Novel Treatment of Hypertension by Specifically Targeting E2F for Restoration of Endothelial Dihydrofolate Reductase and eNOS Function Under Oxidative Stress

Hong Li,* Qiang Li,* Yixuan Zhang, Wenting Liu, Bo Gu, Taro Narumi, Kin Lung Siu, Ji Youn Youn, Peiqing Liu, Xia Yang, Hua Cai

Abstract—We have shown that hydrogen peroxide (H_2O_2) downregulates tetrahydrobiopterin salvage enzyme DHFR (dihydrofolate reductase) to result in eNOS (endothelial NO synthase) uncoupling and elevated blood pressure. Here, we aimed to delineate molecular mechanisms underlying H_2O_2 downregulation of endothelial DHFR by examining transcriptional pathways hypothesized to modulate DHFR expression and effects on blood pressure regulation of targeting these novel mechanisms. H_2O_2 dose and time dependently attenuated DHFR mRNA and protein expression and enzymatic activity in endothelial cells. Deletion of E2F-binding sites, but not those of Sp1 (specificity protein 1), abolished H_2O_2 attenuation of DHFR promoter activity. Overexpression of E2F1/2/3a activated DHFR promoter at baseline and alleviated the inhibitory effect of H_2O_2 on DHFR promoter activity. H_2O_2 treatment diminished mRNA and protein expression of E2F1/2/3a, whereas overexpression of E2F isoforms increased DHFR protein levels. Chromatin immunoprecipitation assay indicated direct binding of E2F1/2/3a to the DHFR promoter, which was weakened by H_2O_2. E2F1 RNA interference attenuated DHFR protein levels, whereas its overexpression elevated tetrahydrobiopterin levels and tetrahydrobiopterin/dihydrobiopterin ratios in vitro and in vivo. In Ang II (angiotensin II)–infused mice, adenovirus-mediated overexpression of E2F1 markedly abrogated blood pressure to control levels, by restoring endothelial DHFR function to improve NO bioavailability and vasorelaxation. Bioinformatic analyses confirmed a positive correlation between E2F1 and DHFR in human endothelial cells and arteries, and downregulation of both by oxidized phospholipids. In summary, endothelial DHFR is downregulated by H_2O_2 transcriptionally via an E2F-dependent mechanism, and that specifically targeting E2F1/2/3a to restore DHFR and eNOS function may serve as a novel therapeutic option for the treatment of hypertension. (***Hypertension***. 2019;73:179-189. DOI: 10.1161/HYPERTENSIONAHA.118.11643.) • Online Data Supplement

Key Words: angiotensin II ■ dihydrofolate reductase ■ E2F ■ eNOS uncoupling ■ hydrogen peroxide ■ hypertension ■ nitric oxide

According to the 2017 report of the American Heart Association, hypertension affected 85.7 million adults (≥20 years of age) in the United States between 2011 and 2014.[1] Hypertensive patients display phenotype of endothelial dysfunction, which is characterized by decreased NO bioavailability and uncoupling of eNOS (endothelial NO synthase).[2,3] Although the molecular mechanisms of hypertension remain to be fully elucidated, a number of studies from our group and others have revealed a central role of eNOS uncoupling in the pathogenesis of hypertension.[4–7] In an Ang II (angiotensin II)–induced hypertensive mouse model, we found that Ang II induces deficiency of DHFR (dihydrofolate reductase) specifically in endothelial cells, which in turn leads to eNOS uncoupling and elevated blood pressure (BP).[5,6] DHFR is the rate-limiting salvage enzyme for the eNOS cofactor tetrahydrobiopterin (H_4B), deficiency of which is responsible for the uncoupling switch of eNOS.[4–6,8,9] Furthermore, we investigated mechanisms of Ang II–induced endothelial DHFR deficiency. We have demonstrated that Ang II downregulates DHFR expression through hydrogen peroxide (H_2O_2).[8] In addition, we

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From the Division of Molecular Medicine, Department of Anesthesiology, Cardiovascular Research Laboratories (H.L., Q.L., Y.Z., B.G., T.N., K.L.S., J.Y.Y., H.C.), Division of Cardiology, Department of Medicine, Cardiovascular Research Laboratories (H.L., Q.L., Y.Z., B.G., T.N., K.L.S., J.Y.Y., H.C.), and Department of Integrative Biology and Physiology (W.L., X.Y.), David Geffen School of Medicine, University of California, Los Angeles; and Department of Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, Guangdong, China (H.L., P.L.).

Current address for H. Li: Department of Biochemistry and Molecular Biology, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, China.

Current address for B. Gu: Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA.

*These authors contributed equally to this work.

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Correspondence to Hua Cai, Division of Molecular Medicine, Department of Anesthesiology and Division of Cardiology, Department of Medicine, Cardiovascular Research Laboratories, David Geffen School of Medicine, University of California, Los Angeles, 650 Charles E Young Dr, Los Angeles, CA 90095. Email hcai@mednet.ucla.edu

have shown that Ang II induces eNOS uncoupling via H_2O_2-dependent DHFR deficiency and consequent reduction in H_4B bioavailability, in cultured endothelial cells, WT (wild type) mice, hph-1 (hyperphenylalaninemia 1) mice, and apoE (apolipoprotein E)-null mice.[5,6,9,10] In these mouse models, modestly uncoupled eNOS causes hypertension, whereas severely uncoupled eNOS promotes formation of abdominal aortic aneurysms.[5,9,10]

Increasing evidence has demonstrated that oxidative stress plays an important role in the development of various vascular diseases.[11–19] Our recent studies have shown that restoration of DHFR function with either folic acid diet or DHFR overexpression recoupled eNOS to attenuate Ang II–induced oxidative stress, NO deficiency, and elevated BP.[4,5,7] Therefore, identification of DHFR modulating mechanisms is of great importance in revealing novel therapeutics for hypertension via restoration of eNOS coupling activity. Several regulatory mechanisms for DHFR have been demonstrated in systems other than endothelial cells. For example, at transcriptional level, the transcriptional factor Sp1 (specificity protein 1) has been shown to bind to the mouse DHFR promoter to upregulate its expression.[20] At post-transcriptional level, miR-24 has been shown to bind to DHFR mRNA to result in reduced DHFR protein expression.[21] In addition, the DHFR inhibitor methotrexate can upregulate DHFR protein expression by attenuating the self-binding of the DHFR protein to its mRNA to allow increased translation of DHFR mRNA.[22,23] Nonetheless, molecular mechanisms underlying regulation of DHFR by Ang II–induced oxidative stress have remained unknown.

The E2Fs are a family of transcription factors, initially identified as a cellular activator that binds to the adenoviral E2 gene promoter.[24] Members of E2F family have been reported to play opposite roles in biological processes, such as transcriptional activation and repression, cell proliferation and apoptosis, and tumor inhibition and carcinogenesis.[25] The E2F family contains several evolutionally conserved domains, including a DNA-binding domain, a dimerization domain, a transcriptional activation domain, and a pocket protein-binding domain.[26,27] In the DHFR promoter region, there are 4 reiterated Sp1-binding sites and 2 overlapping and inverted E2F-binding sites.[28] Therefore, we set out to examine the hypothesis that transcriptional mechanisms involving E2F family members are involved in the H_2O_2 attenuation of DHFR and subsequent regulation of BP.

In this study, we investigated molecular mechanisms underlying oxidative stress downregulation of DHFR by specifically examining transcriptional pathways that are hypothesized to regulate endothelial DHFR expression, especially the E2F family transcriptional factors. We found that H_2O_2 transcriptionally decreased mRNA expression of E2F1/2/3a, which resulted in attenuated protein abundance and their binding to DHFR promoter as assessed by chromatin immunoprecipitation assays. This was accompanied by decreased DHFR mRNA and protein expression. Based on this novel molecular mechanism, adenovirus-mediated in vivo delivery of E2F1 reduced BP via restoration of endothelial DHFR expression, recoupling of eNOS, augmented NO bioavailability, and improved endothelium-dependent vasorelaxation. In addition, bioinformatic analyses confirmed a positive correlation between E2F1 and DHFR in human endothelial cells and arteries, and downregulation of both by oxidized phospholipids (OxPAPC). Taken together, our data indicate that specifically targeting E2F to improve DHFR and eNOS function under oxidative stress may serve as a novel therapeutic option for the treatment of hypertension.

Materials and Methods

Data, analytic methods, and study materials will be made available to other researchers on publication of this research article. These will not be stored online or publicly but can be shared electronically or physically on request (see Materials in the online-only Data Supplement).

Construction of DHFR Reporter Plasmids and Luciferase Assay

WT DHFR promoter (DHFR-1, −771 to +9) was amplified from HEK293T genomic DNA and subcloned into pGL3 basic luciferase reporter plasmid, which is a generous gift from Dr Xiangming Ding (University of California, Los Angeles). The deletions of transcriptional factor–binding sites were generated by polymerase chain reaction. As shown in Figure 1, DHFR-2 is deleted of 2 SP1-binding sites from −553 to −516. DHFR-3 is deleted of 2 SP1-binding sites from −121 to −77. DHFR-4 is deleted of 2 E2F-binding sites from −67 to −56. All mutants were confirmed by sequencing. For luciferase activity assay, bovine aortic endothelial cells (BAECs) were seeded in 48-well plates, and plasmids of pBH-E2F1/2/3a/7/8 (150 ng), pCMV-E2F4/5/6 (100 ng), and equal amount of corresponding empty vectors were cotransfected with WT DHFR promoter or different deletions (200 ng per well), as well as pRL renilla luciferase reporter plasmid (5 ng per well), which was a generous gift from Dr Yibin Wang (University of California, Los Angeles). After incubation for 5 hours, BAECs were starved in medium 199 with 5% fetal bovine serum overnight and subjected to 100 μmol/L H_2O_2 stimulation for 12 hours. Luciferase activity was determined by the dual-luciferase reporter assay system (Promega, Fitchburg, WI). All experiments were performed in triplicates from independent repeats using different passages of cells.

Real-Time Telemetry BP Measurement

Male C57BL/6 mice (12 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Wireless BP transmitters were implanted into the animals as we published previously.[5,7] The catheter of the BP probe was inserted into the left carotid artery, whereas the body of the probe was inserted into the right flank. Animals were given 1 week to recover from the surgery. Then Ad-E2F1 or Ad-Luc (10^9 IFU) in 50 μL sterilized PBS was injected directly into the left ventricle of the heart. After 3 days of recovery, mice were subcutaneously implanted with osmotic pumps (DurectCorp, Cupertino, CA) containing Ang II (0.7 mg/kg per day; Sigma-Aldrich, St. Louis, MO). BP was monitored for the infusion period of 14 days. Measurements were made daily from 11:00 AM to 4:00 PM at a 100-Hz sampling rate. Average BP was calculated daily as the average of the entire recording period. Two weeks after Ang II infusion, mouse aortas were used for measurement of NO bioavailability using electron spin resonance (ESR) or isolation of aortic endothelial cells as described previously.[6,7,9,10] The endothelial cell fraction and the endothelial cell-denuded aortas were lysed for Western blot analysis. The use of animals and experimental procedures were performed based on protocols approved by the University of California, Los Angeles Institutional Animal Care and Use Committee.

Determination of NO Bioavailability Using ESR

Aortic NO bioavailability was determined using ESR as described previously.[5–7,9,10] Briefly, freshly isolated aortas were cut into 2 mm rings and then incubated in freshly prepared NO-specific spin trap Fe^{2+}(DETC [diethyldithiocarbamic acid sodium salt])$_2$ colloid (0.5 mmol/L) in nitrogen bubbled, modified Krebs/HEPES buffer at 37°C for 60 minutes, in the presence of calcium ionophore A23187 (10 μmol/L). The aortic rings were then snap-frozen in liquid nitrogen and loaded

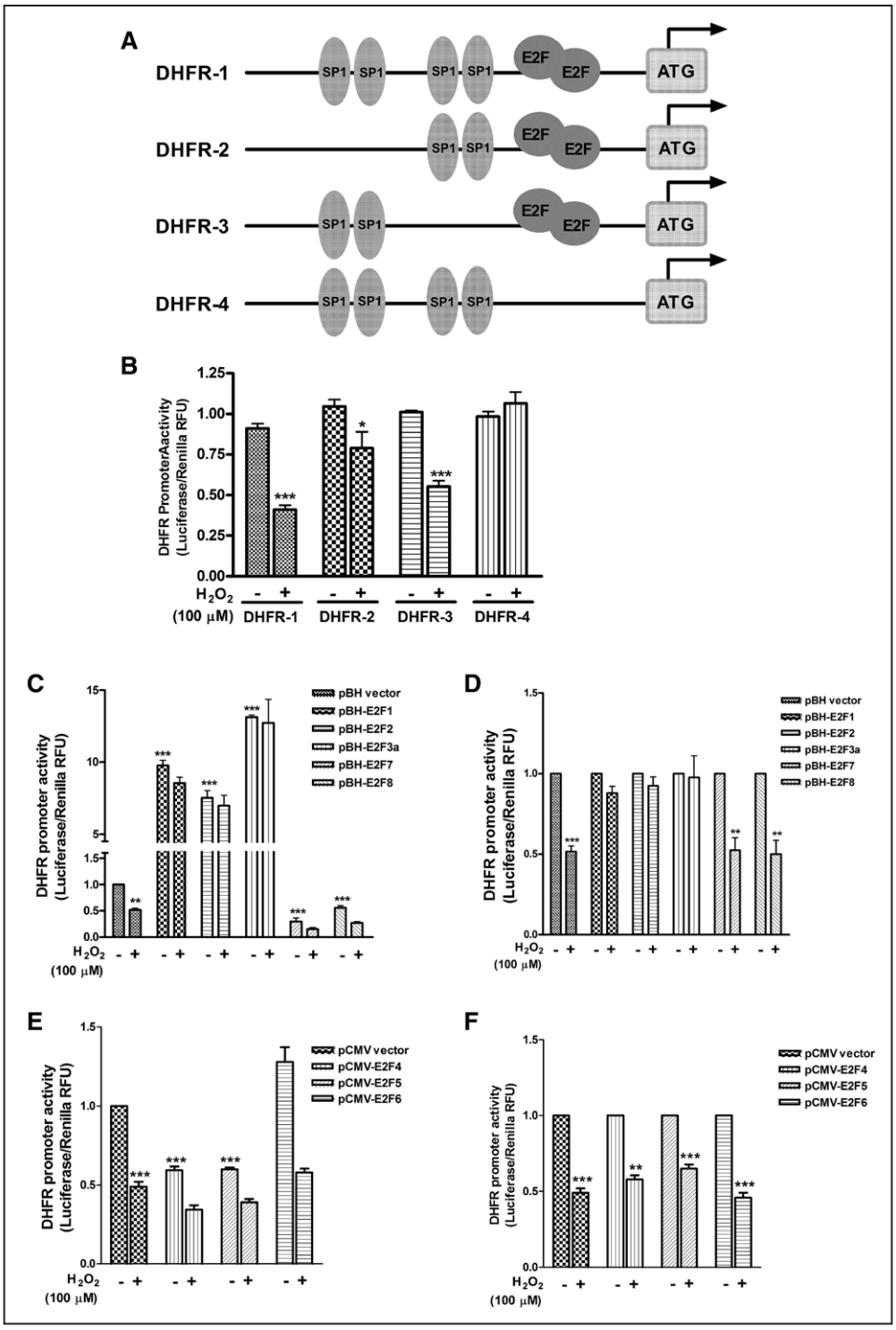

Figure 1. E2F1, E2F2, and E2F3a mediate hydrogen peroxide inhibition of DHFR (dihydrofolate reductase) promoter activity. **A**, Schematic representation of the DHFR promoter and its deletion constructs. DHFR-1 is the WT (wild type) DHFR promoter (–771 to +9) inserted into the pGL3 luciferase reporter vector. DHFR-2/3/4 were deletion constructs containing DHFR promoters missing SP1 (specificity protein 1)- or E2F-binding sites (missing –553 to –516, –121 to –77, or –67 to –56). **B**, Luciferase reporter constructs of WT DHFR promoter and deletion variants were cotransfected with pRL renilla luciferase plasmid into bovine aortic endothelial cells. Twenty-four hours later, cells were exposed to hydrogen peroxide (H_2O_2; 100 μmol/L) for another 12 h in M199 medium containing 5% FBS (fetal bovine serum). Luciferase activity was measured by the dual-luciferase reporter assay system and normalized to the renilla luciferase activity. Data were presented as Mean±SEM. *P<0.05, ***P<0.001 vs ctrl; n=3. **C**, pBH-vector, pBH-E2F1, pBH-E2F2, pBH-E2F3a, pBH-E2F7, or pBH-E2F8 plasmid was cotransfected with WT DHFR luciferase reporter and renilla luciferase reporter. Twenty-four hours later, cells were exposed to H_2O_2 (100 μmol/L) for another 12 h in M199 medium containing 5% FBS. Luciferase activity was measured as described above. The group of empty pBH-vector without H_2O_2 treatment was used as control, and all other groups were shown as the fold differences compared with it. **D**, The data of (**C**) were reanalyzed by normalizing H2O2-treated pBH-E2F groups to their corresponding groups of pBH-E2F plasmid without H_2O_2 treatment. **E**, pCMV-vector, pCMV-E2F4, pCMV-E2F5, or pCMV-E2F6 plasmid was cotransfected with WT DHFR luciferase reporter and renilla luciferase reporter. Then the same treatment was performed as in (**C**). The group of empty pCMV-vector without H_2O_2 treatment was used as control, and all other groups were shown as the fold differences compared with it. **F**, The data of (**E**) were reanalyzed by normalizing H_2O_2-treated pCMV-E2F groups to their corresponding groups of pCMV-E2F plasmid without H_2O_2 treatment. All data are presented as Mean±SEM. **P<0.01, ***P<0.001 vs empty vector (**C** and **E**) or corresponding control group (**D** and **F**); n=3. ATG indicates the start codon of mRNA; and RFU, relative fluorescence units.

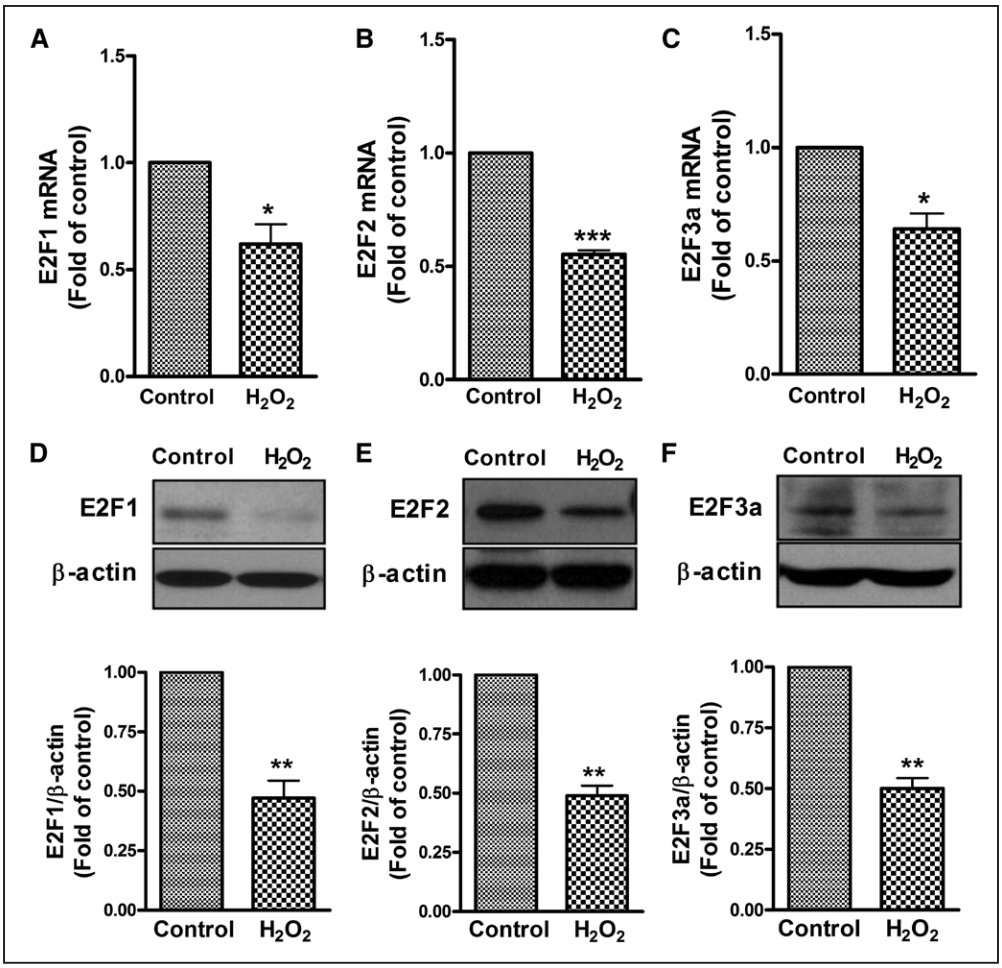

Figure 2. Hydrogen peroxide downregulates E2F1, E2F2, and E2F3a mRNA and protein expression, **A**–**C**, Bovine aortic endothelial cells (BAECs) were harvested after exposure to hydrogen peroxide (H_2O_2; 100 μM, 24 h). E2F1/2/3a mRNA levels were determined by quantitative reverse transcription–polymerase chain reaction with the housekeeping gene *GAPDH* as an endogenous control. Mean±SEM. **P*<0.05, ****P*<0.001 vs ctrl; n=3. **D**–**F**, BAECs were harvested after exposure to H_2O_2 (100 μM, 24 h). E2F1, E2F2, and E2F3a antibodies were used to detect protein levels of E2F1/2/3a. Mean±SEM. ***P*<0.01 vs ctrl; n=3 to 4.

into a finger Dewar for measurement with ESR. The instrument settings were as the following: center field, 3440; sweep width, 100 G; microwave frequency, 9.796 GHz; microwave power, 13.26 mW; modulation amplitude, 9.82 G; 512 points of resolution; and receiver gain, 356.

Determination of eNOS Uncoupling Using ESR

eNOS uncoupling was assessed by ESR analysis as we described previously.[5,7–10,29,30] In brief, freshly isolated aortas were homogenized in lysis buffer supplemented with protease inhibitor cocktail.[31] Then the tissue lysate was incubated with superoxide-specific spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (500 μmol/L) in the presence or absence of L-NAME (N(ω)-nitro-L-arginine methyl ester; 10 μmol/L) and/or PEG-SOD (polyethylene glycol-superoxide dismutase; 20 U/mL). Next, the mixture was loaded into a capillary and analyzed for superoxide production by ESR. The SOD inhibitable superoxide production (normalized by protein concentration) is shown in Figure 5E. The difference in superoxide production in the presence or absence of L-NAME was indicative of eNOS coupling/uncoupling activity. In controls where eNOS is coupled, L-NAME increases measured superoxide production by removing the buffering effects of NO on superoxide. When uncoupled, eNOS produces superoxide instead of NO, and inhibition of eNOS by L-NAME results in a decrease in measured superoxide production.

Bioinformatic Analysis of E2F1 Correlation to DHFR

We collected 4 large-scale human transcriptomic data related to the vasculature, including human aortic endothelial cells,[32] aortic artery, coronary artery, and tibial artery from the GTEx study (Gene by Tissue Expression),[33] to examine the correlation between E2F1 and DHFR. The aortic endothelial cell dataset was retrieved from GSE30169, which contains 629 microarrays of human primary aortic endothelial cells with and without treatment with OxPAPC—a proinflammatory factor involved in atherosclerotic plaque formation.[32] There were 307 control arrays and 322 OxPAPC-treated arrays. Generally, duplicate arrays on 158 cultures for both control and OxPAPC conditions were generated; in 12 cases, expression per condition was based on a single array, and 1 culture had triplicate OxPAPC arrays, and 4 cultures had quadruplicate OxPAPC arrays. The expression intensity values were normalized with the robust multiarray average normalization method using the just robust multiarray average function of R package affy.[34,35] The gene expression level was then obtained by the average expression of probes for the same gene. From the GTEx project v7[33], 53 tissue-specific RNASeq expression data are available (https://gtexportal.org/home/datasets). We collected the expression data on 3 artery-related tissues: aortic artery, coronary artery, and tibial artery, where the sample sizes of these 3 tissues are 299, 173, and 441, respectively. The normalized counts per gene, transcripts per million,[36] were used for correlation analysis.

Pearson correlation was calculated between E2F1 and DHFR in each dataset and various conditions. Differences in the expression levels of E2F1 and DHFR between control and OxPAPC-treated aortic endothelial cells were assessed using 2-sided Wilcoxon rank-sum test.[37]

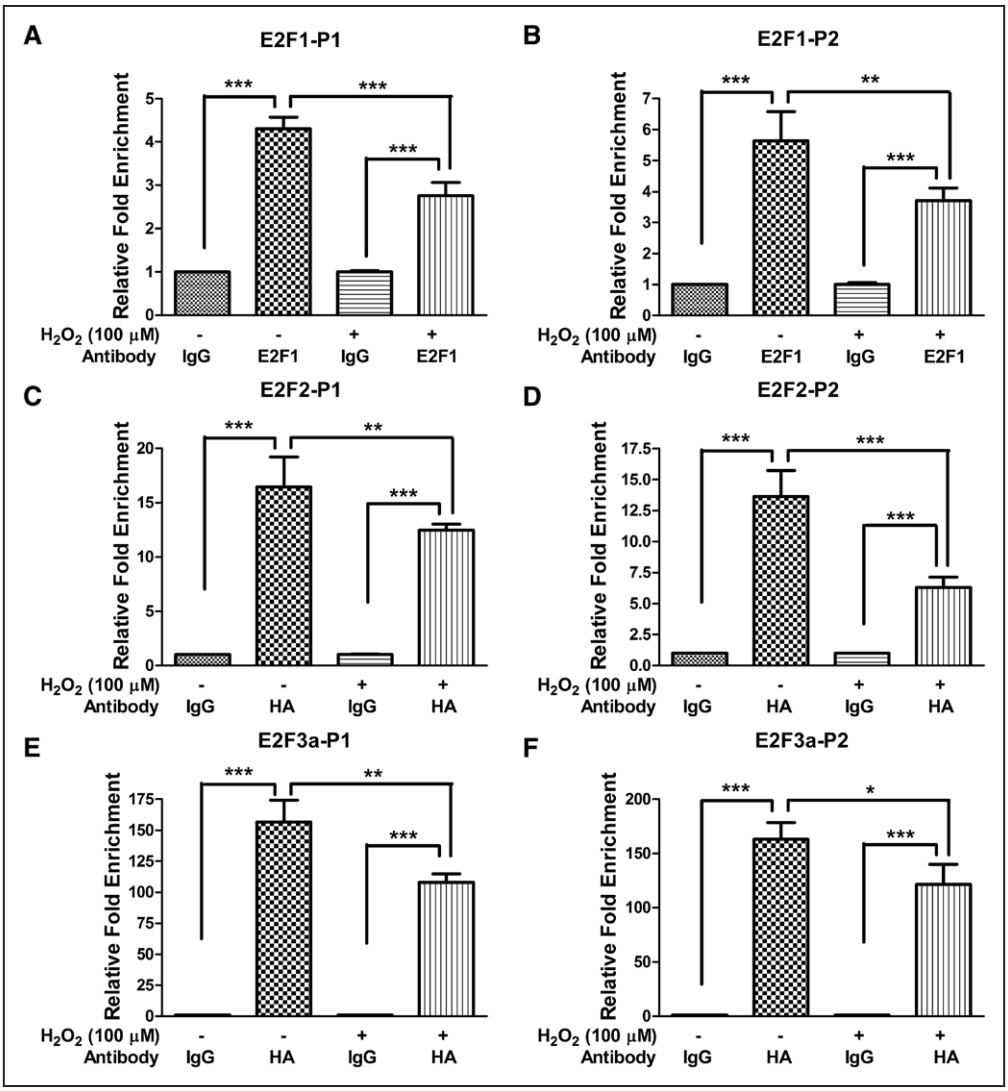

Figure 3. Chromatin immunoprecipitation (ChIP) quantitative real-time polymerase chain reaction (qPCR) assay indicating hydrogen peroxide inhibition of the binding of E2F1, E2F2, and E2F3a to DHFR (dihydrofolate reductase) promoter. **A** and **B**, Bovine aortic endothelial cells (BAECs) were treated with 100 μM hydrogen peroxide (H_2O_2) for 24 h and harvested for ChIP assay. Cross-linked chromatin was immunoprecipitated with normal rabbit IgG or E2F1 antibody and analyzed by qPCR. Two primers (P1 and P2) encompassing the predicted E2F-binding site were used for ChIP-qPCR. **C**–**F**, BAECs were transfected with pCMV-E2F2 (**C** and **D**) or pCMV-E2F3a (**E** and **F**) for 36 h before exposure to H_2O_2 (100 μM, 12 h). Cross-linked chromatin was immunoprecipitated with normal rabbit IgG or HA (hemagglutinin) antibody and analyzed by qPCR using 2 primers. For all data, the level of enrichment was expressed as fold changes relative to IgG immunoprecipitates. Data are presented as Mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001; n=3 to 7.

Results

DHFR Expression and Activity Are Downregulated by H_2O_2 in BAECs

Our previous study has shown that H_2O_2 attenuates DHFR protein expression in endothelial cells,[8] which mediates Ang II–induced eNOS uncoupling to result in development of hypertension in WT mice and formation of abdominal aortic aneurysm in genetic strains.[5–10] To explore detailed molecular mechanisms underlying H_2O_2 regulation of DHFR, BAECs were treated with different doses of H_2O_2 for 24 hours or 100 μmol/L H_2O_2 at indicated time points.[38] As shown in Figure S1A and S1B in the online-only Data Supplement, H_2O_2 dose and time dependently attenuated protein abundance of DHFR. Furthermore, DHFR activity determined by HPLC mirrored this response (Figure S1C and S1D). To further investigate whether H_2O_2-induced changes in protein abundance of DHFR are a result of transcriptional regulation, DHFR mRNA levels were determined by quantitative real-time polymerase chain reaction. As demonstrated in Figure S1E and S1F, H_2O_2 downregulated DHFR mRNA levels in a dose- and time-dependent manner.

H_2O_2 Inhibition of DHFR Promoter Activity Requires E2F-Binding Sites

Next, promoter activity assay was performed using WT DHFR promoter and 3 promoter deletions as shown in Figure 1A (DHFR-2: deletion of 2 Sp1 sites from −553 to −516; DHFR-3: deletion of 2 Sp1 sites from −121 to −77; DHFR-4: deletion of 2 E2F sites from −67 to −56). Of note, H_2O_2 treatment resulted in a decline in WT DHFR promoter activity by 59% (Figure 1B). Deletion of E2F-binding sites (DHFR-4), but not that of Sp1 sites (DHFR-2 and DHFR-3), completely abolished the inhibitory effects of H_2O_2 on DHFR promoter activity

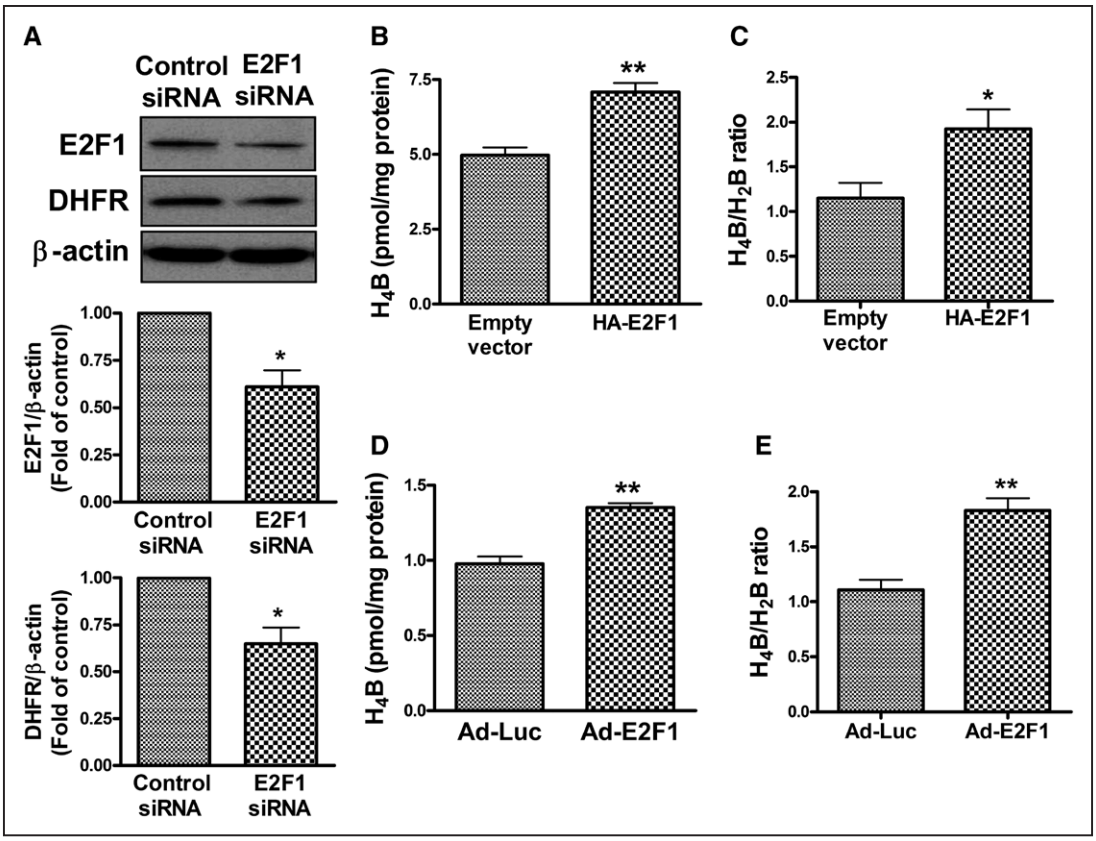

Figure 4. E2F1 increases tetrahydrobiopterin (H_4B) bioavailability via upregulation of DHFR (dihydrofolate reductase) in vitro and in vivo. **A**, Bovine aortic endothelial cells (BAECs) were transfected with 100 nmol/L E2F1 siRNA for 48 h and harvested for Western blotting. The protein abundances of E2F1, DHFR, and β-actin were detected. Mean±SEM. *P<0.05 vs ctrl; n=5. **B** and **C**, BAECs were transfected with pCMV-HA (hemagglutinin) or pCMV-HA-E2F1 for 48 h. H_4B (tetrahydrobiopterin) levels were determined by HPLC, and H_4B:H_2B (dihydrobiopterin) ratio was calculated. Mean±SEM. *P<0.05; n=3 to 4. **D** and **E**, Wild-type mice were injected with Ad-luc or Ad-E2F1 (10^9 IFU) directly into the left ventricle of the heart for 14 d. Aortic H_4B levels were determined by HPLC, and H_4B:H_2B ratio was calculated. Mean±SEM. **P<0.01; n=4.

(Figure 1B). These data indicate that E2F-binding sites are required in H_2O_2 regulation of DHFR promoter activity.

Diminished E2F1, E2F2, or E2F3a Mediates H_2O_2-Induced Reduction in DHFR Promoter Activity

Because E2F-binding sites were found critical for the regulation of DHFR promoter activity by H_2O_2, we next examined which one(s) of the 8 members of the E2F family was involved.[27,39,40] Promoter activity assays indicate that without H_2O_2 treatment, transfection of E2F1, E2F2, and E2F3a activated DHFR promoter activity (Figure 1C), whereas that of E2F4, E2F5, E2F7, and E2F8 inhibited its activity (Figure 1C and 1E). Transfection with E2F6 plasmid had no obvious effect (Figure 1E). Under the treatment with H_2O_2, E2F1, E2F2, or E2F3a overexpression effectively alleviated H_2O_2-induced attenuation in DHFR promoter activity (Figure 1D and 1F). Moreover, H_2O_2 significantly reduced mRNA levels of E2F1, E2F2, and E2F3a (Figure 2A through 2C), which resulted in corresponding decreases in their protein abundances by around 50% (Figure 2D through 2F). These data indicate that deficiencies in E2F1/2/3a are specifically involved in H_2O_2 downregulation of DHFR.

H_2O_2 Weakens Binding of E2F1/2/3a to DHFR Promoter

To further examine the direct roles of E2F1/2/3a in H_2O_2 regulation of DHFR, chromatin immunoprecipitation assay was used to evaluate physical interaction between E2F1/2/3a and DHFR promoter with or without H_2O_2 treatment. Two primers for each E2F isoform were used to amplify targeted DNA sequence harboring E2F1/2/3a. Meanwhile, a negative primer that recognizes 5 kb upstream of E2F-binding sites was also designed. As shown in Figure 3A and 3B, compared with the IgG group, E2F1 antibody significantly pulled down more E2F-binding fragments, implicating that E2F1 physically associates with DHFR promoter. Similar results were found when E2F2 (Figure 3C and 3D) or E2F3a (Figure 3E and 3F) was immunoprecipitated. As is obvious, H_2O_2 significantly inhibited binding to DHFR promoter of all 3 E2F isoforms.

E2F1 Increases H_4B Bioavailability via Upregulation of DHFR In Vitro and In Vivo

Although all 3 E2F isoforms are involved in the downregulation of DHFR by H_2O_2, H_4B levels were determined after overexpression of E2F1 as a representative isoform of the three. As shown in Figure 4A, transfection of endothelial cells with siRNA targeting E2F1 decreased its protein levels to 61.0±8.8% of controls, while it also reduced the protein abundance of DHFR to 64.9±8.7% of controls. On the contrary, overexpression of E2F1 in endothelial cells significantly increased bioavailable H_4B levels (Figure 4B; HA [hemagglutinin]-empty vector: 5.0±0.2 pmol/mg

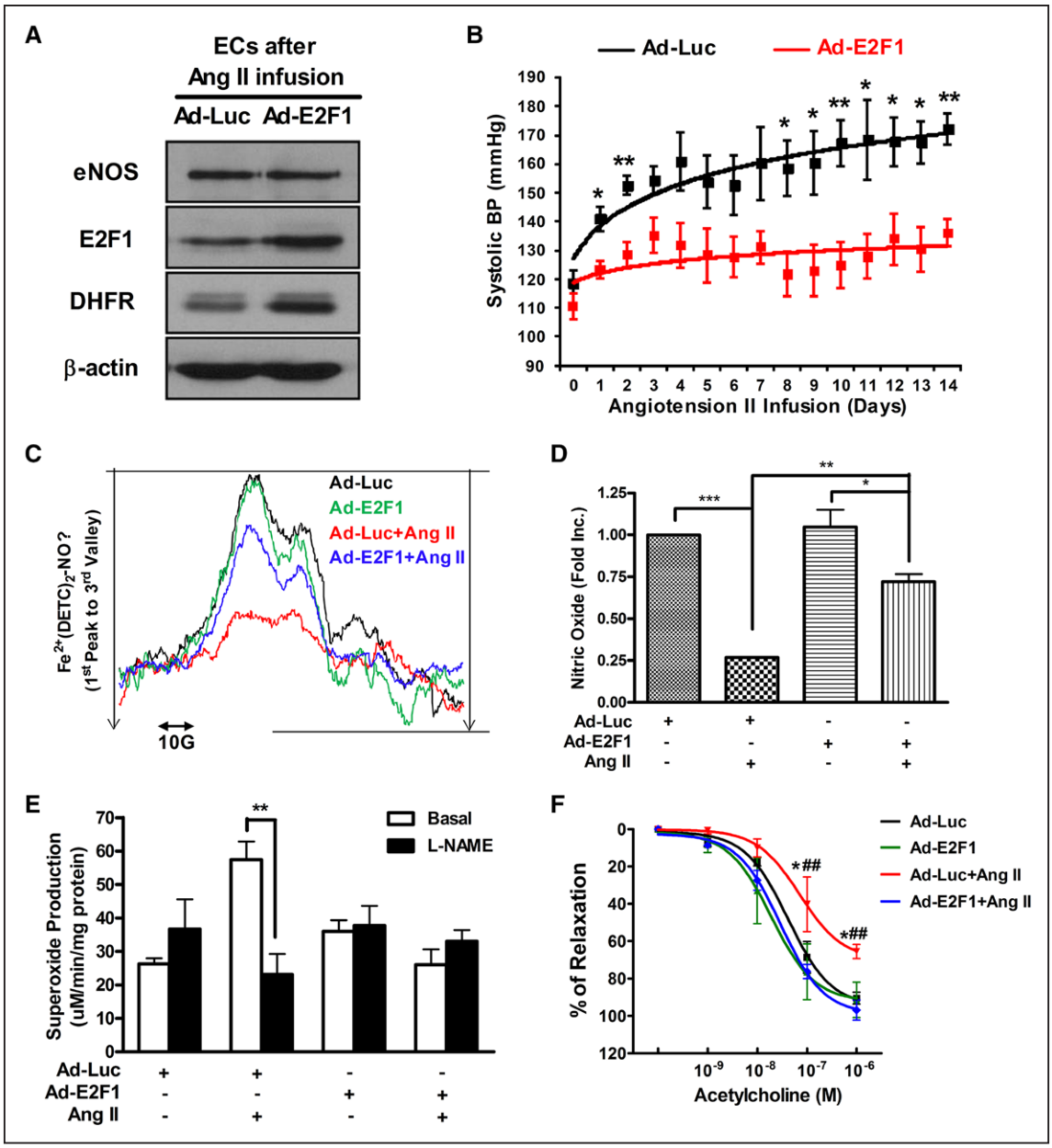

Figure 5. Restoration of NO bioavailability and reduction in blood pressure (BP) by adenovirus-mediated E2F1 overexpression in vivo. Wild-type mice were injected with Ad-luc or Ad-E2F1 (10^9 IFU) directly into the left ventricle of the heart 3 d before being infused with Ang II (angiotensin II; 0.7 mg/kg per day) for 14 d. **A**, Representative Western blots of eNOS (endothelial NO synthase), E2F1, DHFR (dihydrofolate reductase), and β-actin in primarily isolated aortic endothelial cells (ECs) from Ang II–infused animals. **B**, BP responses as monitored by an intracarotid telemetry method. **C** and **D**, The aortic NO bioavailability as determined by electron spin resonance (ESR). **C**, Representative ESR NO spectra. **D**, Quantitative grouped data of bioavailable NO. Mean±SEM. ***P<0.001; n=3 to 5. **E**, eNOS uncoupling activity as determined by ESR: L-NAME (N(ω)-nitro-L-arginine methyl ester)–dependent superoxide production. Mean±SEM. **P<0.01; n=5. **F**, Endothelium-dependent vasorelaxation in response to acetylcholine in precontracted aortic rings. Mean±SEM. *P<0.05, Ad-Luc vs Ad-Luc+Ang II; ##P<0.01, Ad-E2F1+Ang II vs Ad-Luc+Ang II; n=3 to 4.

protein versus HA-E2F1: 7.1±0.3 pmol/mg protein; P<0.05). Adenovirus-mediated overexpression of E2F1 in vivo also resulted in elevated aortic H_4B bioavailability (Figure 4C; control adenovirus Ad-Luc: 0.98±0.05 pmol/mg protein versus Ad-E2F1: 1.35±0.03 pmol/mg protein; P<0.01). In addition, both in vitro (Figure 4D) and in vivo (Figure 4E) overexpression of E2F1 increased H_4B:H_2B ratio by around 1.7-fold. These data indicate that augmentation of the E2F1-DHFR axis is effective in improving H_4B bioavailability.

Overexpression of E2F1 Attenuates Ang II–Induced Hypertension via Restoration of Endothelial DHFR Function to Improve NO Bioavailability and Endothelium-Dependent Vasorelaxation

In view of the intermediate role of E2F1/2/3a deficiency in H_2O_2 attenuation of DHFR protein expression and activity, the effects on BP regulation of E2F1 overexpression in Ang II–infused mice were examined. Ad-E2F1 (or Ad-Luc as the control) was directly injected into the left ventricle of the heart to achieve overexpression of E2F1. As shown in Figure 5A,

transfection of Ad-E2F1 in vivo led to increased protein abundance of E2F1 and DHFR (Figure 5A). This has resulted in markedly abrogated BP in Ang II–infused mice (Figure 5B; systolic BP of Ad-E2F1 group at day 14: 136.0±4.8 mm Hg versus systolic BP of Ad-Luc group at day 14: 172.2±5.4 mm Hg; $P<0.01$; real-time telemetry). Upregulation of endothelial DHFR protein expression is anticipated to improve NO bioavailability.[5,6,8,9] Indeed, Ad-E2F1 overexpression resulted in markedly restored NO bioavailability in Ang II–infused animals (Figure 5C and 5D). Moreover, Ang II–induced eNOS uncoupling activity was also completely attenuated by E2F1 overexpression (Figure 5E). Next, we evaluated endothelium-dependent vasorelaxation in these animals. As shown in Figure 5F, Ad-E2F1 overexpression markedly restored vasorelaxation of aortic rings isolated from Ang II–infused animals.

To demonstrate whether H_2O_2 is involved in Ang II downregulation of E2F1, we pretreated BAECs with PEG-catalase (100 U/mL; 30 minutes) before exposure of the cells to Ang II (100 nmol/L) for 24 hours. As shown in Figure S2A, PEG-catalase reversed Ang II–induced downregulation of E2F1, indicating that Ang II attenuates E2F1 expression through H_2O_2. There is also a small and nonstatistically significant reduction in baseline E2F1 expression with PEG-catalase treatment, which is, however, not surprising as low levels of reaction oxygen species are known to play important roles in cell signaling such as that involved in proliferation and migration.[41] We further examined which signaling pathway(s) is (are) required for H_2O_2-induced E2F1 downregulation. Inhibition of p38 MAPK (mitogen-activated protein kinase) by SB203580 (10 μmol/L) completely prevented H_2O_2 downregulation of E2F1, whereas inhibition of ERK (extracellular regulated MAP kinase, U0126; 50 μmol/L), PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase, Wortmannin; 100 nmol/L), or JNK (c-Jun N-terminal kinase, JNK inhibitor; 10 μmol/L) had no effect (Figure S2B). Taken together, these data indicate that overexpression of E2F1 is highly effective in attenuating Ang II–induced hypertension via restoration of endothelial DHFR expression to improve NO bioavailability and endothelium-dependent vasorelaxation, and that Ang II–induced E2F1 deficiency is mediated by H_2O_2 activation of p38 MAPK.

Bioinformatic Analyses Confirm Positive Correlation Between E2F1 and DHFR in Human Endothelial Cells and Arteries and Downregulation of Both by oxPAPC

We also used bioinformatic approaches (see Methods for details) to further evaluate the relationship between E2F1 and DHFR using existing datasets of human endothelial cells and arteries. As shown in Figure 6A, positive correlation between E2F1 and DHFR was found in human aortic endothelia cells, with (right) or without (middle) treatment of OxPAPC. Correlation was also significant for data that pooled all endothelial cells (Figure 6A, left). We then examined 3 transcriptomic datasets of human arteries of aortas and coronary and tibial arteries. We found that expression of E2F1 positively correlated with expression of DHFR in all these human arteries. Moreover, both E2F1 and DHFR were downregulated by oxPAPC in human aortic endothelial cells (Figure 6C), which is similar to our findings of downregulation of E2F1/DHFR axis in hypertension. Therefore, these bioinformatics analyses further indicate that a loss of E2F1 to result in DHFR deficiency may be an important pathological process to target for vascular diseases.

Discussion

The most significant findings of the present study include the following: (1) downregulation of DHFR expression by H_2O_2 is mediated by decreased mRNA expression of E2F1/2/3a and its protein binding to DHFR promoter; (2) overexpression of E2F1 to restore DHFR expression, H_4B levels, eNOS coupling activity, and NO bioavailability completely attenuated elevated BP in Ang II–infused mice. We have previously shown that H_2O_2 mediates Ang II uncoupling of eNOS to induce hypertension.[5–10] In the current study, we present further evidence that H_2O_2-induced downregulation of E2F1/2/3a expression and binding to DHFR promoter diminishes DHFR transcription and protein abundance. Intriguingly, adenovirus-mediated overexpression of E2F1 to restore DHFR expression in Ang II–infused mice abolished elevation in BP. These data indicate that specifically targeting E2F1 to restore DHFR function and eNOS coupling activity may serve as a novel therapeutic option for the treatment of hypertension.

Oxidative stress has been implicated in the pathogenesis of hypertension.[4–12,16,19,42] We have previously shown that NOX (nicotinamide adenine dinucleotide phosphate oxidase)-derived vascular superoxide production causes eNOS uncoupling, NO deficiency, and increased BP.[4–10] In particular, we have established that H_2O_2 produced from transient activation of endothelial NOX induces subsequent deficiency in DHFR to result in persistent H_4B deficiency and eNOS uncoupling,[8] which in turn mediates development of hypertension in Ang II–infused mice.[5–9] Our current study mainly focuses on the mechanistic details of H_2O_2 downregulation of DHFR and efficacies in reducing BP of strategies targeting these mechanisms. Consistent with previous findings, H_2O_2 downregulated DHFR protein abundance in endothelial cells.[8] The present study further demonstrated reduced mRNA expression and impaired activity of DHFR in response to H_2O_2 exposure. The changes in DHFR mRNA appeared much sooner than that of protein, implicating that DHFR might be transcriptionally regulated by H_2O_2. Indeed, the dual-luciferase reporter assay revealed that H_2O_2 exposure induces an evident decrease in DHFR promoter activity. Deletion of the E2F-binding sites, but not those of Sp1, completely abolished H_2O_2 attenuation of DHFR promoter activity, indicating that E2F is involved in the transcriptional regulation of DHFR by H_2O_2.

E2F family has been categorized into 2 subfamilies[43,44] of activators, which include E2F1, E2F2, and E2F3a, and repressors, which include E2F3b and E2F4-8. However, this classification has been debated because the so-called activating E2Fs have been shown to repress equal amount of genes they activate and that E2F4, E2F5, and E2F6 could actually work as activators,[45,46] whereas E2F2 and E2F3 turned out to be repressors under some circumstances.[47] Our data indicated that E2F1/2/3a takes part in the regulatory process of H_2O_2 attenuation of DHFR, based on data from the luciferase reporter assay (overexpression with reasonable amount of plasmids) and the chromatin immunoprecipitation assay. E2F1/2/3a functioned as the activators in DHFR transcriptional regulation, which could be inhibited by H_2O_2. H_2O_2 mainly decreased E2F1/2/3a at mRNA levels, which resulted in corresponding

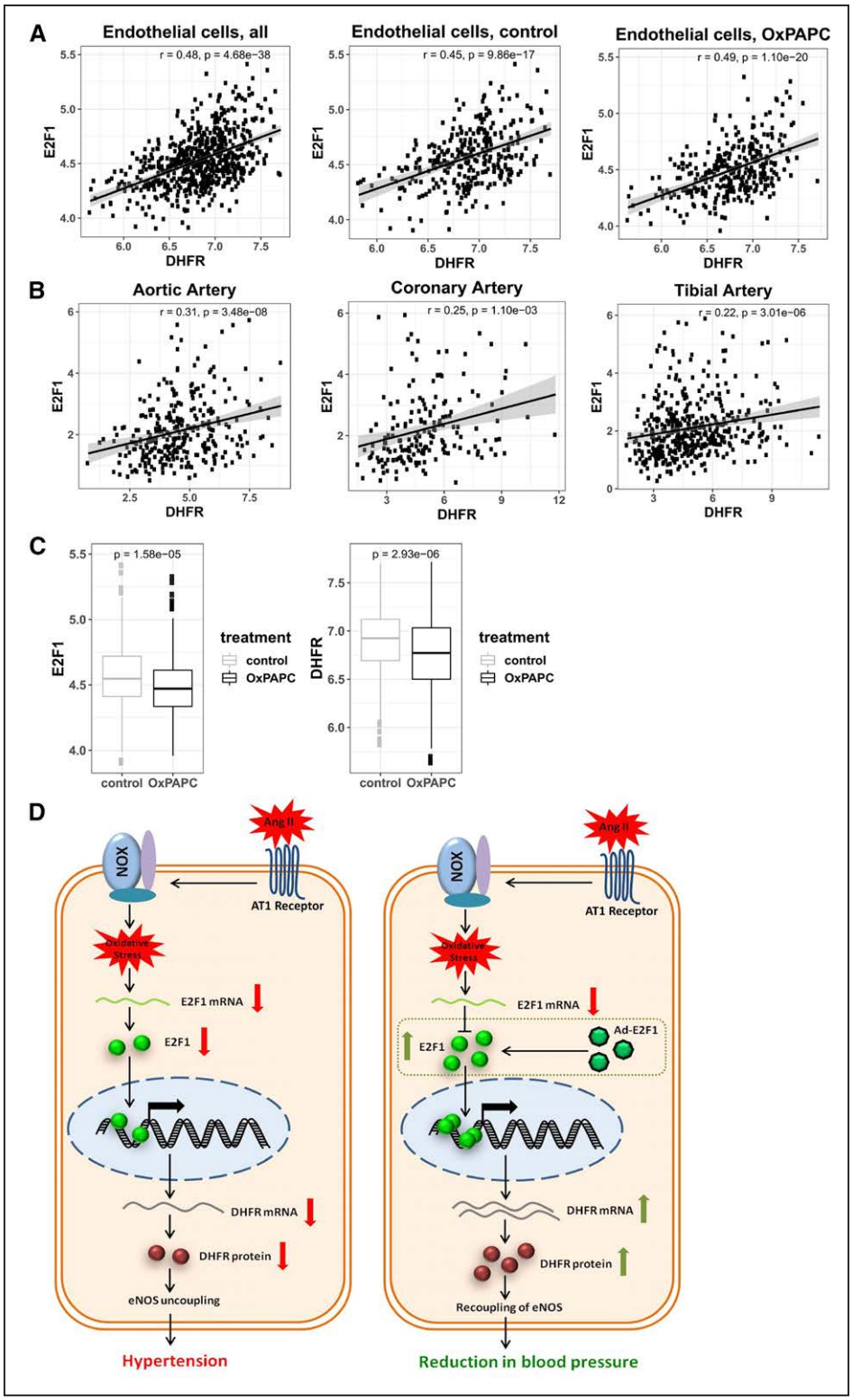

Figure 6. Targeting E2F for the restoration of endothelial DHFR (dihydrofolate reductase) and eNOS (endothelial NO synthase) function to treat hypertension. **A**–**C**, Validation of the relationship between E2F1 and DHFR using large human transcriptomic datasets. **A**, Correlation plots between E2F1 and DHFR in human aortic endothelial cells from all samples (**left**), controls only (**middle**), and oxidized phospholipid (oxPAPC) treatment experiments (**right**). **B**, Correlation plots between E2F1 and DHFR in aortic artery tissues (**left**), coronary artery tissues (**middle**), and tibial artery tissues (**right**). P and r represent the Pearson correlation coefficient and statistical significance of the correlation. **C**, Significant downregulation of E2F1 (**left**) and DHFR (**right**) between control and oxPAPC-treated aortic endothelial cells, where *P* is computed from 2-sided unpaired Wilcoxon test. **D**, Schematic illustration of a role of E2F/DHFR deficiency in oxidative stress–induced hypertension and of targeting E2F as a novel therapeutic option for the treatment of hypertension. **Left**, Ang II (angiotensin II) induces a rapid and transient activation of endothelial NOX (nicotinamide adenine dinucleotide phosphate oxidase), resulting in oxidative stress and hydrogen peroxide-dependent downregulation of E2F1 mRNA expression and protein abundance. Loss of E2F1 binding to DHFR promoter results in reduced DHFR protein expression, uncoupling of eNOS, and development of hypertension. **Right**, Adenovirus-mediated overexpression of E2F1 in vivo transcriptionally upregulates DHFR protein expression to restore eNOS function, which in turn restores NO bioavailability and endothelium-dependent vasodilatation to reduce blood pressure. AT1 indicates angiotensin II type 1.

reduction in protein expression. This led to attenuated amount of E2F1/2/3a that is available for the physical binding to the DHFR promoter. In addition, we have shown that p38 MAPK is required for H_2O_2-induced downregulation of E2F1, whereas ERK, PI3K, and JNK are not involved.

It has been shown that the E2F family members are responsible for controlling the transcription of various genes that are essential for cell cycling, development, differentiation, DNA synthesis and replication, and DNA damage repair.[40] However, little is known about their roles in endothelial cells. In the present study, we found that E2F1/2/3a overexpression increased DHFR protein expression in cultured endothelial cells, and that adenovirus-mediated E2F1 overexpression upregulated endothelial DHFR protein levels in vivo. Our previous studies have shown that infusion of Ang II induces hypertension in WT mice, in which DHFR deficiency-dependent eNOS uncoupling is critically involved.[5–10] Restoration of DHFR by either folic acid diet or DHFR overexpression recoupled eNOS to reduce BP.[4–6,9] Based on the novel mechanisms identified in this study that H_2O_2 induces DHFR deficiency via attenuation of E2F1/2/3a expression and binding to DHFR promoter, E2F1 was overexpressed using an adenoviral system in vivo. Overexpression of E2F1 in vivo restored endothelial DHFR protein abundance, eNOS coupling activity, and NO bioavailability, resulting in improved endothelium-dependent vasorelaxation and reduced BP.

Perspectives

As schematically illustrated in Figure 6D (using E2F1 as a representative E2F isoform of E2F1/2/3a), Ang II induces a rapid and transient activation of endothelial NOX, resulting in oxidative stress and H_2O_2-dependent downregulation of E2F1/2/3a mRNA and protein abundance. Loss of E2F1/2/3a binding to DHFR promoter leads to reduced DHFR protein expression, uncoupling of eNOS, and development of hypertension. Adenovirus-mediated overexpression of E2F1, however, completely attenuated elevated BP in Ang II–infused mice, via upregulation of DHFR protein expression to restore H_4B and NO bioavailability and coupling activity of eNOS. Furthermore, we observed positive correlation between E2F1 and DHFR using 4 independent human transcriptomic datasets and confirmed downregulation of both under oxidative conditions in human aortic endothelial cells. These results strongly support the robustness and translational value of our molecular mechanistic findings. In conclusion, our study indicates that specifically targeting E2F1/2/3a may be used as a novel therapeutic option for the treatment of hypertension.

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Disclosures

None.

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Novelty and Significance

What Is New?

- First identification of an intermediate role of E2F deficiency in hydrogen peroxide downregulation of DHFR (dihydrofolate reductase).
- First identification that targeting E2F to restore DHFR and eNOS (endothelial NO synthase) function is highly effective in reducing blood pressure.

What Is Relevant?

- Our data reveal a novel mechanism underlying oxidative stress–induced hypertension.
- Our data establish a novel therapeutic approach for the treatment of hypertension.

Summary

These new findings indicate that specifically targeting E2F may serve as a novel therapeutic option for the treatment of hypertension.