Reversal of Endothelial Nitric Oxide Synthase Uncoupling and Up-Regulation of Endothelial Nitric Oxide Synthase Expression Lowers Blood Pressure in Hypertensive Rats

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OBJECTIVES

We sought to examine the hypothesis that a pharmacologic up-regulation of endothelial nitric oxide synthase (eNOS) combined with a reversal of eNOS uncoupling provides a protective effect against cardiovascular disease.

BACKGROUND

Many cardiovascular diseases are associated with oxidant stress involving protein kinase C (PKC) and uncoupling of eNOS.

METHODS

Messenger ribonucleic acid (mRNA) expression was analyzed with RNase protection assay or quantitative real-time polymerase chain reaction, vascular nitric oxide (NO) with spin trapping, and reactive oxygen species (ROS) with dihydroethidium fluorescence.

RESULTS

Aortas of spontaneously hypertensive rats (SHR) showed an elevated production of ROS when compared with aortas of Wistar-Kyoto rats (WKY). The aortic expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits (Nox1, Nox2, Nox4, and p22phox) was higher in SHR compared with WKY. In SHR, aortic production of ROS was reduced by the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME), indicating eNOS “uncoupling” in hypertension. Oral treatment with the PKC inhibitor midostaurin reduced aortic Nox1 expression, diminished ROS production, and reversed eNOS uncoupling in SHR. Aortic levels of (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) were significantly reduced in SHR compared with WKY. Midostaurin normalized BH4 levels in SHR. In both WKY and SHR, midostaurin increased aortic expression of eNOS mRNA and protein, stimulated bioactive NO production, and enhanced relaxation of the aorta to acetylcholine. Midostaurin lowered blood pressure in SHR and, to a lesser extent, in WKY; the compound did not change blood pressure in WKY made hypertensive with L-NAME.

CONCLUSIONS

Pharmacologic interventions that combine eNOS up-regulation and reversal of eNOS uncoupling can markedly increase bioactive NO in the vasculature and produce beneficial hemodynamic effects such as a reduction of blood pressure. (J Am Coll Cardiol 2006;47:2536–44) © 2006 by the American College of Cardiology Foundation. 

Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) plays a protective physiological role in the vasculature (1). Besides its vasodilator effects, NO protects the blood vessels from thrombosis by inhibiting platelet aggregation and adhesion. In addition, endothelial NO possesses multiple antiatherosclerotic properties, including: 1) prevention of leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall; 2) decreased endothelial permeability, reduced influx of lipoproteins into the vascular wall, and inhibition of low-density lipoprotein oxidation; and 3) inhibition of deoxyribonucleic acid synthesis, mitogenesis, and proliferation of vascular smooth muscle cells (1). In agreement with this concept, pharmacologic inhibition of eNOS causes accelerated atherosclerosis in rabbits (2) and mice (3), and deficiency in eNOS accelerates the development of atherosclerosis in apolipoprotein E-knockout mice (4).

The NO released from endothelial cells has an important role in controlling blood pressure. Blockade of NO synthesis with pharmacologic NO synthase inhibitors causes significant peripheral vasoconstriction and elevation of blood pressure (5,6). Mice with a disrupted eNOS gene are hypertensive and lack endothelium-dependent NO-mediated vasodilation (7).

Given the beneficial effects of endothelial NO, enhancement of NO production by up-regulating eNOS expression could be of prophylactic or therapeutic interest. However, overexpression of eNOS has been associ-
Animals and treatment with midostaurin. Male SHR and Wistar-Kyoto rats (WKY) were obtained from Charles River Laboratories (Sulzfeld, Germany). The use of animals in this study complies with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the German law on the protection of animals. Midostaurin, formulated as 16% (w/w) preparation in Gelucire 44/14, and Gelucire 44/14 itself were provided by Novartis Pharma AG (Basel, Switzerland). The rats were treated intragastrically by gavage for 5 days with 75 mg/kg/day midostaurin or the control substance Gelucire 44/14. Gelucire 44/14, and Gelucire 44/14 itself were provided by Novartis Pharma AG (Basel, Switzerland). The rats were treated intragastrically by gavage for 5 days with 75 mg/kg/day midostaurin or the control substance Gelucire 44/14.

RNAse protection assay for eNOS messenger ribonucleic acid (mRNA) analyses. Total ribonucleic acid (RNA) was isolated from rat aorta by guanidinium thiocyanate–phenol–chloroform extraction. Endothelial NO synthase mRNA was analyzed with RNase protection assays as described previously (14).
Measurement of vascular content of (6R)-5,6,7,8-tetrahydro-L-biopterin (BH$_4$). After treatment with midostaurin (75 mg/kg/day) for 5 days, aortas were isolated and homogenized in ice-cold lysis buffer (0.1 mol/l Tris-HCl, pH 7.8, containing 5 mmol/l ethylenediamine tetraacetic acid, 0.3 mol/l KCl, 5 mmol/l 1,4-dithioerythritol, 0.5 mM Pefabloc, and 0.01% saponin). Samples were oxidized under either acidic conditions (with 0.2 mol/l HCl containing 50 mmol/l I$_2$) or alkaline conditions (with 0.2 mol/l NaOH containing 50 mmol/l I$_2$). Biopterin content was assessed using high-performance liquid chromatography with fluorescence detection (350 nm excitation,
450 nm emission). BH₄ concentration was calculated as fmol/µg protein by subtracting the biopterin peak resulting from alkaline oxidation (accounting for BH₂) from the biopterin peak resulting from acidic oxidation (accounting for both BH₂ and BH₄).

**Spin trapping of aortic NO using colloid Fe(DETC)₂.** Segments from rat thoracic aorta (10 mm) were cut into 3-mm rings and incubated at 37°C for 30 min in Krebs solution in the presence of 1 µmol/l acetylcholine and 200 µmol/l colloid Fe(DETC)₂ as described previously (11,19). Electron paramagnetic resonance studies were performed on a tabletop X-band spectrometer Miniscope (Magnettech, Berlin, Germany). Recordings were made at 77 K using a Dewar flask (Wilmad, Buena, New Jersey). Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, and 60 s of sweep time. Samples were scanned 10 times.

**Determination of total NO synthesis as nitrite/nitrate in rat serum.** Oxidation products of NO (nitrite and nitrate) were assayed in rat serum as a measure of total NO synthesis using a NOA 280 Nitric Oxide Analyzer (Sievers, Boulder, Colorado) after enzymatic reduction with nitrate reductase (20,21).

**Organ chamber experiments using rat aorta.** After 5 days of treatment with midostaurin or Gelucire 44/14, aortas were isolated from 12 WKY and 18 SHR, cut into 3-mm rings, set up in organ chambers, and precontracted with 100 nmol/l norepinephrine. Endothelium-dependent vasodila-

tor responses to acetylcholine were determined in the absence or presence of L-NAME (1 mmol/l).

**Telemetric measurement of blood pressure.** In 18 conscious WKY and 18 SHR, systolic and diastolic blood pressure as well as heart rate were measured telemetrically with implanted transmitter-coupled pressure transducers in the abdominal aorta as described (22). The rats first received Gelucire 44/14 (vehicle) for one week and, after a washout period of another week, midostaurin for an additional week. Additional WKY were treated with L-NAME 24 mg/kg/day before and during midostaurin treatment.

**Statistics.** Analysis of variance (ANOVA) for repeated measures was used to analyze the curves for the vascular relaxation studies in the organ bath. Other differences were tested for statistical significance by ANOVA followed by Fisher protected least-significant-difference test.

**RESULTS**

Enhanced oxidative stress and increased vascular expression of NADPH oxidases in SHR compared with WKY. Compared with WKY, SHR produced more ROS in the vasculature (Fig. 1A). This increased oxidative stress can in part be explained by an enhanced expression of NADPH oxidases Nox1, Nox2, Nox4, and p22phox in the aorta of SHR compared with WKY (Fig. 2). In SHR, an additional contribution to ROS production came from uncoupled eNOS, because L-NAME markedly reduced ROS production in these hypertensive rats (Figs. 1A and 1B). In WKY, we found no evidence for eNOS uncoupling because L-NAME did not reduce aortic ROS production (Fig. 1A).
Treatment with midostaurin reduced aortic ROS production, diminished vascular Nox1 expression, and reversed eNOS uncoupling in SHR.

Treatment with midostaurin (75 mg/kg/day) for 5 days had no effect on aortic ROS production in WKY but normalized the elevated ROS levels seen in SHR (Fig. 1).

In WKY rats, midostaurin also had no significant effect on the expression of NADPH oxidase (Fig. 2), which mirrored its missing effect on aortic ROS production.

In contrast, midostaurin treatment of SHR for 5 days (75 mg/kg/day) markedly reduced mRNA expression of Nox1 (Fig. 2), a major source of ROS in the vasculature (11,23,24). Midostaurin had no significant effect on the expression of Nox2, Nox4, or p22phox in SHR (Fig. 2).

In midostaurin-treated SHR, L-NAME no longer reduced aortic ROS production (Fig. 1), demonstrating a reversal of eNOS uncoupling by the PKC inhibitor.

**Treatment with midostaurin increased vascular BH$_4$ levels in SHR.** The aortic levels of BH$_4$ were lower in SHR than in WKY. Midostaurin treatment (75 mg/kg/day) for 5 days significantly increased the BH$_4$ content in the aorta of SHR (Fig. 3).

**Treatment of WKY rats and SHR with midostaurin increased eNOS expression.** Oral treatment with 75 mg/kg/day midostaurin for 5 days resulted in a significant increase in eNOS mRNA expression in aorta compared with control rats, as determined with RNase protection assay (Fig. 4). This was true for both SHR and WKY. Immunohistochemical analyses demonstrated the up-regulation of eNOS protein in aortic endothelium of SHR (Fig. 5).

**Treatment with midostaurin increased aortic NO production and serum nitrite/nitrate levels.** Electron paramagnetic resonance studies showed increased amounts of active NO in the aorta of SHR treated with midostaurin (75 mg/kg/day orally for 5 days) (Figs. 6A and 6B). Accordingly, serum levels of nitrite/nitrate, the oxidation products of NO, were also increased by midostaurin (Fig. 6C). The effect in WKY was similar to SHR (Fig. 6C).

**Midostaurin increased endothelium-/eNOS-dependent relaxation of the aorta of WKY and SHR.** In organ chamber experiments with aortas from WKY or SHR, midostaurin enhanced the potency and efficacy of acetylcholine in inducing endothelium-mediated relaxations. The NOS inhibitor L-NAME completely abolished relaxations of control and midostaurin-treated aortas. Figure 7 shows the responses of SHR aortas; aortas from WKY showed closely similar results (n = 6, not shown).

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**Figure 4.** Midostaurin increases eNOS mRNA expression in the aorta of SHR and WKY rats. Rats were treated orally with vehicle or midostaurin (+Mido, 75 mg/kg/day) for 5 days. Endothelial nitric oxide synthase mRNA expression in aortas was analyzed with RNase protection assay. Panels A and C show representative gels of an RNase protection assay. Panels B and D show the results of densitometric analyses. Each column (mean ± SEM) represents data obtained with aortas from nine different rats (***p < 0.001 compared vehicle, tested by ANOVA followed by Fisher protected least-significant-difference test). Abbreviations as in Figures 1 and 2.

**Figure 5.** Midostaurin increases eNOS protein expression in the aorta of SHR. Six SHR were treated orally with vehicle (SHR) and six other SHR with midostaurin (SHR+Mido, 75 mg/kg/day) for 5 days. Endothelial nitric oxide synthase was stained immunohistochemically with an anti-eNOS monoclonal antibody. In the negative control, mouse immunoglobulin G was used instead of the anti-eNOS antibody. Micrographs are shown in 400× magnification. Abbreviations as in Figure 1.
When midostaurin (0.1 to 10 μmol/l) was added acutely to aortas from untreated SHR in organ chambers, it had no effect on vascular tone (n = 4, not shown). Midostaurin treatment lowered blood pressure in SHR and WKY, but not in L-NAME-treated WKY. Vehicle (Gelucire 44/14) treatment had no effect on blood pressure development in SHR (Fig. 8 A; a slight increase was also seen in untreated SHR). Treatment with midostaurin resulted in a reduction of blood pressure (Fig. 8 A). Blood pressure dropped significantly within 24 h of the first dose. The reduction reached a maximum after 48 h and was maintained at this level. This decrease in blood pressure seemed to result from eNOS recoupling and enhanced eNOS expression rather than direct activation of the enzyme, because acute infusion of midostaurin into SHR (for 20 min) produced no decrease in blood pressure (data not shown). The effect of long-term treatment with midostaurin was reversible; blood pressure increased again when midostaurin was withdrawn (Fig. 8 A).

Midostaurin treatment also lowered blood pressure in WKY, although to a lesser extent (Fig. 8B).

In additional experiments, hypertension was induced in WKY with L-NAME. When midostaurin was administered in the presence of L-NAME, the compound had no effect on blood pressure (Fig. 8C).

**DISCUSSION**

The present study demonstrates that a compound with the combined properties of PKC inhibition and eNOS up-regulation leads to a marked elevation of bioactive NO with the expected sequel of vasodilation and blood pressure reduction in SHR.
Cardiovascular diseases, such as hypertension, hypercholesterolemia, and atherosclerosis, are often associated with PKC activation and increased oxidative stress in the vasculature (10). Under these conditions, eNOS can become dysfunctional and uncoupled, producing ROS instead of NO. Such an eNOS uncoupling has been shown to be the major mechanism for endothelial dysfunction observed in vascular diseases, including angiotensin II-induced hypertension, streptozotocin-induced diabetes, and nitrate tolerance (11,14,25). Oxidation of BH4 owing to vascular oxidative stress has been shown to cause BH4 deficiency (26). BH4 is an essential cofactor for eNOS, and a lack of BH4 seems to play a crucial role in eNOS uncoupling (27–29).

Numerous enzyme systems can potentially produce ROS in the vessel wall; however, four enzyme systems seem to predominate. These include the NADPH oxidases, xanthine oxidase, mitochondrial sources, and uncoupled eNOS (30). Interestingly, there appears to be substantial interplay among these sources, such that activation of one can enhance activity of others. Especially the Nox isoforms seem to be upstream of an activation of other ROS-producing enzymes, because the Nox-derived superoxide has been implicated in the activation of xanthine oxidase and in eNOS uncoupling (31).

In the current study, we analyzed the expression of Nox in the aorta of SHR and found that Nox1, Nox2, Nox4, and
p22phox were up-regulated compared with WKY. This may be responsible for the observed oxidative stress in these hypertensive animals. An up-regulation of p22phox in SHR has also been reported in a previous study (32).

In SHR, but not in WKY, treatment with midostaurin markedly reduced the (enhanced) expression of Nox1, but left the other Nox isoforms (subunits) unchanged. Nox1 down-regulation by midostaurin seems to be PKC-dependent, because a similar down-regulation has been observed with other PKC inhibitors such as chelerythrine (11). Nox1 has been established as a major source of ROS in animal models of hypertension (11,23,24). Accordingly, in the current study the down-regulation of Nox1 by midostaurin was associated with a reduced aortic ROS production and a reversal of eNOS uncoupling in SHR. The main reason for this “recoupling” of eNOS could be an elevation of vascular BH4 content (Fig. 3) owing to reduced BH4 oxidation.

Interestingly, midostaurin also increases eNOS gene expression. We have previously demonstrated the same effect in cultured human endothelial cells. Midostaurin-induced eNOS expression is a transcriptional event, because midostaurin increases eNOS promoter activity and has no effect on eNOS mRNA stability (13). The mechanism underlying the midostaurin-induced stimulation of eNOS promoter activity is not clear at this time, but it is independent of the inhibitory effect of the compound on PKC, cAMP-dependent kinase (PKA), CGMP-dependent kinase (PKG), or tyrosine kinases (13). Also, Rho kinase is unlikely to be involved. Rho kinase negatively regulates eNOS mRNA stability by changes in the endothelial actin cytoskeleton (33). Because eNOS mRNA stability is not changed by midostaurin, the effect of midostaurin on eNOS expression is likely to be independent of Rho kinase.

Here we show that such an eNOS up-regulation also occurs in vivo. This also contributes to the observed increase in NO production in vivo and the enhanced vasodilator response to acetylcholine ex vivo. The blood pressure reduction seen in SHR is mediated by a functional and up-regulated eNOS, because the effect is not seen in L-NAME-treated WKY. Also, in apolipoprotein E-knockout mice, treatment with midostaurin increased the expression of eNOS in the vasculature, and the eNOS enzyme was in a functional state (34). The up-regulation of a functional eNOS enzyme was associated with a dilation of resistance arterioles, which is in agreement with the blood pressure lowering effect of midostaurin (34).

Owing to the antithrombotic, antiatherosclerotic, and antihypertensive properties of endothelial NO, the eNOS enzyme could be an interesting target for the prevention or therapy of cardiovascular diseases (10,35). However, a simple up-regulation of eNOS gene expression and/or enzyme activity could be detrimental, because eNOS is often uncoupled under pathologic conditions. An up-regulation of uncoupled eNOS would boost the pre-existing oxidative stress, as has been reported for eNOS-transgenic mice on an apolipoprotein E-knockout background (36).

A more realistic strategy for eNOS-related treatment of vascular disease may be the reversal of eNOS uncoupling. Midostaurin is a compound that achieves this goal by inhibiting PKC. We have previously shown in other animal models that PKC inhibition successfully reduced ROS production, reversed eNOS uncoupling, and reestablished endothelial function (11,14). In a rat model of angiotensin II-induced hypertension, we used the PKC inhibitor chelerythrine (11), and in the rat model of streptozotocin-induced diabetes, we used midostaurin at a dose of 10 mg/kg/day (14). This dose is high enough to inhibit PKC but insufficient to up-regulate eNOS expression.

In cardiovascular diseases, eNOS is often found up-regulated (10). This can be considered an attempt of the organism to compensate for NO deficiency. The attempt usually fails because the up-regulated eNOS uncouples. Activation of PKC seems to play a crucial role in eNOS up-regulation in disease states (as it does in eNOS uncoupling, as shown earlier) (10,37). Consequently, the PKC inhibitors, although restoring eNOS enzymatic function, reduced eNOS expression (14). The result was a functional eNOS but low NO production (14).

This predicament is not encountered with midostaurin. At higher doses, the compound increases eNOS expression in addition to restoring eNOS functionality (via PKC inhibition) (13). Both the (PKC-independent) eNOS up-regulation and the (PKC-dependent) eNOS “recoupling” contribute to the enhanced production of bioactive NO and thus the beneficial effects of midostaurin in vascular pathology. In three previous in vivo studies, three different PKC inhibitors other than midostaurin have been used (11,38,39). None of these compounds had any effect on eNOS expression (13,37), and none changed vascular tone or blood pressure. In a study by Chrissobolis and Sobey (38) with chronically hypertensive rats, PKC inhibition with Ro 31-8220 did not significantly affect basilar artery diameter; in a rat diabetes model, PKC inhibition with LY335351 did not reduce hypertension, although it attenuated the progression of diabetic nephropathy (39).

In conclusion, we postulate the following sequence of events for the action of midostaurin in SHR. Midostaurin reduces Nox1 expression (Fig. 2) and thus ROS generation (Fig. 1). This in turn enhances BH4 levels (Fig. 3) and lets eNOS regain its normal function (“recoupling”) (Fig. 1). Bioactive NO is being produced again, and its production is further increased by the concurrent stimulation of eNOS gene expression (Figs. 4 and 5). This combination of effects leads to blood pressure reduction in SHR.

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