Original Article

Attenuation of Angiotensin II Signaling Recouples eNOS and Inhibits Nonendothelial NOX Activity in Diabetic Mice

Jeong-Ho Oak and Hua Cai

Angiotensin II (Ang II) levels are increased in patients with diabetes, but mechanisms underlying its contribution to diabetic vascular diseases are incompletely understood. We recently reported that in aortic endothelial cells, Ang II induces endothelial nitric oxide synthase (eNOS) uncoupling to produce superoxide ($O_2^{\cdot -}$) rather than nitric oxide (NO·), upon loss of the tetrahydrobiopterin (H_4B) salvage enzyme dihydrofolate reductase (DHFR). Here, we found that streptozotocin-induced diabetic mice had a marked increase in aortic $O_2^{\cdot -}$ production, which was inhibited by *N*-nitro-L-arginine methyl ester hydrochloride, indicating uncoupling of eNOS. Ang II receptor type 1 blocker candesartan or ACE inhibitor captopril markedly attenuated eNOS-derived $O_2^{\cdot -}$ and hydrogen peroxide production while augmenting NO· bioavailability in diabetic aortas, implicating recoupling of eNOS. $O_2^{\cdot -}$ and NO· production were characteristically and quantitatively measured by electron spin resonance. DHFR expression was decreased in diabetic aortas but significantly restored by candesartan or captopril. Either also improved vascular H_4B content and endothelium-dependent vasorelaxation in diabetes. Rac1-dependent NAD(P)H oxidase (NOX) activity was more than doubled in the endothelium-denuded diabetic aortas but was attenuated by candesartan or captopril, indicating that NOX remains active in nonendothelial vascular tissues, although uncoupled eNOS is responsible for endothelial production of $O_2^{\cdot -}$. These data demonstrate a novel role of Ang II in diabetic uncoupling of eNOS and that Ang II–targeted therapy improves endothelial function via the novel mechanism of recoupling eNOS. Dual effectiveness on uncoupled eNOS and NOX may explain the high efficacy of Ang II antagonists in restoring endothelial function. *Diabetes* 56:118–126, 2007

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Ang II, angiotensin II; AT1R, Ang II receptor type 1; CMH, methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine; DETC, diethyldithiocarbamic acid; DHE, dihydroethidium; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; ESR, electron spin resonance; H_4B, tetrahydrobiopterin; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; NOX, NAD(P)H oxidase; ROS, reactive oxygen species; STZ, streptozotocin; VSMC, vascular smooth muscle cell.

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Production of reactive oxygen species (ROS) is increased in both type 1 and type 2 diabetes, contributing not only partially to etiology of diabetes (1–4), but also significantly to diabetic acceleration of vascular diseases (2,4–9). One important mechanism for ROS-induced vascular damage is oxidative degradation of nitric oxide (NO·). This leads to endothelial dysfunction that is characterized by a loss in NO·-dependent vasodilatation (6,9). Endothelium-dependent vasodilatation is impaired in diabetes, and this can be significantly reversed by superoxide dismutase or the antioxidant vitamin C (10–12). In diabetic coronaries, ROS also hinder endothelium-dependent hyperpolarizing factor–mediated vasodilatation (13). What remains to be fully elucidated is the enzyme(s) responsible for production of ROS in diabetes. Recent studies demonstrate that vascular NAD(P)H oxidase (NOX) serves as the predominant source of ROS in the diseased blood vessels (9,14). Uncoupled endothelial nitric oxide synthase (eNOS), however, could be another important source of ROS (see below) in the diabetic endothelium (15), likely downstream of NOX (14,16). Mitochondria as ROS resources have been investigated in nonvascular cells in diabetes. Recent studies suggest that there might be cross talks between different enzymatic sources of ROS, resulting in ROS-dependent self-propagation (16).

By producing NO· to inactivate superoxide ($O_2^{\cdot -}$) and its derivatives, eNOS normally functions as a potent antioxidant enzyme in the vasculature. Together with other anti-inflammatory and antithrombotic properties of NO·, this antioxidant role of eNOS is essential for protection against endothelial dysfunction. Under pathological conditions, however, eNOS can transform into a pro-oxidant, generating $O_2^{\cdot -}$ rather than NO·. This "uncoupling" of eNOS occurs not only in "traditional" vascular diseases such as hypertension and atherosclerosis (14,17–19), but also in diabetic blood vessels (15). The next obvious question is then what is the common mechanism that induces uncoupling of eNOS in different vascular diseases?

Angiotensin II (Ang II) is a circulating vasoconstrictive hormone whose production is often elevated in patients with hypertension and hypercholesterolemia. Infusion of Ang II in apolipoprotein E–deficient or LDL receptor–deficient mice dramatically accelerated atherogenesis (20–22). Of importance, production of Ang II is also increased in patients with diabetes, particularly those with hypertension and renal dysfunction (23,24). It is generally believed that hyperglycemia in the early stages of diabetes

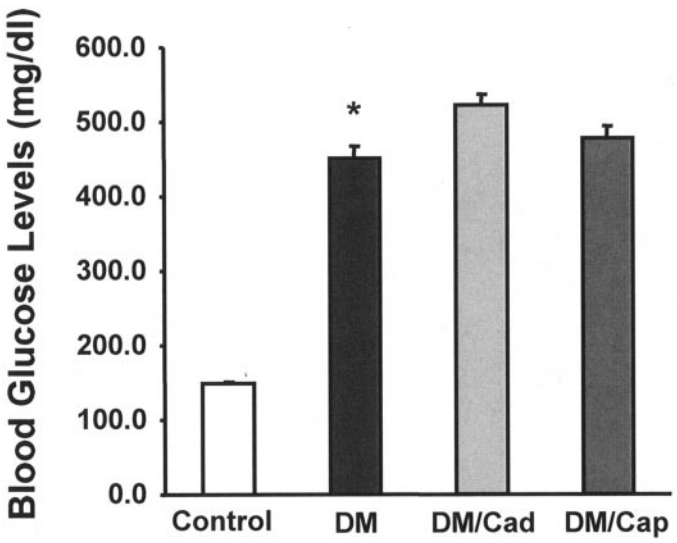

FIG. 1. Blood glucose levels. On day of harvest, blood was collected from tail vein for glucose analysis for each mouse, using OneTouch glucose monitor and matching stripes. $P < 0.05$ vs. control, ANOVA, $n = 36$.

induces hyperfiltration and natriuresis, and this activates Ang II synthesis as a compensatory mechanism to maintain normal blood pressure (25,26). High glucose also potently upregulates expression of the Ang II receptor type 1 (AT1R) (27). This response could sensitize vascular cells to Ang II. It is thus rational to hypothesize that Ang II may mediate common pathological mechanisms involved in the development of different vascular diseases. One possible target could be eNOS.

We recently reported that in cultured aortic endothelial cells, Ang II induces NOX-dependent uncoupling of eNOS (19). Dihydrofolate reductase (DHFR) is the key enzyme responsible for salvation of the eNOS cofactor tetrahydrobiopterin (H_4B) from its inactive, oxidized form. Normal endothelial DHFR is required for basal endothelial H_4B and NO· bioavailability (19). Ang II, however, can induce DHFR deficiency via hydrogen peroxide (H_2O_2)–dependent mechanisms, resulting in a loss of H_4B and, consequently, the uncoupling of eNOS. In keeping with this, scavenging H_2O_2 or overexpressing DHFR was effective in restoring NO· production from eNOS while eliminating eNOS-derived $O_2\cdot^-$ production (19). In view of the potential significance of Ang II in diabetic vascular disease, in the current study, we investigated whether Ang II is involved in diabetic uncoupling of eNOS in vivo, and whether attenuation of Ang II signaling with AT1R blocker candesartan or ACE inhibitor captopril is effective in recoupling eNOS to improve endothelial function. Tissue-specific, differential contribution to total diabetic vascular $O_2\cdot^-$ production of uncoupled eNOS and NOX was also examined.

RESULTS

Hyperglycemia in streptozotocin-induced diabetic mice. Diabetic mice were created by streptozotocin (STZ) administration as described in detail in RESEARCH DESIGN AND METHODS. On day of harvest (7th–8th day after initial STZ injection, same hereafter), blood glucose was elevated to 452.0 ± 15.1 mg/dl in diabetic mice versus 148.4 ± 3.2 mg/dl in the C57BL6 controls (Fig. 1). Candesartan (100 mg · kg^{-1} · day^{-1}, same hereafter) or captopril (100 mg · kg^{-1} · day^{-1}, same hereafter) treatment since day 4 had no significant effect on STZ induction of hyperglycemia (522.5 ± 14.6 or 478.3 ± 14.6 mg/dl, respectively; Fig. 1). For the entire study period, mice were monitored and found comfortable without any signs of severe loss in body weight.

Uncoupling of eNOS in diabetes. Diabetic aortas showed a marked increase in total ROS production, detected by dihydroethidium (DHE) fluorescence (Fig. 2*A*). Whereas *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) (100 μmol/l; inhibitor of NO· synthase) increased ROS production in the controls because of scavenging of NO· from coupled eNOS, it almost completely attenuated ROS in the diabetic mice, strongly suggesting uncoupling of eNOS (Fig. 2*A*). Aortic production of superoxide anion ($O_2\cdot^-$) was also detected specifically using electron spin resonance (ESR) and a cell-permeable $O_2\cdot^-$-specific spin trap methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) (for details, see RESEARCH DESIGN AND METHODS). As shown by representative ESR spectra in Fig. 2*B* and quantitative data in Fig. 2*C* and *D*, aortic $O_2\cdot^-$ production was more than doubled in diabetic mice (3.3 ± 1.6 vs. 7.0 ± 2.6 nmol/l per min/mg wet wt aorta for control vs. diabetic mice), and this response was attenuated to near baseline by L-NAME. L-NAME did not potentiate detectable $O_2\cdot^-$ in control mice, likely because of the rapid degradation of $O_2\cdot^-$ into other species, because L-NAME did enhance total ROS production detected by DHE. Taken together, by directly measuring L-NAME–sensitive aortic ROS in general and $O_2\cdot^-$ production specifically and quantitatively, these data clearly demonstrate that uncoupled eNOS can function as a major enzymatic source for vascular production of ROS.

Candesartan or captopril attenuated ROS and superoxide production in diabetes. In mice treated with agents targeting Ang II signaling, candesartan, or captopril, aortic production of total ROS was significantly attenuated compared with untreated diabetic mice (Fig. 3*A*). Aortic $O_2\cdot^-$ in candesartan- or captopril-treated diabetic mice, detected specifically by ESR, was also significantly reduced to near control levels (Fig. 3*B–D*), strongly suggesting that these agents are effective not only in treating diabetic oxidant stress in general, but also in attenuating eNOS production of ROS. This conclusion is further supported by observations of how these agents modulated eNOS-derived H_2O_2 production (see below).

Candesartan or captopril diminished eNOS-derived H_2O_2 production in diabetes. Aortic H_2O_2 production was detected specifically using an Amplex-Red Assay (for details, see RESEARCH DESIGN AND METHODS). Diabetic mice had a more than fourfold increase in H_2O_2 levels (5.86 ± 1.21 vs. 22.39 ± 3.61 pmol · mg^{-1} protein · min^{-1} for control vs. diabetic mice), which was significantly attenuated by treatment with candesartan or captopril (10.18 ± 3.66 or 8.33 ± 3.62 pmol · mg^{-1} protein · min^{-1}, respectively; Fig. 4*A*). In control aortas, L-NAME increased H_2O_2 production, resulting in negative numbers for L-NAME subtraction (without L-NAME minus with L-NAME) (Fig. 4*B*). By contrast, L-NAME inhibited H_2O_2 production in diabetic mice, confirming eNOS-derived ROS production (Fig. 4*A* and *B*). The eNOS-derived H_2O_2, however, was significantly decreased by treatment with candesartan or captopril to near control levels (Fig. 4*A* and *B*), together with the $O_2\cdot^-$ data described above, strongly suggesting that both agents were effective in "terminating" eNOS production of ROS, in other words, "recoupling" of eNOS.

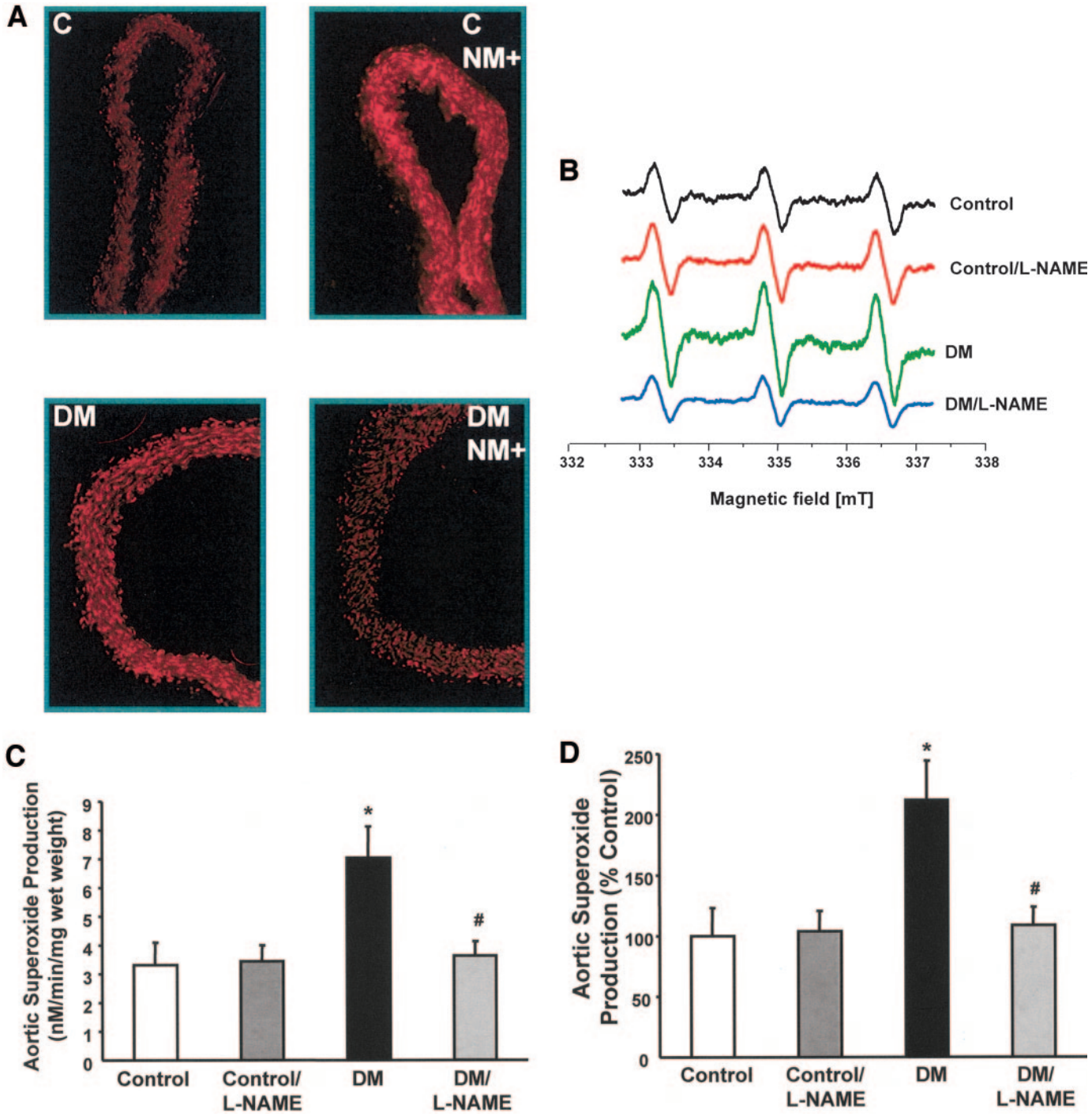

FIG. 2. Uncoupling of eNOS in diabetes (DM). *A*: Aortic ROS production detected by DHE fluorescence. Freshly prepared aortic OCT sections were incubated on glass cover slides with fresh DHE solution (2 μmol/l) in dark for 1 h, in the presence or absence of L-NAME (100 μmol/l) (NM). Fluorescent images of aortic sections were captured using a Zweiss Axioskop fluorescent microscope. C, control. *B*: Representative spectra for aortic $O_2\cdot^-$ detected by ESR and $O_2\cdot^-$-specific spin trap CMH (0.5 mmol/l). Freshly isolated aortic segments (~3 mm) were incubated with spin trapping solution in the presence or absence of L-NAME and then analyzed using ESR. *C* and *D*: Grouped data of aortic $O_2\cdot^-$ production expressed as nanomoles per liter per minute per milligram wet weight or fold increase from control. *$P < 0.05$ vs. control and #$P < 0.05$ vs. diabetic, ANOVA, $n = 6–8$.

Candesartan or captopril restored DHFR expression in diabetes. We recently reported that a loss of endothelial DHFR is involved in Ang II uncoupling of eNOS (19). We next examined whether DHFR expression was regulated in diabetes. As is evident in Fig. 5*A*, aortic DHFR protein expression was clearly downregulated in diabetic mice but was significantly restored to near control levels by candesartan or captopril. Captopril treatment also resulted in a modest increase in eNOS expression (Fig. 5*B*).

Candesartan or captopril improved aortic H_4B content in diabetes. A separate group of mice was subjected to high-performance liquid chromatography quantitation of the eNOS cofactor H_4B as described previously (19). Aortic H_4B content was reduced in diabetes, consistent with the loss of DHFR (Fig. 6*A*). Candesartan or captopril

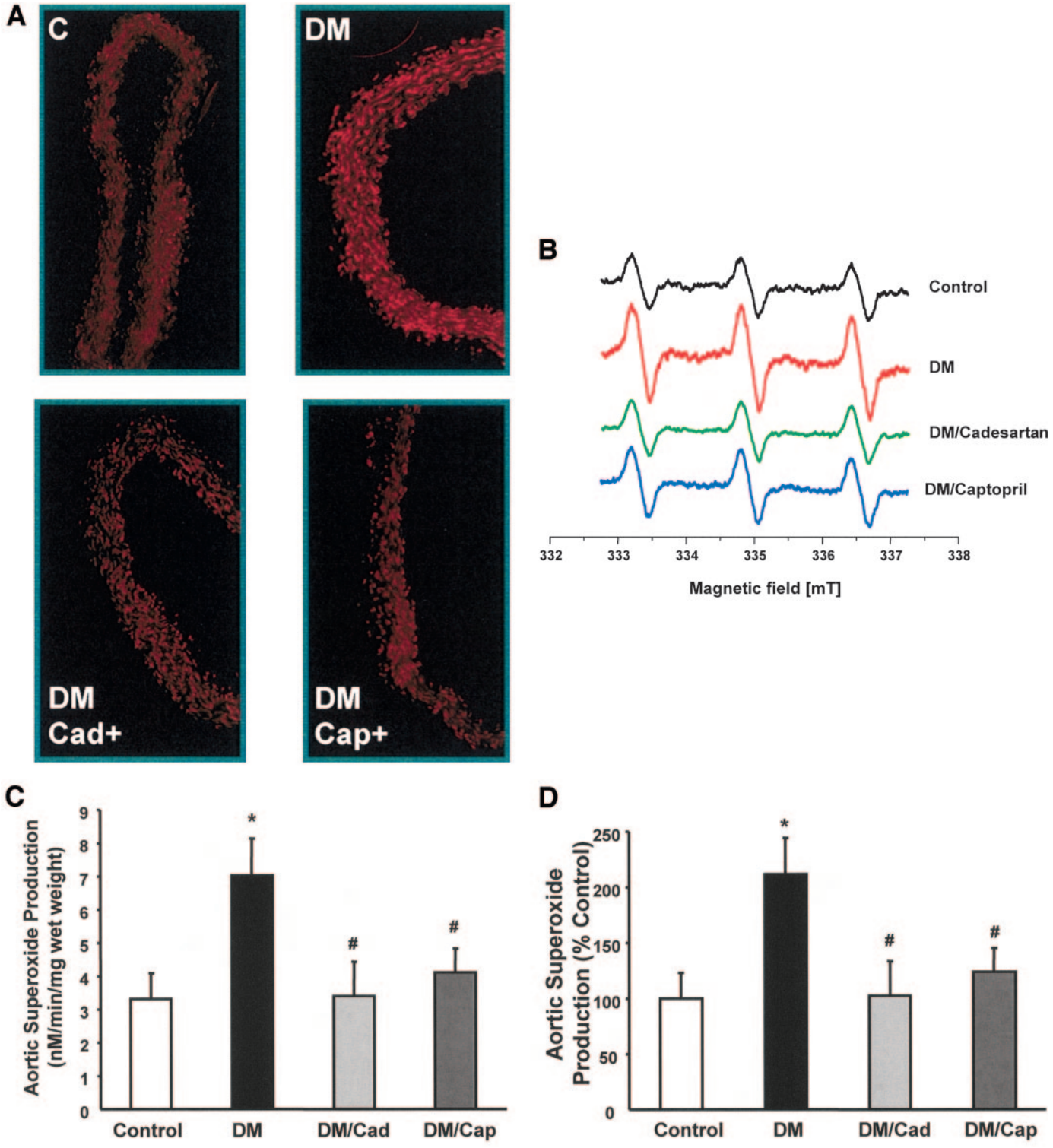

FIG. 3. Candesartan or captopril diminished ROS and superoxide production in diabetes. *A*: **Aortic ROS production detected by DHE fluorescence in control mice (C) and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) (Cad) or captopril (100 mg · kg^{-1} · day^{-1}) (Cap) treatment.** *B*: **Representative spectra for aortic $O_2\cdot^-$ detected by ESR and $O_2\cdot^-$-specific spin trap CMH in control mice and diabetic mice with or without candesartan or captopril treatment.** *C* **and** *D*: **Grouped data of aortic $O_2\cdot^-$ production expressed as nanomoles per liter per minute per milligram wet weight or fold increase from control. *P < 0.05, #P < 0.05 vs. diabetic, ANOVA, n = 6–8.**

treatment significantly restored aortic H_4B content to the control levels (Fig. 6*B*). Of note, both agents also increased total biopterin content (including H_4B and its oxidized forms), although diabetes had little effect on its own (Fig. 6*A*), indicating that attenuation of Ang II signaling might also augment the synthetic pathway of H_4B.

Candesartan or captopril restored bioavailable NO· in diabetes. Aortic NO· production was directly and characteristically detected using ESR (for details, see RESEARCH DESIGN AND METHODS). As shown in representative ESR spectra and grouped data (Fig. 7), diabetic mice had a marked decrease in bioavailable NO· (0.50 ± 0.08 in

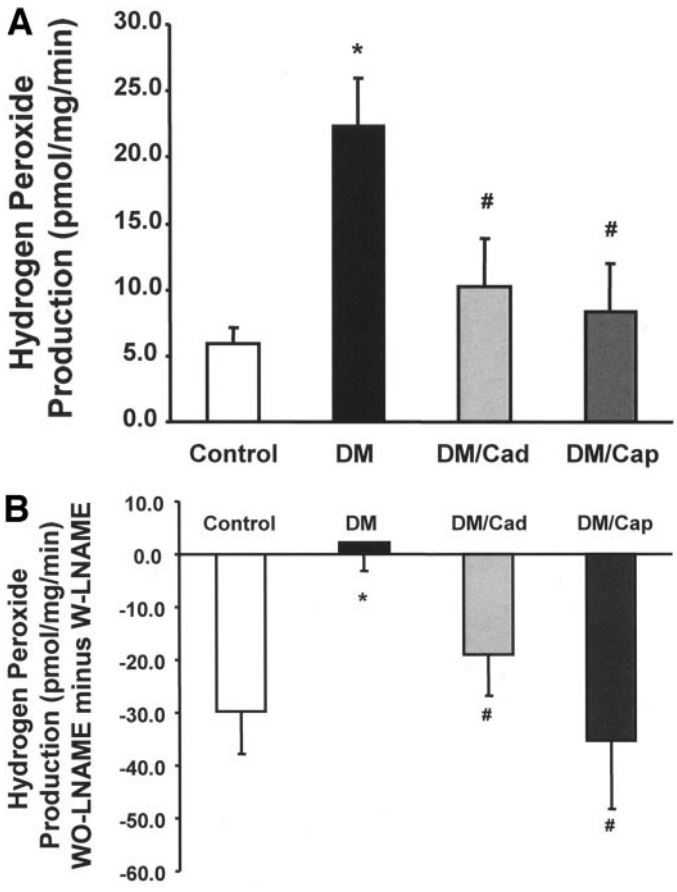

FIG. 4. Candesartan (Cad) or captopril (Cap) diminished eNOS-derived hydrogen peroxide production. *A*: Hydrogen peroxide production determined by Amplex-Red assay in control mice and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) or captopril (100 mg · kg^{-1} · day^{-1}) treatment. *B*: L-NAME–sensitive hydrogen peroxide production. **P* < 0.05 vs. control, #*P* < 0.05 vs. diabetic, ANOVA, *n* = 8.

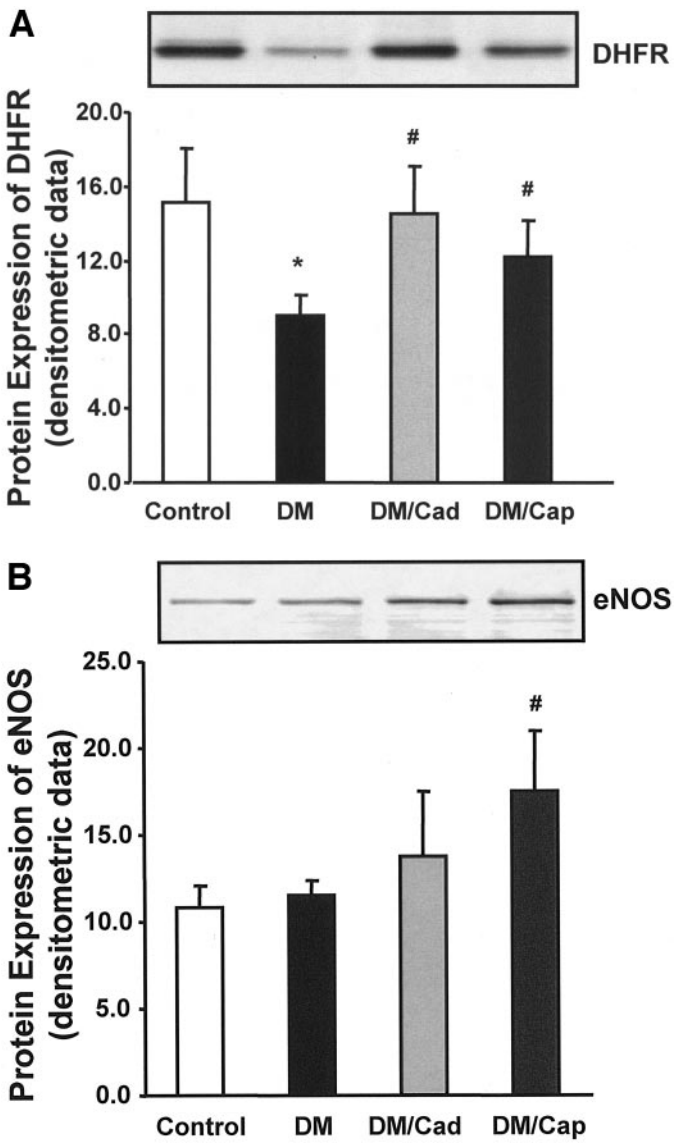

FIG. 5. Effects of candesartan (Cad) and captopril (Cap) on diabetes regulation of DHFR and eNOS. *A*: Aortic DHFR expression in control mice and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) or captopril (100 mg · kg^{-1} · day^{-1}) treatment. Forty micrograms aortic proteins was separated in SDS-PAGE and probed with a specific monoclonal antibody for DHFR. *Top panel* shows a representative Western blot, and *bottom panel* shows grouped densitometric data. *B*: Aortic eNOS expression in control mice and diabetic mice without treatment or treated with candesartan or captopril. **P* < 0.05, #*P* < 0.05 vs. diabetic, ANOVA, *n* = 10–12.

diabetic mice vs. 0.72 ± 0.10 nmol/mg dry wt), and this response was significantly attenuated by treatment with candesartan or captopril (restored NO· to 0.66 ± 0.14 or 0.73 ± 0.13 nmol/mg dry wt, respectively). Together with the inhibitory effects of these agents on eNOS-derived ROS production (Fig. 5*A* and *B*), these data further established the effectiveness of candesartan or captopril in recoupling eNOS.

Candesartan or captopril improved endothelium-dependent vasorelaxation. As expected, diabetic mice had an impaired endothelium-dependent vasorelaxation (Fig. 8). Treatment with candesartan or captopril, however, significantly restored vasodilatation in response to acetylcholine (Fig. 8), indicating that in our model system, these agents are beneficial in improving endothelial function. Data described earlier seem to suggest that this phenomenon is at least partially mediated by a novel mechanism of recoupling eNOS.

Rac1-dependent NOX activity was increased in endothelium-denuded diabetic aortas but attenuated by candesartan or captopril. Data described in Fig. 2 and those reported by Guzik et al. (28) demonstrate that L-NAME completely inhibited $O_2\cdot^-$ production in mouse and human diabetic arteries, respectively, indicating a potent response of eNOS uncoupling. On the other hand, it is possible that the spin trap cannot penetrate into subendothelial cell layers of the blood vessels, i.e., vascular smooth muscle cells (VSMCs). If it only reached endothelium, complete blockade of $O_2\cdot^-$ only means that uncoupled eNOS is the major or sole source of ROS in the diabetic endothelium. Whether NOX remains active in nonendothelial diabetic vascular tissues requires further investigation. Thus, we detected $O_2\cdot^-$ production using ESR in intact or endothelium-denuded aortas. Whereas NSC23766 (200 nmol/l, Rac1 antagonist)-sensitive $O_2\cdot^-$ production in the intact aortas was not different between control and diabetes, it was more than doubled in the endothelium-denuded diabetic aortas compared with the controls. These observations seem to suggest that the spin trap was absorbed better in VSMCs after endothelium removal and, more importantly, that NOX remained active in nonendothelial diabetic vascular tissues (Fig. 9). In

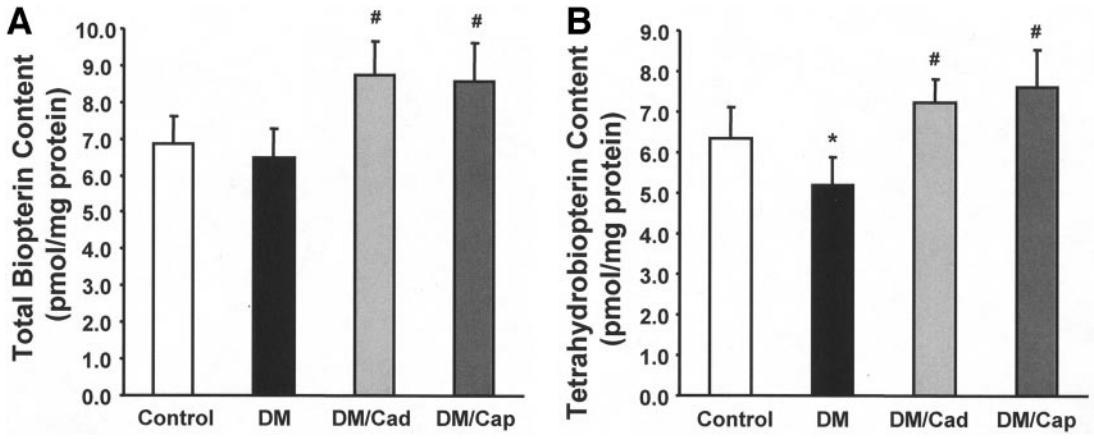

FIG. 6. Candesartan (Cad) or captopril (Cap) restored vascular H_4B content in diabetes. Fresh aortic homogenates from control mice and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) or captopril (100 mg · kg^{-1} · day^{-1}) treatment were subjected to high-performance liquid chromatography analysis of cellular biopterin contents as described in RESEARCH DESIGN AND METHODS. *A*: Total biopterin content. *B*: H_4B content. *$P < 0.05$, #$P < 0.05$ vs. diabetic, ANOVA, $n = 12$.

addition, this difference was abolished in mice treated in vivo with candesartan or captopril, suggesting that these agents are effective in blocking VSMC NOX activity (Fig. 9).

DISCUSSION

Data from the present study clearly demonstrate a novel role of Ang II in diabetic uncoupling of eNOS in vivo and that attenuation of Ang II signaling improves endothelial function by restoring NO· and eliminating eNOS-derived ROS production. Candesartan or captopril, Ang II signaling antagonist, is extremely effective in recoupling eNOS. These agents also markedly attenuated nonendothelial NOX activity in diabetic mice. The dual effectiveness on both enzymatic systems of uncoupled eNOS and NOX may underlie the high efficacy of Ang II–targeting therapies in treating endothelial dysfunction.

Using three different methods to measure L-NAME–sensitive productions of ROS in general, or $O_2\cdot^-$ or H_2O_2 specifically, uncoupling of eNOS in vivo was fully characterized. Our observations are consistent with the earlier study by Hink et al. (15), in which lucigenin chemiluminescent signal of $O_2\cdot^-$ was first found to be sensitive to L-NAME in diabetes. The important novel findings, however, are at mechanistic levels, demonstrating that diabetic uncoupling of eNOS is at least partially attributed to Ang II. Disruption of Ang II signaling was effective in restoring eNOS function from its uncoupled state, accounting for a

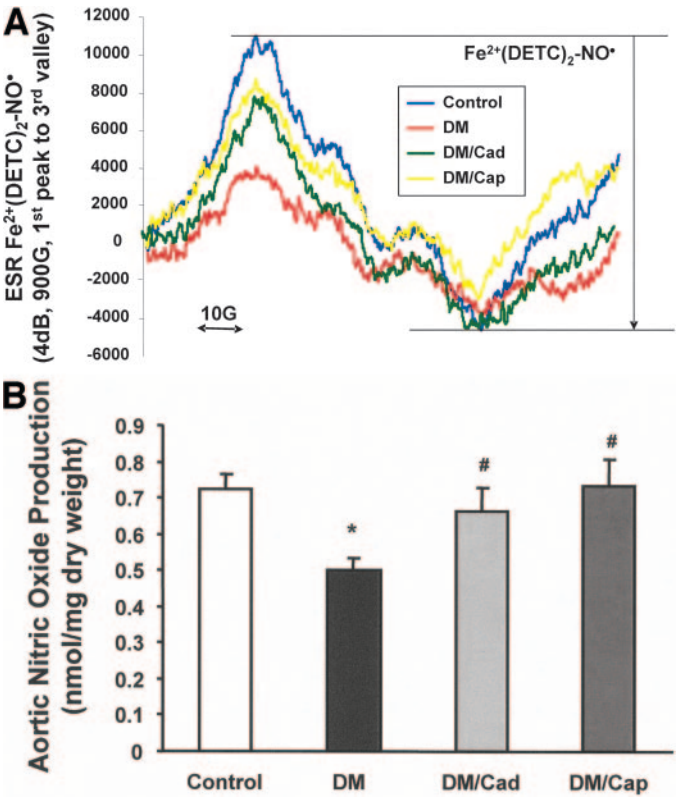

FIG. 7. Candesartan (Cad) or captopril (Cap) restored aortic NO· bioavailability in diabetes. *A*: Representative ESR spectra for aortic NO· production in control mice and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) or captopril (100 mg · kg^{-1} · day^{-1}) treatment, detected using the NO·-specific spin trap $Fe^{2+}(DETC)_2$. *B*: Grouped data for NO· production. *$P < 0.05$, #$P < 0.05$ vs. diabetic, ANOVA, $n = 8$.

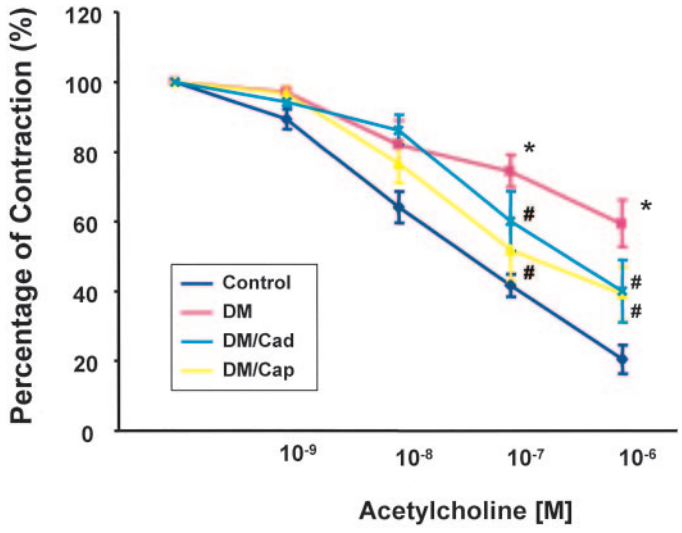

FIG. 8. Candesartan (Cad) or captopril (Cap) improved endothelium-dependent vasodilatation in diabetes. Aortas were precontracted with phenylephrine (5 μmol/l) before vasodilatation analysis in response to acetylcholine in control mice and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) or captopril (100 mg · kg^{-1} · day^{-1}) treatment. *$P < 0.05$, #$P < 0.05$ vs. diabetic, ANOVA, $n = 8$.

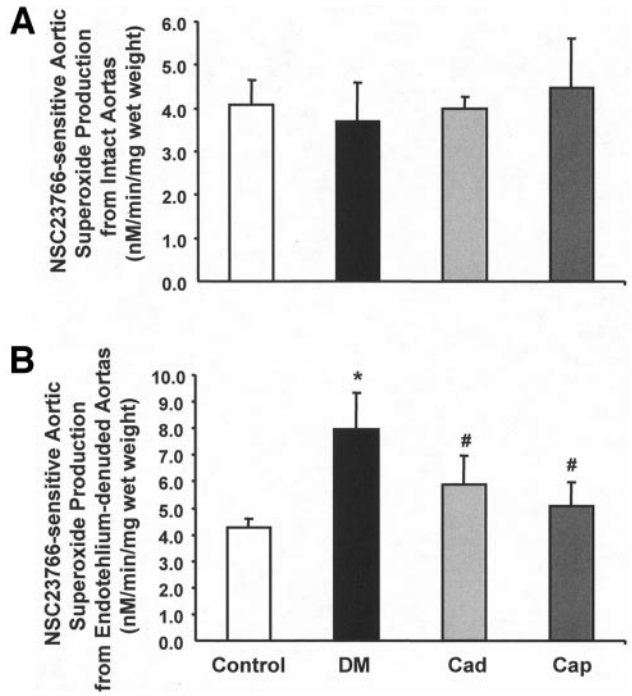

FIG. 9. Rac1-dependent NOX activity was increased in nonendothelial diabetic vascular tissues but attenuated by candesartan (Cad) or captopril (Cap). Aortic $O_2\cdot^-$ production from intact (*A*) or endothelium-denuded (*B*) aortas was detected by ESR in control mice and diabetic mice (DM) with or without candesartan (100 mg · kg^{-1} · day^{-1}) or captopril (100 mg · kg^{-1} · day^{-1}) treatment. $\Delta O_2\cdot^-$ levels with or without preincubation with Rac1 antagonist NSC23766 (200 nmol/l, 90 min) was presented. *$P < 0.05$, #$P < 0.05$ vs. diabetic, ANOVA, $n = 6$.

novel mechanism whereby ACE inhibitors or AT1R blockers improve endothelial function.

Many of the pathological effects of Ang II are mediated by ROS (9). For example, besides oxidative inactivation of NO·, redox-sensitive activations of p38 mitogen-activated protein kinase and Akt mediate Ang II induction of VSMC hypertrophy (29). On the other hand, attenuation of Ang II signaling improved endothelium-dependent vasorelaxation (30) and slowed the progressive intima-media thickening of the common carotid artery (an index of early atherosclerosis) in diabetic patients (31,32). Diabetic acceleration of atherosclerosis in apolipoprotein E–deficient mice was also prevented by inhibition of ACE (33). These beneficial effects of attenuating Ang II signaling have been mostly attributed to their inhibitory effects on NOX activity (9,34). In cultured aortic endothelial cells, we found that Ang II activation of endothelial NOX precedes uncoupling of eNOS (19). Data in the present study further demonstrate that by restoring H_4B contents via its salvage enzyme DHFR, agents attenuating Ang II signaling are directly effective in recoupling eNOS. So these agents not only "stop" uncoupling of eNOS by interfering with endothelial activation of NOX but also "reverse" eNOS uncoupling by upregulating H_4B salvage pathway. It is thus interesting to speculate that attenuation of Ang II signaling is more beneficial than simple scavenging ROS with nonspecific antioxidants.

In addition, the Ang II signaling attenuators also significantly inhibited nonendothelial vascular NOX activity in diabetic mice. In intact aortas where spin traps may only penetrate to endothelial layer, L-NAME abolished $O_2\cdot^-$ signal in diabetic mice, suggesting that uncoupled eNOS is the sole source of ROS in the diabetic endothelium. In the endothelium-denuded aortas, however, Rac1-dependent NOX activity ($O_2\cdot^-$ production sensitive to NSC23766) was more than doubled in diabetes but abolished by candesartan or captopril, indicating that these agents are also effective in shutting down nonendothelial NOX activity. These observations seem to share similarity with studies by Wendt et al. (35) in which activity of NOX isoform 1 was found increased in membrane fraction of diabetic aortic homogenates.

Of note, captopril is modestly more beneficial than candesartan in terms of improving NO· bioavailability. This may be related to the additional antioxidant capacity of captopril. In addition, Ang II signaling antagonism was previously found to inhibit bradykinin breakdown (36). This pathway may interact with the "recoupling of eNOS" mechanism to contribute to correction of endothelial dysfunction.

This study also presents additional in vivo evidence that endothelial DHFR is important for eNOS function. We reported earlier that upon RNAi silencing of DHFR expression, endothelial H_4B content and NO· bioavailability are significantly reduced (19). Loss of DHFR also mediates Ang II uncoupling of eNOS in cultured aortic endothelial cells (19). The present study indicates that in "prevascular disease" condition, namely STZ-induced diabetes, DHFR downregulation can occur to result in H_4B deficiency. Candesartan or captopril had no effect on blood glucose levels, although they both restored DHFR expression. These data suggest Ang II–dependent regulation of DHFR that is reversible by attenuation of Ang II signaling. It is interesting to speculate that loss of DHFR may also occur in other pathophysiological conditions in which Ang II production is elevated.

In addition to oxidant-generating pathways characterized in the current study, accumulating studies have suggested that other pathways, such as PKC-dependent signal transduction (37), aldose reductase (38), and peroxisome proliferator–activated receptor activation (39), may also contribute to development of diabetic vascular diseases. Potential interactions among these pathways are of potential significance and warrant future investigation. Mitochondrial oxidant generation may occur either downstream or upstream of NOX and uncoupled eNOS in different organ systems, contributing to total oxidant stress in diabetes (16). Current literature seems to suggest that mitochondrion-derived oxidants are more relevant to diabetic cardiomyopathy (40,41).

In summary, data in the present study demonstrate a novel role of Ang II in diabetes uncoupling of eNOS and that improvement in endothelial function by Ang II signaling attenuation is at least partially attributed to recoupling of eNOS. Ang II uncoupling of eNOS may represent a common mechanism whereby eNOS dysfunction occurs in vascular diseases. Attenuation of Ang II signaling is also effective in blocking nonendothelial NOX activity, suggesting that the high efficacy of these agents are likely due to their dual effectiveness on uncoupled eNOS and NOX.

RESEARCH DESIGN AND METHODS

Diabetic mice and drug interventions. Male C57BL/6J mice (6–8 weeks old) were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were housed in a pathogen-free condition. The use of animals and experimental procedure were approved by the Institutional Animal Care and Usage Committee at the University of Chicago. Diabetes was induced by tail vein injection of STZ (100 mg/kg) dissolved in 50 μl 0.9% saline immediately before use, once a day for 3 days (15,42,43). Blood glucose was determined using the

OneTouch Ultra blood glucose meter (Lifescan) at baseline and on day 4 after STZ injection for each individual mouse. On day 4, STZ-induced diabetic mice were injected with candesartan (44) or captopril (45) dissolved in 0.9% saline via tail vein at 100 mg · kg^{-1} · day^{-1} for 3 days. By days 7–8, animals were killed using CO_2 inhalation and whole aorta was removed, cleared from surrounding connective tissues, and cut transversely into 2-mm rings for subsequent experiments. This model of diabetes is characterized by acute hyperglycemia. No renal dysfunction occurs during the study period of 7 days (46).

DHE detection of aortic ROS production. Frozen sections of aortic rings were incubated with DHE (2 μmol/l; Sigma) for 1 h and rinsed off excessive reagent, and the fluorescent images were captured using a Zweiss Axioskop fluorescent microscope. In some cases, vessels were incubated with the NOS inhibitor L-NAME (100 μmol/l; Sigma) before incubation with DHE.

ESR measurement of aortic superoxide production. Freshly isolated aortas were placed into chilled modified Krebs/HEPES buffer (99.01 mmol/l NaCl, 4.69 mmol/l KCl, 2.50 mmol/l $CaCl_2$, 1.20 mmol/l $MgSO_4$, 1.03 mmol/l KH_2PO_4, 25.0 mmol/l $NaHCO_3$, 20.0 mmol/l Na-HEPES, and 5.6 mmol/l glucose [pH 7.4]) and cleaned of excessive adventitial tissue, with care taken not to injure the endothelium. The specific $O_2{\cdot}^-$ spin trap CMH (500 μmol/l; Noxygen) solution was prepared freshly in nitrogen gas bubbled Krebs/HEPES buffer containing diethyldithiocarbamic acid (DETC; 5 μmol/l; Sigma) and deferoxamine (25 μmol/l; Sigma). Aortic segment (~3 mm) was then mixed with the spin trap solution and loaded into glass capillary (Fisher Scientific) for analysis of $O_2{\cdot}^-$ signal (CM· formed after trapping $O_2{\cdot}^-$) using the ESR spectrometer (Miniscope MS200; Magnettech, Berlin, Germany). Some of the aortic segments were preincubated with freshly prepared L-NAME (100 μmol/l) for 1 h at 4°C before addition of spin trap solution. The ESR settings used were as follows: bio-field 3,350, field sweep 45.00 G (1 G = 0.1 mT), microwave frequency 9.78 GHz, microwave power 7 dB (20 mW), modulation amplitude 3,000 mG, 4,096 points of resolution, and receiver gain 700.

In additional experiments, identical $O_2{\cdot}^-$ measurements were performed using intact or endothelium-denuded aortas in the presence or absence of Rac1 antagonist NSC23766 (200 nmol/l, 90 min). NSC23766-sensitive $O_2{\cdot}^-$ levels are presented in Fig. 9.

Amplex-Red assay for hydrogen peroxide production. Freshly isolated aortic rings (4 × 2 mm) were used for assessment of hydrogen peroxide production using a fluorometric horseradish peroxidase assay (Amplex-Red assay; Molecular Probes). Fluorescence was measured (excitation 530 nm and emission 590 nm) after 1 h of incubation at 37°C in the dark against background fluorescence of buffer. Polyethylene glycol–conjugated catalase (300 units/ml; Sigma) inhibitable fraction reflects specific hydrogen peroxide signal. Some of the aortic segments were preincubated with freshly prepared L-NAME (100 μmol/l). The rate of hydrogen peroxide production was presented as picomoles per milligram protein per minute after calculation, according to a standard curve generated using fresh hydrogen peroxide in reaction buffer (47).

Western analysis of eNOS and DHFR. Freshly isolated aortas were homogenized in buffer containing protease inhibitors and 1% Triton (48). Forty micrograms protein was separated in 10% SDS-PAGE and transferred to nitrocellulous membranes (Amersham Biosciences). The membranes were then probed with eNOS or DHFR antibodies as shown previously (19,49).

ESR of aortic nitric oxide production. Freshly isolated aortic rings (6 × 2 mm) were incubated with freshly prepared NO·-specific spin trap $Fe^{2+}(DETC)_2$ (0.5 mmol/l) in modified Krebs/HEPES buffer at 37°C for 60 min (for spin trap and buffer recipe, see above and previous publication [19]), in the presence or absence of calcium ionophore A23187 (10 μmol/l). After the incubation, the aorta in Krebs/HEPES buffer was snap frozen in liquid nitrogen and loaded into a finger Dewar for analysis with ESR spectrophotometer. The instrument settings were as follows: bio-field 3,280, field sweep 77.54 G (1 G = 0.1 mT), microwave frequency 9.78 GHz, microwave power 4 dB (40 mW), modulation amplitude 10 G, 4,096 points of resolution, and receiver gain 900.

Assessment of vascular reactivity. Freshly prepared aortic rings (2 mm) were placed in organ baths containing modified Krebs/HEPES buffer (for recipe, see above), aerated with a mixture of 95% oxygen/5% carbon dioxide, and maintained at 37°C. After being kept under 5 mN tension for 90 min to stabilize, cumulative tension was measured by a Graz Tissue Bath System (Hugo Sachs Elektronik/Harvard Apparatus, March Hugstetten, Germany) connected to an MP100 workstation (BioPac Systems). Relaxation curve to acetylcholine (10^{-9}–10^{-6} mol/l) was assessed in aortic segment after contraction by phenylephrine (5 μmol/l). Data acquisition process and postacquisition calculations were performed with AcqKnowledge software (BioPac Systems).

Quantification of aortic H_4B content by high-performance liquid chromatography. Aortas were lysed by using trichloroacetic acid containing 10 mmol/l dithiothreitol. Lysates were subjected to differential oxidation in acidic (0.2 mol/l trichloroacetic acid) or alkalytic (0.1 mol/l NaOH) solutions containing 2.5% I_2/10% KI or 0.9% I_2/1.8% KI as shown previously (14,19). After centrifugation, 20 μl supernatant was injected into a high-performance liquid chromatography system with a fluorescent detector (Schimadzu model RF-10Axl; Fisher). A 250- × 4.6-mm C18 column (Alltech, Deerfield, IL) was used with a 5% methanol solution as a mobile phase at a flow rate of 1.5 ml/min. Fluorescent detection is carried out using excitation and emission wavelengths of 350 and 450 nm, respectively. Total biopterin and H_4B content was calculated according to a standard curve generated using the same preparation procedure and presented as picomoles per milligram cellular protein.

Statistical analysis. Differences among different groups of means were compared with ANOVA. When differences were indicated, the Dunnet's post hoc test was used. Statistical significance was set for $P < 0.05$. All grouped data shown in the figures were presented as means ± SE.

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