Abstract—Incubation of endothelial cells in vitro with high concentrations of glucose activates protein kinase C (PKC) and increases nitric oxide synthase (NOS III) gene expression as well as superoxide production. The underlying mechanisms remain unknown. To address this issue in an in vivo model, diabetes was induced with streptozotocin in rats. Streptozotocin treatment led to endothelial dysfunction and increased vascular superoxide production, as assessed by lucigenin- and coelenterazine-derived chemiluminescence. The bioavailability of vascular nitric oxide (as measured by electron spin resonance) was reduced in diabetic aortas, although expression of endothelial NOS III (mRNA and protein) was markedly increased. NOS inhibition with N\textsuperscript{G}-nitro-L-arginine increased superoxide levels in control vessels but reduced them in diabetic vessels, identifying NOS as a superoxide source. Similarly, we found an activation of the NADPH oxidase and a 7-fold increase in gp91\textsuperscript{phox} mRNA in diabetic vessels. In vitro PKC inhibition with chelerythrine reduced vascular superoxide in diabetic vessels, whereas it had no effect on superoxide levels in normal vessels. In vivo PKC inhibition with N-benzoyl-staurosporine did not affect glucose levels in diabetic rats but prevented NOS III gene upregulation and NOS-mediated superoxide production, thereby restoring vascular nitric oxide bioavailability and endothelial function. The reduction of superoxide in vitro by chelerythrine and the normalization of NOS III gene expression and reduction of superoxide in vivo by N-benzoyl-staurosporine point to a decisive role of PKC in mediating these phenomena and suggest a therapeutic potential of PKC inhibitors in the prevention or treatment of vascular complications of diabetes mellitus. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001; 88:e14-e22.)

Key Words: diabetes ■ nitric oxide synthase ■ protein kinase C ■ uncoupling ■ NADPH oxidase

Myocardial infarction and stroke constitute major causes of death in patients with diabetes mellitus. Long-term diabetes is associated with macroangiopathy and microangiopathy. Several hypotheses have been put forth to explain the adverse effects of hyperglycemia on the vasculature. These include the activation of the polyol pathway, nonenzymatic glycation, redox potential alterations, and stimulation of the diacylglycerol (DAG)–protein kinase C (PKC) pathway (for review, see Ishii et al\textsuperscript{1}). Although studies on the polyol pathway and nonenzymatic glycation remain inconclusive so far, more recent studies strongly point to a decisive role of the DAG-PKC pathway for the vascular complications associated with diabetes.\textsuperscript{1}

Incubation of vascular tissue with high concentrations of glucose increases intracellular DAG levels, which ultimately lead to PKC activation.\textsuperscript{1} High glucose-induced endothelial dysfunction can be corrected with PKC inhibitors.\textsuperscript{2} These in vitro observations are supported by studies demonstrating that in vivo treatment with PKC inhibitors ameliorates vascular complications in diabetic rats.\textsuperscript{3} The mechanisms underlying PKC-mediated endothelial dysfunction remain poorly understood. In vitro experiments have shown that PKC-mediated phosphorylation of nitric oxide synthase (NOS) III protein may reduce the activity of the enzyme.\textsuperscript{4} Stimulation of endothelial cells with phorbol esters (direct activators of PKC)\textsuperscript{5} or glucose\textsuperscript{6} increases the expression of NOS III. Glucose also greatly enhances endothelial superoxide production,\textsuperscript{6} leading to increased vascular formation of the nitric oxide (NO)/superoxide reaction product peroxynitrite.\textsuperscript{7} Peroxynitrite in turn has been recently shown to oxidize avidly tetrahydrobiopterin, an NOS III cofactor, to dihydrobiopterin.\textsuperscript{8} Under conditions of BH\textsubscript{4} deficiency, NOS III is in an...
uncoupled state, which means that electrons flowing from the NOS III reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to l-arginine, resulting in production of superoxide rather than NO. Indeed, there is indirect evidence for a dysfunctional, uncoupled NOS in experimental and clinical studies showing that the administration of the NOS III cofactor tetrahydrobiopterin improves endothelial dysfunction in the setting of diabetes mellitus.

Increased superoxide production in diabetes is not restricted to endothelial cells and was also demonstrated to be improved endothelial-dependent relaxations without altering superoxide production of vascular smooth muscle cells, an observation that may point to a significant contribution of a dysfunctional NOS to endothelial dysfunction in diabetes.

In the present study, we used the animal model of streptozotocin (STZ)-induced diabetes, which has been shown to be associated with vascular PKC activation. To determine whether (1) STZ-induced diabetes mellitus in rats is associated with an altered NOS III gene expression; (2) NOS III may uncouple and produce superoxide; (3) other superoxide-producing enzymes such as the NADPH oxidase may be activated; and (4) PKC inhibition in vitro and in vivo can inhibit oxidative stress and therefore improve endothelial dysfunction.

Materials and Methods
The present study was conducted in accordance with the guidelines for animal experimentation at the University Hospital Eppendorf, Hamburg, Germany.

Animals
One hundred fifty Wistar rats, 8 weeks old and weighing 220 to 250 g, received a single intravenous injection of STZ (65 mg/kg) or placebo into the tail vein. Two weeks later, the animals were killed by an overdose of pentobarbital. Four groups of animals were used: (1) controls (n = 25), (2) STZ-treated animals was measured with three different chemiluminescence substrates, vessels were incubated with chelerythrine and L-NNA to test whether their effect on superoxide production may be additive. To demonstrate specificity for superoxide, some of the vessels were incubated with the superoxide dismutase (SOD) mimetic Mn(III)tetraakis(4-benzoic acid) porphyrin chloride (MnTBAP) (100 μmol/L for 30 minutes).

NADPH oxidase activity was measured using lucigenin-enhanced chemiluminescence as described using lucigenin at a concentration of 5 μmol/L. In all experiments, NADPH was added to the particulate fraction of the vessel homogenate at a concentration of 100 μmol/L.

Electron Spin Resonance (ESR) Studies: Detection of Vascular NO and Superoxide
Concentrations of NO in rat aorta in the presence of superoxide were assayed using ESR spectroscopy and the spin-trap iron (II)-proline-dithio-carbamate [Fe(PrTC) 2], which has been shown to trap NO with high efficacy by forming an ESR-deetectable paramagnetic complex Fe(NO) 6PTC 3, as described.13 Stimulation of rat aorta with acetylcholine results in the doubling of the Fe(PrTC) signal, and treatment with L-NNA (1 mmol/L) as well as endothelial removal abolishes the ESR signal, indicating that we are actually trapping NO with this method.

To quantify superoxide in vessels from animals with and without STZ treatment, the formation of CP' radicals was monitored using ESR spectroscopy and 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl-pyrroline hydrochloride (CPH, 1 mmol/L).18

RNA Isolation and Competitive RT-PCR
In aortic tissue from diabetic rats and control animals, mRNA expression of NAD(P)H oxidase subunit gp91phox was quantified. Total RNA from rat aortic sections was isolated by guanidinium thiocyanate/phenol chloroform extraction (RNAzolTMB, WAK-Chemie). The gp91phox mRNA expression was determined by standard, calibrated, competitive reverse transcriptase–polymerase chain reaction (RT-PCR). To generate an internal standard for competitive RT-PCR, a human gp91phox-specific cDNA fragment of 706 bp (position 814–1519) was amplified from RNA of human endothelial cells by RT-PCR using the following primers: gp91phox sense primer, 5'-CCATGACTTGAAATGGAT-3' and gp91phox antisense primer, 5'-TCATCTTTCCTCCATCAT-3', respectively. The gp91phox-specific cDNA fragment was subsequently cloned into the pCR-Script Amp SK (-) cloning vector (Stratagene) and its identity confirmed by DNA sequencing (ABI PRISM TM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS [Perkin-Elmer Co]; ABI 373 DNA sequence). DNA sequence analysis was performed using the Gene Runner software (Hastings Software, Inc.). Database searches of GenBank were performed using BLAST. Subsequently, an internal deletion of 198 bp was introduced into this fragment by linker primer PCR. The internal-deleted gp91phox cDNA standard plasmid template was linearized with XhoI, which cleaves downstream of the insert, in vitro–transcribed into cRNA with T3 polymerase (RNA transcription kit, Stratagene), and standard cRNA was quantified spectrophotometrically.

In RT-PCR experiments with RNA from rat aortic tissue, the indicated human primers amplified a gp91phox-specific cDNA fragment of the expected size (706 bp). The identity of the rat gp91phox fragment was confirmed by DNA sequencing. In competitive RT-PCR experiments, equal amounts of total RNA (250 ng) were mixed with defined amounts of gp91phox standard cRNA molecules, respectively. The samples were incubated for 3 minutes at 70°C and subsequently reverse-transcribed into cDNA using random hexamer primers and SuperScript II RNase H reverse transcriptase (Life Technologies) for 1 hour at 42°C. Twenty percent of each reverse

gate the effect of PKC inhibition on vascular superoxide, aortic rings were incubated at 37°C for 30 minutes with the specific PKC inhibitor chelerythrine (3 μmol/L).

To test for an involvement of the xanthine oxidase or mitochondrial superoxide sources, vessels were incubated with oxypurinol (100 μmol/L) or rotenone (100 μmol/L). In separate experiments, vessels were incubated with chelerythrine and L-NNA to test whether their effect on superoxide production may be additive. To demonstrate specificity for superoxide, some of the vessels were incubated with the superoxide dismutase (SOD) mimetic Mn(III)tetraakis(4-benzoic acid) porphyrin chloride (MnTBAP) (100 μmol/L for 30 minutes).
transcription reaction was then amplified in separate reaction with 20 pmol/L gp91phox− sense and antisense primers by the following PCR protocol: 30 seconds at 95°C, 30 seconds at 58°C, and 45 seconds at 72°C (38 cycles). PCR primers compete for sample-specific and standard molecules in the amplification reaction. The PCR reactions were separated by standard agarose gel electrophoresis, stained with ethidium bromide, and documented by photography using Polaroid film type 667. The optical density of standard and sample-specific PCR fragment was estimated densitometrically (ScanPack 3.0, Bio-Imeta). The logarithm of the quotient of standard and sample-specific PCR fragment density was graphically plotted versus the amount of standard cRNA molecules. As presented in the graph, equal amounts of RNA molecules in sample and standard were present at the equivalence point.

Cloning of a Rat NOS III cDNA Fragment and RNase Protection Analysis

A cDNA fragment of rat NOS III was generated with RT-PCR as described24 using 2 μg of total RNA from rat aorta. Oligonucleotide primers were 5'-GACATTGAGCAAAAGGCTGC-3' (sense) and 5'-CGGCTTGTCACTCTGG-3' (antisense). The cloning of the rat γ-actin probe (used for normalization of the RNase protection assays) was done as previously described.25 RNase protection assays were performed with a mixture of RNase A and RNase T1 as described.16 The protected RNA fragments of NOS III and γ-actin were 425 nt and 110 nt, respectively.

Western Blot Analyses

Western blot analyses for the detection of NOS III and Cu/Zn SOD were performed as described previously.16,26 Quantification of the expression of soluble guanylyl cyclase (sGC; β subunit) was performed using a polyclonal antibody to sGC-β (Transduction Laboratories). To test for the effects of in vivo PKC inhibition on tyrosine phosphorylation, a monoclonal antibody to phosphotyrosine was used (Oncogene). For quantification, the density of all tyrosine MS-phosphorylated bands of control vessels was set as 100%, and changes in tyrosine phosphorylation in the other three groups were expressed as a percentage from control.

Oxidative Fluorescent Microtopography

The oxidative fluorescent dye hydroethidine was used to evaluate the in situ concentration of superoxide as described previously.27 Unfixed frozen rings of aortic segments were cut into 30-μm-thick sections and placed onto a glass slide. Hydroethidine (2×10−5 mol/L) was topically applied to each tissue section and coverslipped. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope with a krypton/argon laser. Fluorescence was detected by staining with nuclear Fast Red. First, images of aortas from sham-treated rats were measured. After adjusting the basal settings of the confocal microscope, images of aortas from STZ-treated rats in vivo with and without PKC inhibition or in vitro with L-NNA (1 mmol/L) were measured.

Statistical Analyses

Results are expressed as mean±SEM. The EC50 value for each experiment was obtained by logit transformation. To compare superoxide and NO bioavailability in normal and diabetic vessels as well as changes in the expression of NOS III, Cu/Zn SOD, and tyrosine phosphorylation, a one-way ANOVA was used. Comparisons of vascular responses were performed using multivariate analysis of variance with percentage of relaxation and EC50 as dependent variables. The Scheffé post hoc test was used to examine differences between groups when significance was indicated. A probability value of <0.05 was considered significant.

Results

Effect of STZ-Induced Diabetes on Endothelium-Dependent Vascular Relaxation and Vascular NO Bioavailability

As an initial approach to examine vascular reactivity in diabetic vessels, cumulative concentrations of acetylcholine and nitroglycerin were applied after preconstriction with phenylephrine. Vessels from diabetic animals showed an impaired relaxation to acetylcholine whereas relaxations to nitroglycerin were not different (Table 1).

To directly examine vascular production and release of NO+, ESR using Fe(PrTC)2 was used. As shown in Figure 1, basal NO production, as detected using this method, was markedly reduced in diabetic vessels compared with normals.

Effect of Diabetes on NOS III Expression

One explanation for impaired endothelium-dependent vascular relaxation and decreased production of NO in diabetic vessels could be a reduction of NOS III expression. To examine this possibility, NOS III mRNA expression was measured by RNase protection assay, and NOS III protein was measured using Western blot analysis. Paradoxically, NOS III expression was not decreased but was increased, both at the mRNA and protein level, by more than 3-fold in vessels from diabetic animals (Figures 2A and 2B).

Effects of Diabetes on gp91phox mRNA

In the setting of diabetes, expression of the NAD(P)H oxidase subunit gp91phox, as assessed by quantitative RT-PCR, was increased 7-fold compared with controls (Figure 3B).

Effect of Diabetes on Vascular O2− Production

Because both NOS III and the NAD(P)H oxidase are potential sources of superoxide, which has been shown to inactivate NO and impair endothelium-dependent relaxation, we measured steady-state levels of superoxide using three different methods. Using lucigenin-enhanced chemiluminescence, superoxide production was increased by >3-fold in diabetic vessels (Figure 4). Using CLA chemiluminescence, similar increases in vascular superoxide were also observed (CLA control tissue: 24±3 counts×104/mg per minute, CLA diabetic tissue: 40±6 counts×104/mg per minute). We also confirmed that superoxide production was increased in diabetic vessels using ESR. The intensity of the ESR signal of CP′ radicals increased from 11.5±1.5 CP′ in control aortas to 27.7±2 CP′ in aortas from diabetic animals. Thus, using three techniques, we demonstrated that superoxide production is increased in STZ-induced diabetes.

We next investigated the possibility that NOS III itself might serve as a source of superoxide in diabetes. In control vessels, treatment with the NOS inhibitor L-NNA increased the lucigenin-derived chemiluminescence (LDCL) signal (Figure 4). In striking contrast, in vessels from diabetic rats, L-NNA markedly reduced the lucigenin signal, identifying NOS as an important superoxide source.

Using coelenterazine as a chemiluminescent probe, we found significant increases in vascular superoxide production and diabetic vessels and similar L-NNA effects. Oxyurinol and rotenone had no significant effect on coelenterazine-derived chemiluminescence. The coelenterazine-derived chemiluminescence was nearly abolished by the SOD mimetic MnTBAP.

PKC has been shown to be activated in diabetes, and PKC activation has been found to increase vascular superoxide production. To examine whether PKC had a role in superox-
ide production, we incubated aortic rings at 37°C for 30 minutes with the specific PKC inhibitor chelerythrine (3 μmol/L). This had no effect on LDCL in aortas from control animals whereas markedly inhibiting LDCL in vessels from diabetic animals (Figure 4). Similarly, PKC inhibition markedly inhibited superoxide production in vessels from diabetic animals as measured with coelenterazine chemiluminescence (Table 2). Adding L-NNA to chelerythrine-exposed vessels had no additional effects.

To investigate a role for the NADPH oxidase in vascular superoxide production, particulate fractions from control and diabetic vessels were stimulated with NADPH (100 μmol/L). In diabetic vessels, NADPH oxidase activity was significantly higher compared with controls (Figure 3A).

Effect of In Vivo Treatment With N-Benzoyl-Staurosporine on Vascular Function

Given the above results showing a beneficial effect of inhibition of PKC in vitro, we sought to examine the effect of in vivo PKC inhibition. For these studies, N-benzoyl-staurosporine, an orally active inhibitor of PKC was used. In vivo PKC inhibition with N-benzoyl-staurosporine had no effect on blood glucose levels in either controls or diabetic animals (Table 3). Of note, in vivo PKC inhibition prevented the development of abnormal endothelium-dependent vascular relaxation (Table 1). In keeping with this physiological effect, PKC inhibition also markedly increased the ability to detect basal NO production in isolated vessel segments using ESR (Figure 5) at levels comparable to control animals (Figure 1).

In vivo N-benzoyl-staurosporine treatment prevented up-regulation of NOS III and CuZn SOD and downregulation of sGC(βi) in vessels of diabetic animals (Figure 6). It also markedly reduced vascular superoxide levels (Figure 4). Incubation of vessels from diabetic rats (cotreated with N-benzoyl-staurosporine) with L-NNA increased the LDCL signal rather than decreasing it (from 1799 ± 97 to 2866 ± 150 counts/mg per minute), indicating that in vivo PKC inhibition may prevent NOS uncoupling.

Using hydroethidine, we found that in vivo PKC inhibition markedly decreased endothelial as well as smooth muscle-derived superoxide, as shown in Figure 7. Incubation experiments with L-NNA confirmed the results obtained with the lucigenin assay. In control vessels, L-NNA increased hydroethidine staining but markedly decreased it in the endothelium (and also to some extent in the adventitia) of diabetic vessels, indicating NOS uncoupling in the setting of diabetes mellitus.

To test whether N-benzoyl-staurosporine inhibits other kinases (eg, tyrosine kinase) in addition to PKC, tyrosine phosphorylation of vessels from control and diabetic animals was assessed with Western blot analysis. We found that tyrosine phosphorylation did not change significantly in response to in vivo PKC inhibition (PKCI) (control: 100%, control + PKCI: 105 ± 4%, diabetes: 110 ± 3%, and diabetes + PKCI: 102 ± 6%). This indicates that the concentration of N-benzoyl-staurosporine chosen may specifically inhibit PKC and not tyrosine kinases.

Diabetes was also associated with a marked increase in phenylephrine-induced constriction, a phenomenon that was completely inhibited by in vivo PKC inhibition (data not shown).

Discussion

The present studies have defined a marked perturbation of NO production and NOS III function in diabetes. Paradoxically, we found that NOS III expression was not decreased but, in fact, increased by 3-fold in diabetic vessels. In contrast, either the bioavailability or production of NO in these vessels was markedly reduced, as demonstrated by studies of endothelium-dependent vascular relaxation and ESR measurements of NO bioavailability (%P<0.05).

Table 1. Effects of In Vivo PKC Inhibition With N-Benzoyl-Staurosporine (10 mg · kg⁻¹ · d⁻¹ for 14 Days) on the Potency and Efficacy of Acetylcholine (ACh) or Nitroglycerin (NTG) to Produce Relaxations in Rat Aortic Ring In Vitro

<table>
<thead>
<tr>
<th>In Vivo Treatment</th>
<th>Potency (ED₅₀, -logM) NTG</th>
<th>ACh</th>
<th>Efficacy (% Maximal Relaxation) NTG</th>
<th>ACh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control aorta</td>
<td>7.47±0.13</td>
<td>7.69±0.09</td>
<td>94±2</td>
<td>91±2</td>
</tr>
<tr>
<td>Control aorta and N-benzoyl-staurosporine</td>
<td>7.54±0.14</td>
<td>7.57±0.11</td>
<td>90±5</td>
<td>94±3</td>
</tr>
<tr>
<td>Diabetic aorta</td>
<td>7.48±0.12</td>
<td>6.80±0.11*</td>
<td>95±1</td>
<td>72±4*</td>
</tr>
<tr>
<td>Diabetic aorta and N-benzoyl-staurosporine</td>
<td>7.58±0.14</td>
<td>7.34±0.10</td>
<td>93±3</td>
<td>89±2</td>
</tr>
</tbody>
</table>

Data are mean±SEM from 7 to 20 independent experiments. *P<0.05 vs control.
NADPH (100 μmol/L) resulted in a significantly stronger superoxide production compared with controls (left). Data are mean±SEM from 6 separate experiments. gp91-phox mRNA as assessed with quantitative RT-PCR showed a 7-fold increase in vessels from STZ-treated animals compared with controls (right). Data are mean±SEM from 4 separate experiments.

**Figure 3.** Effect of STZ treatment on NADPH oxidase activity (A) and on the NADPH oxidase subunit mRNA gp91phox (B). With use of lucigenin-enhanced chemiluminescence, stimulation of the membrane fraction of vascular tissue homogenates by NADPH (100 μmol/L) resulted in a significantly stronger superoxide production compared with controls (left). Data are mean±SEM from 6 separate experiments. gp91phox mRNA as assessed with quantitative RT-PCR showed a 7-fold increase in vessels from STZ-treated animals compared with controls (right). Data are mean±SEM from 4 separate experiments.

**Figure 4.** Effects of in vitro and in vivo PKC inhibition on basal superoxide production in control and diabetic animals as measured by LDCL. Diabetes for 2 weeks markedly increased vascular superoxide (column 1 versus column 5). These elevated levels were reduced by the NOS inhibitor Nω-nitro-L-arginine (L-NNA, column 6 versus column 5), identifying the NOS III as a significant superoxide source under these conditions. In vitro as well as in vivo PKC inhibition (with chelerythrine and N-benzoylstaurosporine, respectively) normalized the elevated superoxide in diabetic vessels. In contrast, incubation of control vessels with L-NNA increased the LDCL signal (column 2 versus column 1), whereas chelerythrine and N-benzoyl-staurosporine had no effect on LDCL of control vessels (columns 3 and 4 versus column 1). Data are presented as mean±SEM from 6 to 8 experiments. *P<0.05 vs column 1 (untreated control vessel); †P<0.05 vs column 5 (diabetic vessel without inhibitors).

**Oxidative Stress and Endothelial Dysfunction in Diabetest**

High concentrations of glucose have been shown to be associated with endothelial dysfunction in vivo and in vitro. Mechanisms underlying this endothelial dysfunction could include a decreased activity and/or expression of NOS III or an increased degradation of NO secondary to an enhanced superoxide production. More recent data support the concept of NO degradation, because treatment of vessels from diabetic animals with SOD improved endothelial-dependent relaxations and the use of vitamin C in patients with non–insulin-dependent diabetes markedly increased endothelial-dependent relaxations in forearm arteries. The present in vivo study also strengthens the concept of oxidative stress of diabetes as being responsible for endothelial dysfunction. We demonstrate that vascular steady-state superoxide production was about twice as high in vessels from

**TABLE 2. Effects of Diabetes on Vascular Superoxide Production as Determined With Coelenterazine (1 μmol/L)**

<table>
<thead>
<tr>
<th>Interventions</th>
<th>n</th>
<th>Control</th>
<th>STZ Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>E+</td>
<td>7</td>
<td>1.8±0.2</td>
<td>4.6±0.8</td>
</tr>
<tr>
<td>L-NNA (1 mmol/L)</td>
<td>6</td>
<td>2.5±0.3†</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>Oxyuripinol (100 μmol/L)</td>
<td>3</td>
<td>2.1±0.4</td>
<td>4.5±0.5</td>
</tr>
<tr>
<td>Rotenone (100 μmol/L)</td>
<td>3</td>
<td>1.5±0.2</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>Chelerythrine (10 μmol/L)</td>
<td>4</td>
<td>1.4±0.1</td>
<td>1.9±0.4†</td>
</tr>
<tr>
<td>Chelerythrine+L-NNA</td>
<td>4</td>
<td>1.3±0.3</td>
<td>1.8±0.2‡</td>
</tr>
<tr>
<td>MnTBAP (1 mmol/L)</td>
<td>5</td>
<td>0.4±0.1†</td>
<td>0.9±0.2‡</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Superoxide is expressed as counts×10³/mg of dry weight of vessels/min. E+ indicates endothelium present.

*P<0.05 vs controls; †P<0.05 vs E+ without intervention.
diabetic animals compared with control animals. Increased superoxide production was measured with LDCL and confirmed with CLA- and coelenterazine-induced chemiluminescence,18 and CP formation was assessed with ESR.17

Effects of Diabetes on NOS III Gene Expression and NOS-Mediated Superoxide Production

With the present studies, we can exclude that decreases in NOS III gene expression contribute to endothelial dysfunction. At both the RNA level and the protein level, the enzyme was upregulated more than 2- to 3-fold in vessels from diabetic animals compared with controls (Figure 2). Nevertheless, there was considerable endothelial dysfunction in the STZ-treated animals. On the basis of these observations, we hypothesized that the superoxide formed within diabetic tissue may either overwhelm the NO production of the upregulated NOS III or that the upregulated NOS III itself may be uncoupled, thereby contributing itself to superoxide production. The assumption that NOS III is uncoupled in the setting of diabetes is strengthened by experiments with the NOS inhibitor L-NNA using lucigenin- as well as coelenterazine-derived chemiluminescence. In control vessels with an intact endothelium, NOS inhibition with L-NNA increased vascular superoxide, indicating that basal production of endothelium-derived NO quenches the baseline lucigenin and coelenterazine signals. In contrast, incubation of aortas from STZ-treated animals with L-NNA markedly reduced the chemiluminescent signals identifying NOS III as an important superoxide source. The concept of an uncoupled NOS inhibitor L-NNA using lucigenin- as well as coelenterazine-derived chemiluminescence.

![Figure 5](image-url) Effect of the inhibition of PKC in vivo (PKCI) with N-benzoyl-staurosporine on the vascular NO bioavailability assessed with ESR spectroscopy. Left, Original spectra obtained with an aorta from STZ-treated diabetic rat (top) and a vessel from a diabetic animal treated in vivo with N-benzoyl-staurosporine (bottom). Right, Average amount of NO trapped by Fe(PrTC)2 in aortas from diabetic animals and in vessels from diabetic animals treated with N-benzoyl-staurosporine (PKCI; n=4 each). N-benzoyl-staurosporine treatment markedly improved the bioavailability of vascular NO (*P<0.05).

![Figure 6](image-url) Effect of the inhibition of PKC in vivo (PKCI) with N-benzoyl-staurosporine on NOS III, sGC (β subunit), and SOD expression in the aorta of untreated control rats (C) and STZ-treated diabetic animals (D). Top, Three Western blots for NOS III, sGC, and SOD with protein from 3 aortas in each group. NOS III protein from human umbilical vein endothelial cells and conventional Cu/Zn SOD protein from bovine erythrocytes were used as positive controls (pos. con.). Bottom, Densitometric analyses (mean±SEM) from 8 to 10 separate experiments per group (*P<0.05 vs control, C).

![Figure 7](image-url) Effect of the inhibition of PKC in vivo on vascular superoxide production in rat aorta as detected with hydroethidine. Fluorescent photomicrographs of confocal microscopic sections of aortas from rats receiving sham treatment with and without N-benzoyl-staurosporine (top) or diabetic rats with and without N-benzoyl-staurosporine (bottom). Vessels were labeled with the dye hydroethidine, which produces a red fluorescence when oxidized to ethidium bromide by superoxide. E indicates endothelium; M, media; and A, adventitia. Data are representative for n=4 experiments.
NOS in diabetes is also supported by the observation that L-NNA markedly decreased dihydroethidine staining in the endothelium of diabetic vessels (Figure 7).

Potential Mechanisms Underlying NOS Uncoupling

It has become clear from studies of both NOS I and NOS III that these enzymes may become uncoupled in the absence of L-arginine or tetrahydrobiopterin. In this uncoupled state, electrons flowing from the reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine, resulting in production of superoxide rather than NO. Recent in vitro studies proposed that oxidized LDL in particular is able to decrease endothelial L-arginine uptake, ultimately leading to both local depletion of L-arginine and NOS III uncoupling. Diabetes has been shown to increase the vascular formation of the NO/superoxide reaction product peroxynitrite. Peroxynitrite in turn rapidly oxidizes the active NOS cofactor tetrahydrobiopterin to cofactor inactive molecules such as dihydrobiopterin, leading to NOS III uncoupling. Indeed, experimental as well as clinical observations indicate that the NOS cofactor BH₄ or the NOS substrate L-arginine is able to improve endothelial dysfunction in the setting of diabetes.

Role for PKC and the NAD(P)H Oxidase in Increased Superoxide Production

The present studies also provide evidence for an involvement of PKC in NOS-mediated superoxide production. The association of diabetes mellitus and activation of PKC is well established. Activation of the DAG-PKC pathway has been shown to occur predominantly in vascular tissue. Incubation of endothelial cells and smooth muscle cells with high glucose increases intracellular DAG levels, subsequently leading to PKC activation, a phenomenon that has also been shown to occur in vessels from STZ-treated rats. A role for PKC in mediating endothelial dysfunction has also been postulated by Tesfamariam et al. In their studies, these investigators demonstrated that incubation of isolated aortic rings with high glucose caused endothelial dysfunction, which was improved by a simultaneous incubation of the tissue with the PKC inhibitor H2222. Incubation of cultured endothelial cells with glucose has also been shown to increase mitochondrial reactive oxygen species, leading subsequently to an activation of PKC. In addition, in vivo treatment of diabetic rats with an inhibitor of the PKC isoform β₁, ameliorated glomerular filtration rate, albumin excretion rate, and retinal circulation in a dose-dependent manner. In agreement with these observations, we found that an in vitro incubation of aortic tissue from diabetic animals with the PKC inhibitor chelerythrine had a marked inhibitory effect on vascular superoxide production. The same concentration of chelerythrine had no significant effect on the superoxide production of vessels from control animals. This suggests an important role for PKC in mediating increased superoxide production in diabetes.

With the present studies, we were able to demonstrate that the activity of the NADPH oxidase was increased in diabetic vessels, which was paralleled by a marked increase in mRNA of the NADPH oxidase subunit gp91phox. gp91phox is expressed in endothelial cells, the adventitia, and inflammatory cells such as macrophages and neutrophils. It seems therefore very likely that a large part of the increases in gp91phox mRNA measured in diabetic vessels is secondary to infiltration with inflammatory cells. Because chelerythrine blocks increased superoxide production in diabetic vessels and PKC has been shown to be involved in the activation of the NADPH oxidase in vascular tissue and neutrophils (PKCβ isoform), it is likely that the increased NADPH oxidase-mediated superoxide production in the endothelial layer, the adventitia, and/or inflammatory cells in diabetic vessels is at least in part mediated by PKC.

Incubation of diabetic vessels with L-NNA led to a reduction in the lucigenin- and coelenterazine-enhanced chemiluminescence signals, which did not differ significantly from L-NNA–treated control vessels, suggesting that NOS-dependent superoxide production rather than increased NADPH oxidase activity mainly accounts for oxidative stress in diabetic tissue. Recent studies from Miller et al. showed that endothelial removal normalized lucigenin-enhanced chemiluminescence signals in vessels from hyperlipidemic animals although a significant increase in superoxide was also observed in the media (using hydroethidine staining). Increased superoxide in smooth muscle cells was detectable only by studying these cells in culture. Given that L-NNA eliminated superoxide mainly in the endothelium and not in the media in the present studies (see Figure 7), it is conceivable that the observed increase in NADPH oxidase activity may originate from smooth muscle cells, which may not be sufficiently detected with lucigenin or coelenterazine. We also speculate that uncoupling of NOS III requires a priming event such as superoxide produced by the NADPH oxidase.

Effects of In Vivo PKC Inhibition on Endothelial Function, NOS III Expression, and NOS-Mediated Superoxide Production

To further address a potential role for PKC in NOS III expression and vascular superoxide production, we treated control rats as well as diabetic animals in vivo with the PKC inhibitor N-benzoyl-staurosporine. The effectiveness of this treatment was verified by its effect on phenylephrine-induced contractions of aortic rings ex vivo. PKC inhibition in vivo markedly improved endothelial dysfunction, normalized vascular superoxide levels, and increased vascular NO bioavailability significantly. These changes cannot be attributed to effects on blood glucose because N-benzoyl-staurosporine did not affect blood glucose levels in control or STZ-treated animals.

The upregulation of NOS III in the setting of diabetes mellitus may represent a counterregulatory effort to increase NO production. This finding is compatible with our previous in vitro observation that PKC activation can increase NOS III expression. Recently, it has also been shown that hydrogen peroxide potently stimulates NOS III expression. It is conceivable that this plays a role in upregulation of NOS III in diabetes, where hydrogen peroxide levels are likely increased as a result of dismutation of the increased levels of superoxide. Of note, we have also found that expression of
the Cu/Zn SOD is increased in diabetic vessels, and this could also serve to increase hydrogen peroxide levels.

As pointed out above, we were able to demonstrate increased superoxide production not only in the endothelial layer but also in the media. Interestingly, recent studies have shown that superoxide may inhibit sGC activity. Thus, it is tempting to speculate that chronic inhibition of sGC due to increased superoxide production may ultimately lead to decreased sGC expression. PKC inhibition in vivo in diabetic rats markedly decreased smooth muscle superoxide production and subsequently normalized sGC expression.

A critical question is whether the effects observed with N-benzoyl-staurosporine treatment are specific for PKC inhibition. Staurosporine has an IC$_{50}$ for PKC inhibition that is almost equal with the IC$_{50}$ which inhibits, for example, tyrosine kinases. Previous studies, however, have demonstrated that N-benzoyl-staurosporine (CGP 41251) has an IC$_{50}$ for PKC inhibition in the range of 6 nmol/L whereas tyrosine kinase inhibition is achieved with an IC$_{50}$ of 3 μmol/L, indicating a high degree of selectivity of this compound. In addition, using Western blot technique and monoclonal antibodies against tyrosine phosphorylation, we were able to demonstrate that with the particular concentration chosen, N-benzoyl-staurosporine had no effect on tyrosine phosphorylation in either control or diabetic vessels. These observations suggest that specific PKC inhibition rather than nonspecific inhibition of tyrosine kinases accounts for observed beneficial vascular actions of this compound. We cannot exclude, however, that N-benzoyl-staurosporine effects are at least in part mediated by inhibitory effects on extracellular signal–regulated protein kinase 2 and activity against platelet-derived growth factor and vascular endothelial growth factor receptors.

Conclusions
The present study demonstrates increased expression of a dysfunctional NOS III as well as increased superoxide production in an experimental model of diabetes mellitus. As potential superoxide sources, we identified an uncoupled dysfunctional NOS as well as increased superoxide production via inhibition of vascular peroxynitrite formation and thus less oxidation of the redox-sensitive NOS III cofactor tetrahydrobiopterin. The reduction of oxidative stress within vascular tissue may also decrease oxidation of LDL, which in turn may favorably influence intracellular L-arginine availability. The reduction of superoxide in vitro by chelerythrine and in vivo by N-benzoyl-staurosporine points to a decisive role for PKC in mediating these phenomena and suggests a therapeutic potential of PKC inhibitors in the prevention or treatment of vascular complications of diabetes mellitus.

Acknowledgments
This work was supported by the Deutsche Forschungsgemeinschaft (Mu 1079/3-1, Hi 712/1-1, and SFB 553/A1). N-benzoyl-staurosporine was kindly provided by Novartis, Basel, Switzerland.

References
18. Munzel T, Kurz S, Rajagopal S, Thoenes M, Berrington WR, Thompson JA, Freeman BA, Harrison DG. Hydralazine prevents nitro-
Mechanisms Underlying Endothelial Dysfunction in Diabetes Mellitus
Ulrich Hink, Huige Li, Hanke Mollnau, Mathias Oelze, Edi Matheis, Mark Hartmann, Mikhail Skatchkov, Friedrich Thaiss, Rolf A. K. Stahl, Ascan Warnholtz, Thomas Meinertz, Kathy Griendling, David G. Harrison, Ulrich Forstermann and Thomas Munzel

_Circ Res._ 2001;88:e14-e22
doi: 10.1161/01.RES.88.2.e14

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/88/2/e14