean±SEM. *P < 0.05; †P < 0.01 vs chow-fed WKYs; and §P < 0.05 vs chow-fed SHRs.
part by an oxidant-sensitive transcriptional pathway mediated by nuclear factor-κB. To identify whether MCP-1 expression was elevated in hypertensive and hyperglycemic rats, immunohistochemical studies were performed. Aortas isolated from WKY control rats displayed no immunohistochemical evidence of MCP-1 in any section investigated (Figure 3A). By contrast, punctate staining for MCP-1 was observed scattered throughout both WKY and SHR aortas from animals treated with STZ. MCP-1 staining was located in both ECs as well as medial smooth muscle cells. These observations are consistent with in vitro studies that have demonstrated MCP-1 expression induced by oxidative stress in both ECs and smooth muscle cells. Moreover, MCP-1 was dramatically enhanced in STZ-treated SHR animals, with the majority of positive staining occurring in the smooth muscle layer (Figure 3B). Negative controls were performed with isotype-matched antibodies on serial sections of aortas. AGEs have been localized in vascular lesions of diabetic humans and animals. AGEs can interact with their specific receptor (RAGE) and induce oxidative stress, increase vascular cell adhesion molecule-1 expression, and enhance monocyte binding. Therefore, we investigated whether AGEs may be playing a role in the current study by immunohistochemistry. In contrast to MCP-1, there was no evidence of AGEs in any of the sections studied.

### TABLE 2. NO<sub>x</sub> Production by Thoracic Aortas Derived From WKYs and SHRs

<table>
<thead>
<tr>
<th>Group</th>
<th>WKYs (NO&lt;sub&gt;x&lt;/sub&gt;, pmol·mg&lt;sup&gt;-1&lt;/sup&gt;·h&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>SHR (NO&lt;sub&gt;x&lt;/sub&gt;, pmol·mg&lt;sup&gt;-1&lt;/sup&gt;·h&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>412±133</td>
<td>576±83</td>
</tr>
<tr>
<td>Fructose</td>
<td>389±111</td>
<td>670±108</td>
</tr>
<tr>
<td>STZ+fructose</td>
<td>400±63</td>
<td>612±79</td>
</tr>
</tbody>
</table>

Figure 3. Immunohistochemistry demonstrating increased expression of MCP-1 in vascular endothelium and subjacent smooth muscle cells in thoracic aorta from an SHR+STZ animal (B) compared with a chow-fed WKY (A).

### Discussion

The salient findings of this study are the following: (1) aortic endothelium derived from diabetic or hypertensive rats displays increased adhesiveness for monocytes, (2) this abnormality is associated with elevated vascular superoxide anion production and expression of MCP-1, and (3) there is a positive interaction between hypertension and hyperglycemia to enhance endothelial adhesiveness and its determinants.

In this study we have employed several rodent models in an effort to evaluate the impact of a variety of potential factors that might modulate endothelial adhesiveness and thus accelerate atherogenesis. Although the experimental manipulations used led to a variety of metabolic and hemodynamic changes, a surprisingly simple story has emerged. To fully understand the implications of the results presented, it is important to discuss the models themselves. In addition to being hypertensive, SHRs are relatively insulin resistant and hyperinsulinemic compared with WKYs. These differences were also observed in the current study (see Table 1) and were associated with an increase in endothelial adhesiveness as shown in Figure 1. Feeding rats a fructose-enriched diet leads to insulin resistance, hyperinsulinemia, hypertriglyceridemia, and an increase in blood pressure; these findings are corroborated by the results in Table 1. However, despite the increase in plasma TG and insulin levels, endothelial adhesiveness did not increase in thoracic aortas from WKYs. Similarly, endothelial adhesiveness did not increase further in SHRs (Figure 1), despite substantial fructose-induced increases in plasma TGs and insulin concentrations. Therefore, it appears that hypertension per se was responsible for the increased adhesion of monocytes to thoracic endothelium from SHRs as shown in Figure 1, with little or no effect of hypertriglyceridemia or hyperinsulinemia.

The conclusion that hyperinsulinemia itself does not affect the binding of mononuclear cells to thoracic aortas is further
supported by the changes seen after STZ injection. The results in Table 1 indicate that plasma insulin concentrations fell in both SHR and WKY animals injected with STZ, whereas endothelial adhesiveness increased significantly (Figure 1). Thus, hyperglycemia, or some unknown abnormality associated with the development of diabetes, seems to play an independent role in determining the degree to which thoracic aortas from SHRs and WKYs bind mononuclear cells.

The current study focused on the effects of diabetes and hypertension on endothelial adhesiveness for monocytes. In addition, it is likely that this endothelial dysfunction will result in increased adherence of other blood cell elements, such as neutrophils and platelets.53,54 Furthermore, these risk factors may also have important direct effects on circulating cells to enhance their activity. Indeed, we have previously observed that mononuclear cells isolated from patients with non–insulin-dependent diabetes mellitus bind more avidly to cultured ECs than do mononuclear cells derived from control subjects.27

Along with demonstrating that hypertension and hyperglycemia affect endothelial adhesiveness, the results presented add evidence to the mechanism(s) responsible for these phenomena. As such, this study may provide insight into the mechanism by which diabetes and hypertension accelerate atherogenesis. Vascular free-radical production has been implicated in several pathophysiological conditions that contribute to atherogenesis, including hypercholesterolemia, hypertension, and diabetes mellitus. Schmid-Shönbein and colleagues28 have demonstrated increased vascular superoxide anion production in the SHR compared with normotensive controls through the use of an oxidant-sensitive fluorophore in vivo; treatment with dimethylurea or a xanthine oxidase inhibitor attenuated the oxidative stress. Interestingly, fluorescent staining was considerably higher along the arterial wall than the venular wall. Endothelium-dependent vasodilator dysfunction in hypertensive as well as diabetic vessels is reversed by SOD, further implicating spontaneous oxidative stress in both conditions.53,54 Increased oxidative stress can negatively affect endothelial adhesiveness for monocytes. Interestingly, these investigators found no change in the surface expression of the endothelial adhesion molecules intercellular adhesion molecule-1 or vascular cell adhesion molecule-1.35 Elevated angiotensin II levels associated with hypertension may also stimulate an NADH/NADPH oxidase system in vascular smooth muscle cells to produce superoxide anion.36

Increased oxidative stress can negatively affect endothelial-dependent vasodilation by inactivation of NO. Although NO synthesis was not affected in our models of diabetes or hypertension, it is likely that bioactive NO was degraded by the excess superoxide anion. (It is important to note that our assay for NO production does not differentiate between bioactive NO and its inactive oxidation products.) Interestingly, all of the hypertensive groups tended to have higher NO production than the WKY groups, consistent with reports by Nava et al57 suggesting that NO synthesis may be upregulated in chronic hypertension as a countervailing force. However, with enhanced superoxide anion production, there would likely be a decrease in local NO activity. Indeed, Malinski and colleagues51 have recently demonstrated that aortic production of bioactive NO in SHRs, as measured by a porphyrinic microsensor, is increased by treatment with SOD. The decreased activity of NO may also play a role in MCP-1 expression. Studies by Zeiher et al52 indicate that NO may inhibit endothelial transcription of MCP-1 in a cGMP-independent fashion. Therefore, decrements in NO activity, as seen in diabetes or hypertension, can exacerbate the expression of MCP-1.

Recent studies have also demonstrated that elevated levels of glucose may adversely affect endothelial function by nonenzymatic protein glycosylation.54 A complex array of chemical reactions that can reach equilibrium over several
weeks results in insoluble, cross-linked complexes called AGEs. Schmidt et al. have demonstrated that exposure of ECs to AGEs can enhance expression of vascular cell adhesion molecule-1 and increase monocyte binding. Interestingly, this effect was inhibited by the antioxidant N-acetylcysteine, indicating an important regulatory role of oxidant stress. Although free radicals may play a significant role in the current investigation, we found no immunohistochemical evidence of AGEs in our diabetic or hypertensive aortas. Therefore, AGEs may play a more important role later in the time course of diabetes-accelerated occlusive vascular disease.

Oxidant-sensitive transcription of adhesion molecules and chemokines increases leukocyte adhesion in vivo. We observed increased endothelial adhesiveness for monocytes induced by diabetes or hypertension. Electron microphotographic studies indicate enhanced leukocyte adhesion to the aortic endothelium in diabetic New Zealand White rabbits. Furthermore, ischemia/reperfusion elicits greater leukocyte adhesion and albumin leakage in diabetic rats. These alterations in the determinants of endothelial adhesiveness can ultimately lead to increased atherosclerosis. Indeed, STZ-induced diabetes accelerates the development of atherosclerotic lesions in hyperlipidemic BALB/c mice and human primates, whereas Goldblatt clip–induced hypertension promotes greater aortic lesions in genetically hypercholesterolemic rabbits.

In summary, the present investigation revealed that genetic hypertension and STZ-induced diabetes are each associated with increased aortic free-radical production, MCP-1 expression, and endothelial adhesiveness for monocytes. These metabolic disorders act additively to increase the elaboration of superoxide anion and endothelial adhesiveness for monocytes. The present study adds to accumulating evidence that implicates oxidant-sensitive transcriptional activation as a common pathway for vascular disease induced by metabolic disorders such as hypertension, hyperlipidemia, and diabetes mellitus.

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