# Endothelial dihydrofolate reductase: Critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase

**Karel Chalupsky and Hua Cai***

Section of Cardiology, Department of Medicine, Division of Biological Sciences and Pritzker School of Medicine, University of Chicago, Chicago, IL 60637

Edited by Salvador Moncada, University of London, London, United Kingdom, and approved May 9, 2005 (received for review December 21, 2004)

**Recent studies demonstrate that oxidative inactivation of tetrahydrobiopterin ($H_4B$) may cause uncoupling of endothelial nitric oxide synthase (eNOS) to produce superoxide ($O_2^{\bullet-}$). $H_4B$ was found recyclable from its oxidized form by dihydrofolate reductase (DHFR) in several cell types. Functionality of the endothelial DHFR, however, remains completely unknown. Here we present findings that specific inhibition of endothelial DHFR by RNA interference markedly reduced endothelial $H_4B$ and nitric oxide ($NO^{\bullet}$) bioavailability. Furthermore, angiotensin II (100 nmol/liter for 24 h) caused a $H_4B$ deficiency that was mediated by $H_2O_2$-dependent down-regulation of DHFR. This response was associated with a significant increase in endothelial $O_2^{\bullet-}$ production, which was abolished by eNOS inhibitor *N*-nitro-L-arginine-methyl ester or $H_2O_2$ scavenger polyethylene glycol-conjugated catalase, strongly suggesting $H_2O_2$-dependent eNOS uncoupling. Rapid and transient activation of endothelial NAD(P)H oxidases was responsible for the initial burst production of $O_2^{\bullet}$ (Rac1 inhibitor NSC 23766 but not an *N*-nitro-L-arginine-methyl ester-attenuated ESR $O_2^{\bullet-}$ signal at 30 min) in response to angiotensin II, preceding a second peak in $O_2^{\bullet-}$ production at 24 h that predominantly depended on uncoupled eNOS. Overexpression of DHFR restored $NO^{\bullet}$ production and diminished eNOS production of $O_2^{\bullet-}$ in angiotensin II-stimulated cells. In conclusion, these data represent evidence that DHFR is critical for $H_4B$ and $NO^{\bullet}$ bioavailability in the endothelium. Endothelial NAD(P)H oxidase-derived $H_2O_2$ down-regulates DHFR expression in response to angiotensin II, resulting in $H_4B$ deficiency and uncoupling of eNOS. This signaling cascade may represent a universal mechanism underlying eNOS dysfunction under pathophysiological conditions associated with oxidant stress.**

hydrogen peroxide | tetrahydrobiopterin | superoxide

It has become apparent over the past decade that excessive production of reactive oxygen species (ROS) contributes to cardiovascular pathogenesis (1–3). Indeed, scavenging ROS restored endothelial function in animal models of hypertension and atherosclerosis (3). Antioxidant trials however produced mixed results in patients regarding disease prevention or regression, possibly because of incomplete understanding of antioxidant chemistry *in vivo*, inappropriate criteria for patient recruitment (4, 5), or nonspecific nature of most antioxidants. The latter is an important issue to consider because it has remained unclear whether one particular ROS is dominantly important in pathological signaling (1). Additionally, mixed results may suggest that some downstream pathological events are not readily reversible by removal of ROS. Recent studies demonstrate that endothelial nitric oxide synthase (eNOS) (and other isoforms of NOS) is capable of transforming into a prooxidant, $O_2^{\bullet-}$-generating enzyme *in vitro* and *in vivo* (1, 3, 6–15). This phenomenon is now referred to as "eNOS uncoupling." It is interesting to speculate that unsatisfactory outcomes of some antioxidant therapies are partially due to their ineffectiveness in restoring eNOS function in the vasculature or, in other words, "recoupling" of eNOS.

Although molecular mechanisms underlying eNOS uncoupling remain to be fully elucidated, a deficiency in eNOS cofactor tetrahydrobiopterin ($H_4B$) appears to be a major cause (6, 8, 10–14). The precise mechanisms as to how $H_4B$ became persistently deficient under pathological conditions, however, remain obscure. Dihydrofolate reductase (DHFR) catalyzes regeneration of $H_4B$ from its oxidized form, dihydropterin, in several cell types (16, 17). Genes encoding DHFR have also been cloned. Functionality of the endothelial DHFR, however, remains completely unknown. Here we found that specific inhibition of endothelial DHFR through RNA interference (RNAi) led to a marked reduction in endothelial $H_4B$ and $NO^{\bullet}$ bioavailability. In hypertension- or diabetes-related atherosclerosis, angiotensin II is a major pathological player (5). Many of the untoward effects of angiotensin II have been attributed to its ability to stimulate ROS production from vascular NAD(P)H oxidases (1, 2). Here we found that angiotensin II rapidly and transiently activated endothelial NAD(P)H oxidases to produce $O_2^{\bullet-}$ within 30 min, leading to $H_2O_2$-dependent down-regulation of DHFR, $H_4B$ deficiency, and a second phase $O_2^{\bullet-}$ production from uncoupled eNOS after 24 h. Overexpression of DHFR restored $NO^{\bullet}$ production and abolished eNOS production of $O_2^{\bullet-}$ in angiotensin II-stimulated cells. These data characterize an essential role of endothelial DHFR in maintaining $H_4B$ and $NO^{\bullet}$ bioavailability in the endothelium. The observation that NAD(P)H oxidase-derived $H_2O_2$ mediates angiotensin II uncoupling of eNOS via down-regulation of DHFR may have general applicability to other pathological conditions in which eNOS dysfunction occurs.

## Experimental Procedures

**Materials.** Monoclonal antibodies for DHFR and eNOS were purchased from Research Diagnostics (Flanders, NJ) and Transduction Laboratories (San Jose, CA), respectively. Fluorescent, double-stranded small interfering RNA (siRNA) targeting human DHFR (nucleotides 35–54, 5′-cccagaacatgggcatcggc-3′; GenBank accession no. NM_000791) and scrambled control siRNA recognizing none of the known human or bovine sequences were customized at Dharmacon Research (Lafayette, CO). $H_4B$ was obtained from Sigma–Aldrich and Schircks Laboratories (Jona, Switzerland). Rac1 inhibitor NSC 23766 was obtained from EMD Biosciences (San Diego). Other chemicals were purchased from Sigma at the highest purity available.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ROS, reactive oxygen species; $H_4B$, tetrahydrobiopterin; NOS, nitric oxide synthase; eNOS, endothelial NOS; DHFR, dihydrofolate reductase; RNAi, RNA interference; siRNA, small interfering RNA; ACE, angiotensin converting enzyme; GTP-CH I, GTP cyclohydrolase I; PEG, polyethylene glycol; PEG-CAT, PEG-conjugated catalase; L-NAME, *N*-nitro-L-arginine-methyl ester.

*To whom correspondence should be addressed at: Section of Cardiology, Department of Medicine, Division of Biological Sciences and Pritzker School of Medicine, University of Chicago, 5841 South Maryland Avenue, MC6088, Chicago, IL, 60637. E-mail: hcai@medicine.bsd.uchicago.edu.





www.pnas.org/cgi/doi/10.1073/pnas.0409594102

Human DHFR cDNA in pcDNA3.1 vector was a kind gift from Edward Chu and Ningwen Tai (Yale University, New Haven, CT). Aorta from mice deficient in angiotensin converting enzyme ($ACE^{-/-}$) were kindly provided by Kenneth E. Bernstein and Hong Xiao (Emory University, Atlanta).

**Cell Culture and Western Analysis.** Bovine aortic endothelial cells (Cell Systems, Kirkland, WA) were cultured to confluence as described in refs. 18–20 and starved in 5% media overnight before experiments. For Western analysis, 20–40 $\mu$g of protein was separated by 10% SDS/PAGE, transferred to nitrocellulose membranes, and probed with DHFR (1:250) or eNOS (1:1,000) antibodies after standard Western procedure (21).

**Transfection of Endothelial Cells with DHFR siRNAs or pcDNA3.1-DHFR.** Proliferating endothelial cells at 85% confluency were incubated with siRNA-oligofectamine mixtures for 4 h before addition of growth media. Cells were harvested 48 h later for analysis of DHFR expression, $H_4B$ content, and $NO^{\bullet}$ production. Additionally, proliferating cells were transfected with 8 $\mu$g of pcDNA3.1-DHFR plasmid by using Lipofectamine for 48 h before angiotensin II stimulation and analysis of endothelial $O_2^{\bullet-}$ and $NO^{\bullet}$ productions.

**ESR Detection of NO Radical.** Bioavailable $NO^{\bullet}$ radical was directly measured by using ESR (18, 20). In brief, endothelial cells cultured on Petri dishes were rinsed with modified Kreb's/Hepes buffer and incubated with freshly prepared, $NO^{\bullet}$-specific spin trap $Fe^{2+}$(diethyldithiocarbamate)$_2$ colloid (0.5 mmol/liter) for 60 min. Gently collected suspensions of cells were snap-frozen in liquid nitrogen and loaded into a finger Dewar for analysis with a Miniscope 200 ESR spectrophotometer (Magnettech, Berlin) at the following settings: biofield, 3267; field sweep, 100 G (1 G = 0.1 mT); microwave frequency, 9.78 GHz; microwave power, 40 mW; modulation amplitude, 10 G; 4,096 points of resolution; and receiver gain, 900.

**ESR Detection of Superoxide Radical.** Gently collected endothelial cells were suspended in modified Kreb's/HEPEs buffer containing deferoximine (25 $\mu$mol/liter, metal chelator). Approximately $1 \times 10^6$ cells were mixed with 1 mmol/liter $O_2^{\bullet-}$-specific spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine in the presence or absence of 100 units/ml polyethylene glycol (PEG)-conjugated $O_2^{\bullet-}$ dismutase (22). The cell mixture loaded in glass capillaries was immediately analyzed for $O_2^{\bullet-}$ production kinetically for 10 min. The ESR settings were as follows: biofield, 3350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; 4,096 points of resolution; receiver gain, 500; and kinetic time, 10 min. The $O_2^{\bullet-}$ dismutase-inhibitable $O_2^{\bullet-}$ signals at a 10 min time point and normalized by protein concentrations were compared among different experimental groups.

**HPLC Determination of Endothelial $H_4B$ Content.** Endothelial cells were lysed by using trichloroacetic acid containing 10 mmol/liter DTT. Lysates were subjected to differential oxidation in acidic (0.2 M trichloroacetic acid) or alkalytic (0.1 M NaOH) solutions containing 2.5% $I_2$/10% KI or 0.9% $I_2$/1.8% KI. After centrifugation, 20 $\mu$l of supernatant was injected into a HPLC system equipped with a 250- × 4.6-mm C18 column (Alltech, Deerfield, IL) and a highly sensitive fluorescent detector (Schimadzu model RF-10Axl, Fisher). Excitation and emission wavelengths of 350 nm and 450 nm were used to detect fluorescent $H_4B$ and its oxidized species (15). Because $H_4B$ is protected under acidic conditions and converted to pterin under alkalytic conditions, the difference in peak intensity reflects $H_4B$ content. The absolute $H_4B$ contents were calculated against a standard curve prepared by using purified $H_4B$ that went through identical extraction procedures and presented as pmol/mg protein.

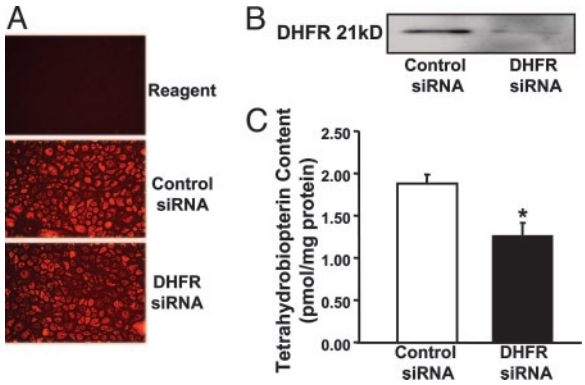


**Fig. 1.** Effects of DHFR siRNA on endothelial $H_4B$ bioavailability. Proliferating endothelial cells were transfected with 25 nmol/liter control or DHFR siRNA for 48 h before analysis of DHFR expression and $H_4B$ content. (*A*) Fluorescent images of transfected cells. (*B*) Western blot of DHFR protein expression. (*C*) Endothelial $H_4B$ content determined by HPLC ($n = 6$). Data are presented as mean ± SEM. *, $P < 0.05$.

**Statistical Analysis.** Data are presented in mean ± SEM from six to 10 independent experiments. Differences in $H_4B$ and $NO^{\bullet}$ levels between control or DHFR siRNA-transfected cells were compared by using a paired Student *t* test. Likewise, protein levels of DHFR and DHFR/eNOS ratio in cells treated with angiotensin II or $H_2O_2$ in aortae of $ACE^{-/-}$ were compared with the controls by using Student's *t* test. $H_4B$ or $O_2^{\bullet-}$ levels among control, angiotensin II- or $H_2O_2$-stimulated cells, and cells pretreated with PEG-conjugated catalase (PEG-CAT) or *N*-nitro-L-arginine-methyl ester (L-NAME) was compared by using ANOVA, followed by Dunnett's post hoc test. Similarly, $O_2^{\bullet-}$ and $NO^{\bullet}$ productions in pcDNA3.1-DHFR transfected cells with or without angiotensin II stimulation were compared with the controls by using ANOVA. Differences in $O_2^{\bullet-}$ production in the presence or absence of L-NAME or NSC 23766 at different time points after angiotensin II stimulation was also analyzed with ANOVA. Statistical significance was set for $P < 0.05$.

## Results

**RNAi Silencing of DHFR Reduces Endothelial $H_4B$ and $NO^{\bullet}$ Bioavailability.** DHFR recycles $H_4B$ in several cell types (16, 17). Its functionality within endothelium particularly regarding $H_4B$ and $NO^{\bullet}$ bioavailability, however, remains unclear. We therefore first examined the consequences of specific disruption of endothelial DHFR by using RNAi. As demonstrated by the fluorescent images in Fig. 1*A*, siRNAs were successfully transfected into ≈95% of the cells. DHFR-targeting siRNA markedly decreased DHFR protein expression by >90% (Fig. 1*B*), consistent with the findings by Tai *et al.* (23). Endothelial $H_4B$ content was 1.89 ± 0.11 pmol/mg protein in control siRNA-transfected cells and was significantly decreased to 1.26 ± 0.11 pmol/mg protein in cells transfected with DHFR siRNA (Fig. 1*C*). Importantly, RNAi-induced specific disruption of DHFR was associated with a substantial decline in endothelial cell production of $NO^{\bullet}$ (Fig. 2).

**$H_2O_2$ Down-Regulation of DHFR Expression.** Because DHFR recycles $H_4B$ from its oxidized form, regulation of DHFR under oxidant stress is particularly relevant to vascular pathophysiology. $H_2O_2$ directly produced or derived from $O_2^{\bullet-}$ has emerged as a signaling intermediate in vascular cells, mediating a variety of gene regulatory responses (1, 2). Interestingly, exogenously applied

PHYSIOLOGY

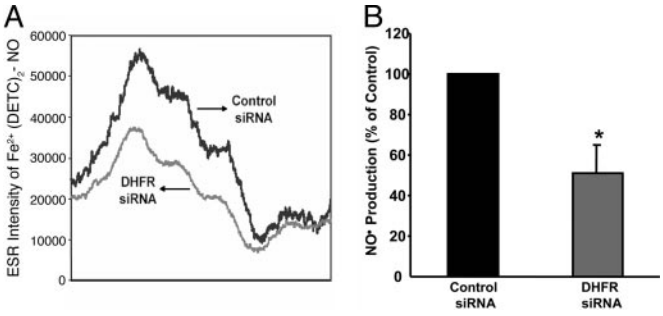


**Fig. 2.** Effects of DHFR siRNA on endothelial $NO^{\bullet}$ bioavailability. Proliferating endothelial cells were transfected with 25 nmol/liter control or DHFR siRNA for 48 h before analysis of $NO^{\bullet}$ production by using ESR. (*A*) Representative ESR spectra. (*B*) Grouped data presented as mean ± SEM ($n = 6$). *, $P < 0.05$.

$H_2O_2$ (at 50, 100, or 200 $\mu$mol/liter for 24 h) dose-dependently down-regulated endothelial DHFR protein expression (Fig. 3 *A* and *B*). $H_2O_2$ decreased the DHFR/eNOS ratio from 1.6 ± 0.28 to 0.6 ± 0.10 in the controls (Fig. 3*C*), implying that substantially less DHFR *per se* is available to supply recycled $H_4B$ to eNOS.

**Angiotensin II Down-Regulation of DHFR Expression.** Elevated angiotensin II levels have been observed in patients with atherosclerotic risk factors, including hypertension, diabetes, and renal dysfunction (2, 5). Interestingly, angiotensin II stimulation (100 nmol/liter for 24 h) of endothelial cells decreased DHFR expression by >50%, as reflected by representative Western blot and grouped data (Fig. 4 *A* and *B*). Similar to $H_2O_2$, angiotensin II shifted DHFR/eNOS ratio from 1.5 ± 0.17 to 0.6 ± 0.16 in the controls (Fig. 4*C*). In $ACE^{-/-}$ mice, for which angiotensin II production was diminished, aortic expression of DHFR was more than doubled compared with wild-type controls (Fig. 4*D*). Taken together, these data strongly suggest that angiotensin II down-regulates DHFR expression under physiological circumstances and that a pathological level of angiotensin II further reduces DHFR abundance.

**Angiotensin II-Induced $H_4B$ Deficiency Is Mediated by $H_2O_2$ Down-Regulation of DHFR.** Loss of DHFR is anticipated to blunt $H_4B$ recycling. Thus, total biopterin and $H_4B$ contents in angiotensin

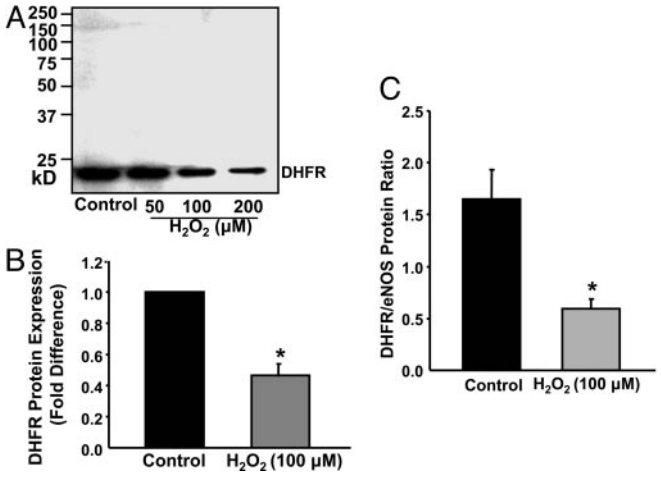


**Fig. 3.** $H_2O_2$ down-regulation of DHFR expression. Confluent endothelial cells were exposed to $H_2O_2$ (50–200 $\mu$mol/liter) for 24 h before Western analysis of DHFR expression. (*A*) Representative Western blot of DHFR protein expression. (*B*) Grouped densitometric data of DHFR expression in response to 100 $\mu$mol/liter $H_2O_2$ ($n = 8$). (*C*) Grouped data on DHFR/eNOS ratio ($n = 8$). Data are presented at mean ± SEM. ·, $P < 0.05$.

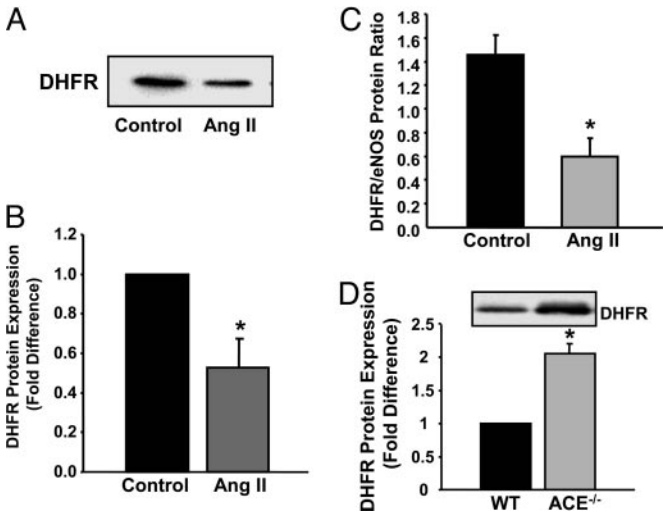


**Fig. 4.** Angiotensin II (Ang II) down-regulation of DHFR expression. Confluent endothelial cells were exposed to angiotensin II (100 nmol/liter) for 24 h before analysis of DHFR and eNOS protein expression. (*A*) Representative Western blot of DHFR protein expression. (*B*) Grouped densitometric data of DHFR expression ($n = 6$). (*C*) Grouped data on DHFR/eNOS ratio ($n = 6$). (*D*) Representative Western blot and grouped data on aortic DHFR expression from wild-type and $ACE^{-/-}$ mice ($n = 6$). Data are presented at mean ± SEM. *, $P < 0.05$.

II-stimulated (100 nmol/liter for 24 h) or $H_2O_2$-stimulated (100 $\mu$mol/liter for 24 h) cells were determined by HPLC. Angiotensin II had little effect on endothelial total biopterin content (Fig. 5*A*). $H_2O_2$ however increased biopterin content modestly by 30% (Fig. 5*A*). These data seem consistent with the previous report that $H_2O_2$ up-regulated $H_4B$ synthetic enzyme GTP cyclohydrolase I (GTP-CH I) (24). Interestingly, endothelial $H_4B$ levels were significantly decreased by angiotensin II or $H_2O_2$ (1.38 ± 0.22 in angiotensin II- vs. 1.24 ± 0.27 in $H_2O_2$-treated cells, compared with 2.42 ± 0.34 pmol/mg protein in confluent control cells) (Fig. 5*B*). Importantly, scavenging of intracellular $H_2O_2$ with a pretreatment of 100 units/ml PEG-CAT for 1 h preserved $H_4B$ content (1.93 ± 0.15 pmol/mg protein) while having no effect on total biopterin, clearly impli-

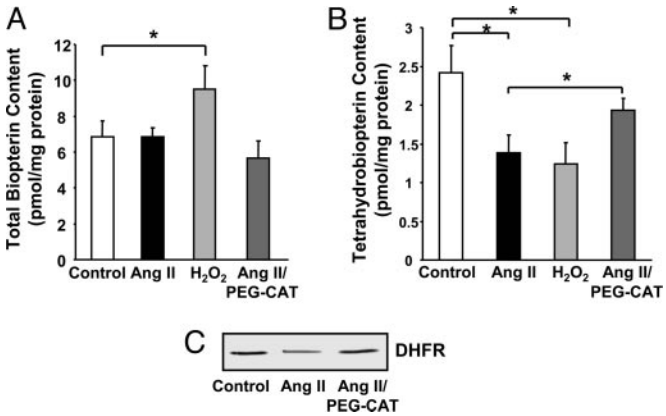


**Fig. 5.** Role of $H_2O_2$ in angiotensin II (Ang II) induction of $H_4B$ deficiency. Confluent endothelial cells were exposed to angiotensin II (100 nmol/liter) or $H_2O_2$ (100 $\mu$mol/liter) for 24 h before analysis of endothelial $H_4B$. Some cells were pretreated with 100 units/ml PEG-CAT for 1 h before angiotensin II stimulation. (*A*) Total biopterin content ($n = 6$). (*B*) $H_4B$ content. (*C*) DHFR expression in angiotensin II- or $H_2O_2$-stimulated cells with or without PEG-CAT preincubation ($n = 6$). Data are presented as mean ± SEM. *, $P < 0.05$.

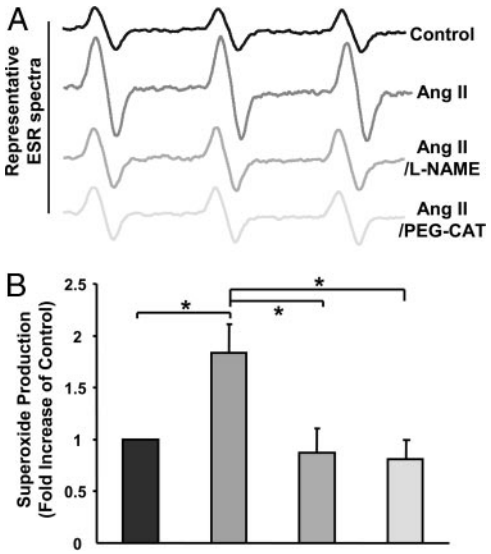


**Fig. 6.** Role of $H_2O_2$ in angiotensin II (Ang II) uncoupling of eNOS. Confluent endothelial cells were exposed to angiotensin II (100 nmol/liter) for 24 h in the presence or absence of preincubation with NOS inhibitor L-NAME (100 $\mu$mol/liter for 1 h) or PEG-CAT (100 units/ml for 1 h) and subsequently analyzed for $O_2^{\bullet -}$ production by using ESR. (*A*) Representative ESR spectra. (*B*) Grouped data presented as mean ± SEM ($n = 6$). *, $P < 0.05$.

cating an intermediate role of $H_2O_2$ in angiotensin II induction of $H_4B$ deficiency (Fig. 5*B*). In addition, PEG-CAT prevented angiotensin II down-regulation of DHFR (Fig. 5*C*).

**Angiotensin II Uncoupling of eNOS.** Role of $H_2O_2$ and DHFR. Because $H_2O_2$ mediates angiotensin II induction of $H_4B$ deficiency, we hypothesized that $H_2O_2$ may contribute to eNOS uncoupling by angiotensin II. As illustrated in Fig. 6, angiotensin II (100 nmol/liter for 24 h) induced a significant increase in endothelial $O_2^{\bullet -}$ production. This response was completely attenuated by the eNOS inhibitor L-NAME (100 $\mu$mol/liter) or PEG-CAT (100 units/ml), strongly implying $H_2O_2$-dependent uncoupling of eNOS.

To directly examine the role of DHFR in angiotensin II uncoupling of eNOS, we transfected endothelial cells with mammalian expression vector containing human DHFR, pcDNA3.1-DHFR. Whereas angiotensin II (100 nmol/liter for 24 h) consistently stimulated $O_2^{\bullet -}$ production in reagent-only-transfected cells, it failed to evoke endothelial production of $O_2^{\bullet -}$ in DHFR-transfected cells (Fig. 7*A*). DHFR overexpression also restored $NO^{\bullet}$ production in angiotensin II-stimulated cells (Fig. 7*B*). These data strongly suggest that DHFR availability is critical in maintaining the coupling state of eNOS.

**Role of Endothelial NAD(P)H Oxidases.** To examine whether a transient or continuous production of $O_2^{\bullet -}$ is required for $H_2O_2$-dependent eNOS uncoupling and to determine the enzymatic source responsible for the initial $O_2^{\bullet -}$ production, cells were exposed to angiotensin II for 30 min, 6 h, or 24 h. As is evident in Fig. 8*A*, angiotensin II stimulated a rapid production of $O_2^{\bullet -}$ at 30 min, which was unaffected by 100 $\mu$mol/liter L-NAME but abolished by 50 $\mu$mol/liter Rac1 inhibitor NSC 23766, indicating a role of endothelial NAD(P)H oxidases. This initial production of $O_2^{\bullet -}$ was transient because it diminished at 6 h (Fig. 8*A*). Twenty-four hours after angiotensin II, there was a potent second peak in $O_2^{\bullet -}$ production, which was completely and consistently attenuated by L-NAME and unaffected by NSC 23766, implicating that uncoupled eNOS was predominantly responsible for $O_2^{\bullet -}$ production after prolonged exposure to

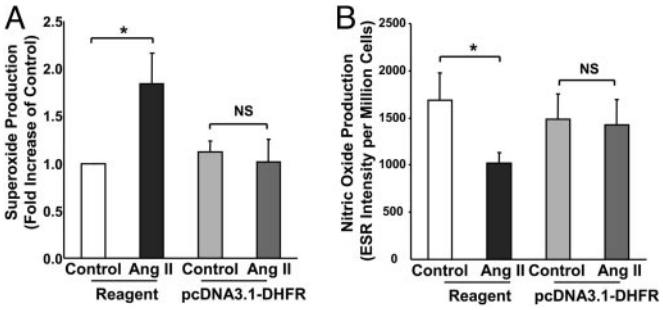


**Fig. 7.** Role of DHFR in angiotensin II-induced $H_4B$ deficiency and eNOS uncoupling. Proliferating endothelial cells were transfected with pcDNA3.1-DHFR for 48 h before exposure to 100 nmol/liter angiotensin II (Ang II) for 24 h. (*A*) Endothelial $O_2^{\bullet -}$ production determined by ESR ($n = 4$). (*B*) Endothelial $NO^{\bullet}$ production determined by ESR ($n = 6$). Data are presented as mean ± SEM. *, $P < 0.05$.

angiotensin II (Fig. 8*A*). Of note, DHFR expression did not change at 30 min, showed a slight downward trend at 6 h, but was markedly diminished at 24 h (Fig. 8*B*). Take together, these data demonstrate that an initial and transient activation of endothelial NAD(P)H oxidases in response to angiotensin II lies upstream of $H_2O_2$-dependent down-regulation of DHFR and eNOS uncoupling. The overall findings of the study are summarized thematically in Fig. 9.

## Discussion

The present study elucidated that DHFR is critical for $H_4B$ and $NO^{\bullet}$ bioavailability in the endothelium. Angiotensin II down-regulates DHFR in a $H_2O_2$-dependent manner, and this response is involved in angiotensin II-induced $H_4B$ deficiency and eNOS uncoupling. The initiating source of $H_2O_2$ appears to be the endothelial NAD(P)H oxidases, which lie upstream of uncoupled eNOS in response to angiotensin II. This cascade of signaling events, as illustrated in Fig. 9, may represent a common mechanism underlying eNOS dysfunction under situations associated with oxidant stress.

$H_4B$ belongs to a large pterin family. Pterins are 2-amino-4-oxo derivatives of pteridines, which, in turn, represent a large and structurally varied group of natural compounds involved in the biosynthetic pathways of cofactors and vitamins (25). Although both oxidized and reduced pterins are detectable in physiological fluid and tissues, only $H_4B$ is biologically active (25). The *de novo*

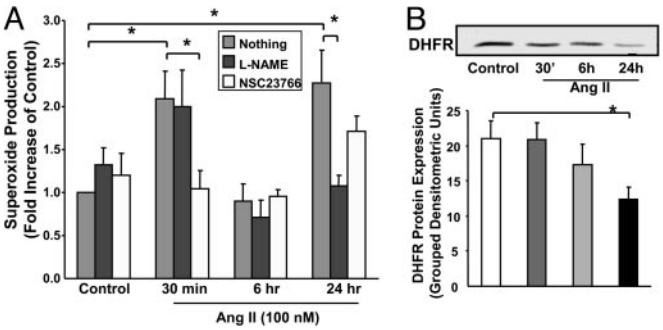


**Fig. 8.** Rapid and transient activation of endothelial NAD(P)H oxidases precedes angiotensin II (Ang II) uncoupling of eNOS. Confluent endothelial cells were exposed to angiotensin II (100 nmol/liter) for 30 min, 6 h, and 24 h before ESR analysis of $O_2^{\bullet -}$ production in the presence or absence of Rac1 antagonist NSC 23766 (50 $\mu$mol/liter) or L-NAME (100 $\mu$mol/liter). (*A*) Grouped data on $O_2^{\bullet -}$ production at each time point ($n = 8$–10). (*B*) Representative Western blot and grouped data of DHFR protein expression at each time point ($n = 10$). Data are presented as mean ± SEM. *, $P < 0.05$.

PHYSIOLOGY

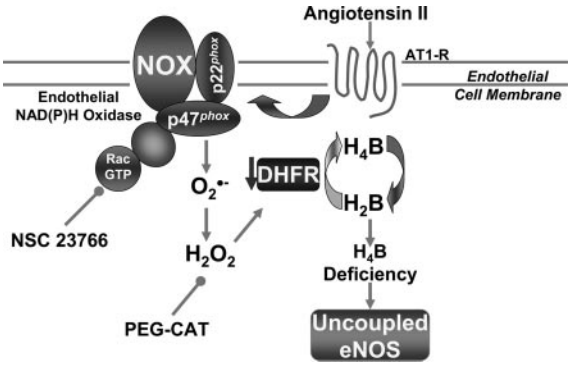


**Fig. 9.** Schematic mechanisms underlying angiotensin II uncoupling of eNOS. Angiotensin II induces a rapid and transient activation of endothelial NAD(P)H oxidases (attenuatable by Rac1 inhibitor NSC 23766), leading to an initial burst production of $O_2^{\bullet-}$ and formation of $H_2O_2$. $H_2O_2$ in turn down-regulates DHFR to induce $H_4B$ deficiency and eNOS uncoupling. These findings may represent a universal mechanism whereby eNOS dysfunction occurs under conditions associated with oxidant stress.

synthesis of $H_4B$ involves the rate-limiting GTP-CH I, 6-pyruvoyl-$H_4B$ synthase and sepiapterin reductase (16, 17). When $H_4B$ is oxidized to dihydropterin, it is recycled by DHFR in several cell types (16, 17). Previous studies demonstrated that inhibition of GTP-CH I impaired endothelium-dependent relaxation (26). Overexpression of GTP-CH I was found partially effective in improving endothelial function in diabetic (27) and hypertensive rodents (28). However, functional regulation of the salvage enzyme DHFR, which could be more crucial in determining $H_4B$ and $NO^{\bullet}$ bioavailability under pathophysiological conditions associated with oxidant stress, remains obscure. Here we found that RNAi silencing of DHFR substantially reduced $H_4B$ and $NO^{\bullet}$ levels in cultured endothelial cells, implying an important role of DHFR in maintaining $NO^{\bullet}$ bioavailability under physiological conditions.

Furthermore, endothelial DHFR expression was significantly down-regulated by pathological concentrations of angiotensin II. The $ACE^{-/-}$ mice had a doubling in aortic expression of DHFR compared with the wide-type controls. These data indicate that angiotensin II modulates DHFR under pathophysiological circumstances. Of caution, these observations do not necessarily rule out potential compensatory regulations of DHFR in angiotensin II-associated hypertension, where mechanical forces or inflammatory substances may regulate the enzyme differentially. Angiotensin II may also regulate DHFR differently in other vascular cells, such as vascular smooth muscle, whose $H_4B$ bioavailability, however, is unrelated to endothelial $NO^{\bullet}$ production. Of note, angiotensin II down-regulation of endothelial DHFR was $H_2O_2$-dependent. This observation shares similarity with previous findings that angiotensin II modulates expression of endothelial adhesion molecules by means of $H_2O_2$-dependent mechanisms (29).

In addition, $H_2O_2$-dependent down-regulation of DHFR mediated angiotensin II induction of $H_4B$ deficiency and eNOS uncoupling. Angiotensin II stimulation led to a significant reduction in endothelial $H_4B$ content, which was preventable by removal of $H_2O_2$. It was previously reported that exogenous $H_2O_2$ up-regulates GTP-CH I expression (23), which likely explains a modest increase in total biopterin content observed in the current study. Endogenously produced $H_2O_2$ by angiotensin II, however, did not seem to modulate GTP-CH I because angiotensin II had no effect on total biopterin content. These data may suggest that DHFR is much more sensitive to lower endogenous $H_2O_2$ compared to GTP-CH I, which was found regulated only in response to exogenous $H_2O_2$. Furthermore, we found that endothelial $O_2^{\bullet-}$ production after prolonged angiotensin II (24 h) was completely dependent on uncoupled eNOS and was correctable by scavenging $H_2O_2$ or overexpressing DHFR. It is interesting to speculate that this $H_2O_2$ down-regulation of DHFR may partially underlie eNOS uncoupling in atherosclerotic or hypertension blood vessels.

These data also suggest that, although angiotensin II potently activates vascular NAD(P)H oxidases in endothelial cells and vascular smooth muscle cells within minutes, uncoupled eNOS is the major enzymatic source of ROS within endothelium after prolonged angiotensin II. Indeed, we found that angiotensin II induced an initial burst production of $O_2^{\bullet-}$ at 30 min which was abolished by Rac1 inhibitor NSC 23766 but unaffected by L-NAME. This increase in $O_2^{\bullet-}$ diminished at 6 h. At 24 h, however, endothelial cells had a second peak in producing $O_2^{\bullet-}$, which was completely attenuated by L-NAME but unaffected by NSC 23766. These data clearly suggest that a rapid and transient activation of endothelial NAD(P)H oxidases precedes $H_2O_2$-dependent eNOS uncoupling. These observations seems consistent with the earlier observation by Mollnau *et al.* (30) that ROS production in angiotensin II infused rat aorta was markedly reduced by inhibiting eNOS. However, these data are not contradictory to the findings that vascular NAD(P)H oxidases remain the predominant sources for vascular smooth muscle-derived ROS in response to prolonged angiotensin II (31). The latter finding seems to also involve up-regulation of NAD(P)H oxidases subunits (32).

In summary, the present study provided evidence that endothelial DHFR critically regulates endothelial $H_4B$ and $NO^{\bullet}$ bioavailability under physiological and pathological conditions. By using angiotensin II as a model system, we found that a rapid and transient activation of endothelial NAD(P)H oxidases precedes $H_2O_2$-dependent down-regulation of DHFR and eNOS uncoupling in the endothelium. This signaling cascade, as illustrated in Fig. 9, may represent a universal mechanism whereby eNOS uncouples under conditions associated with oxidant stress, such as atherosclerosis, hypertension, and diabetes, for which activation of vascular NAD(P)H oxidases has been documented.

This work was supported by American Heart Association Scientist Development Grant 0435189N, an American Diabetes Association Research Award, a Schweppe Foundation Career Development Award, and a start-up fund from the University of Chicago (all to H.C.).

1. Cai, H. (2005) *Circ. Res.* **96,** 818–822.
2. Cai, H., Griendling, K. K. & Harrison, D. G. (2003) *Trends Pharmacol. Sci.* **24,** 471–478.
3. Cai, H. & Harrison, D. G. (2000) *Circ. Res.* **87,** 840–844.
4. Lassegue, B. & Griendling, K. K. (2004) *Am. J. Hypertens.* **17,** 852–860.
5. Harrison, D. G., Cai, H., Landmesser, U. & Griendling, K. K. (2003) *J. Renin Angiotensin Aldosterone Syst.* **4,** 51–61.
6. Heinzel, B., John, M., Klatt, P., Bohme, E. & Mayer, B. (1992) *Biochem. J.* **281,** 627–630.
7. Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H. & Rosen, G. M. (1992) *J. Biol. Chem.* **267,** 24173–24176.
8. Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H. & Zweier, J. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6770–6774.
9. Xia, Y. & Zweier, J. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6954–6958.
10. Xia, Y., Tsai, A. L., Berka, V. & Zweier, J. L. (1998) *J. Biol. Chem.* **273,** 25804–25808.
11. Wever, R. M., van Dam, T., van Rijn, H. J., de Groot, F. & Rabelink, T. J. (1997) *Biochem. Biophys. Res. Commun.* **237,** 340–344.
12. Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P. & Pritchard, K. A., Jr. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9220–9225.
13. Milstien, S. & Katusic, Z. (1999) *Biochem. Biophys. Res. Commun.* **263,** 681–684.

14. Laursen, J. B., Somers, M., Kurz, S., McCann, L., Warnholtz, A., Freeman, B. A., Tarpey, M., Fukai, T. & Harrison, D. G. (2001) *Circulation* **103,** 1282–1288.
15. Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E. & Harrison, D. G. (2003) *J. Clin. Invest.* **111,** 1201–1209.
16. Thony, B., Auerbach, G. & Blau, N. (2000) *Biochem. J.* **347,** 1–16.
17. Werner-Felmayer, G., Golderer, G. & Werner, E. R. (2002) *Curr. Drug Metab.* **3,** 159–173.
18. Cai, H., Li, Z., Dikalov, S., Holland, S. M., Hwang, J., Jo, H., Dudley, S. C., Jr., & Harrison, D. G. (2002) *J. Biol. Chem.* **277,** 48311–48317.
19. Cai, H., Li, Z., Davis, M. E., Kanner, W., Harrison, D. G. & Dudley, S. C., Jr. (2003) *Mol. Pharmacol.* **63,** 325–331.
20. Cai, H., McNally, J. S., Weber, M. & Harrison, D. G. (2004) *J. Mol. Cell. Cardiol.* **37,** 121–125.
21. Cai, H., Li, Z., Goette, A., Mera, F., Honeycutt, C., Feterik, K., Wilcox, J. N., Dudley, S. C., Jr., Harrison, D. G. & Langberg, J. J. (2002) *Circulation* **106,** 2854–2858.
22. Landmesser, U., Cai, H., Dikalov, S., McCann, L., Hwang, J., Jo, H., Holland, S. M. & Harrison, D. G. (2002) *Hypertension* **40,** 511–515.
23. Tai, N., Schmitz, J. C., Chen, T. M. & Chu, E. (2004) *Biochem. J.* **378,** 999–1006.
24. Shimizu, S., Shiota, K., Yamamoto, S., Miyasaka, Y., Ishii, M., Watabe, T., Nishida, M., Mori, Y., Yamamoto, T. & Kiuchi, Y. (2003) *Free Radical Biol. Med.* **34,** 1343–1352.
25. Blau, N., Thony, B., Cotton, R. G. H. & Hyland, K. (2003) in *The Metabolic and Molecular Bases of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Childs, B. & Vogelstein, B. (McGraw–Hill, New York), pp. 1725–1776.
26. Kinoshita, H., Milstien, S., Wambi, C. & Katusic, Z. S. (1997) *Am. J. Physiol.* **273,** H718–H724.
27. Alp, N. J., Mussa, S., Khoo, J., Cai, S., Guzik, T., Jefferson, A., Goh, N., Rockett, K. A. & Channon, K. M. (2003) *J. Clin. Invest.* **112,** 725–735.
28. Zheng, J. S., Yang, X. Q., Lookingland, K. J., Fink, G. D., Hesslinger, C., Kapatos, G., Kovesdi, I. & Chen, A. F. (2003) *Circulation* **108,** 1238–1245.
29. Pueyo, M. E., Gonzalez, W., Nicoletti, A., Savoie, F., Arnal, J. F. & Michel, J. B. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 645–651.
30. Mollnau, H., Wendt, M., Szocs, K., Lassegue, B., Schulz, E., Oelze, M., Li, H., Bodenschatz, M., August, M., Kleschyov, A. L., *et al.* (2002) *Circ. Res.* **90,** E58–E65.
31. Griendling, K. K. & Ushio-Fukai, M. (2000) *Regul. Pept.* **91,** 21–27.
32. Fukui, T., Ishizaka, N., Rajagopalan, S., Laursen, J. B., Capers, Q., IV, Taylor, W. R., Harrison, D. G., de Leon, H., Wilcox, J. N. & Griendling, K. K. (1997) *Circ. Res.* **80,** 45–51.