ONLINE SUPPLEMENT

Androgen-dependent hypertension is mediated by 20-HETE-induced vascular dysfunction: Role of IkappaB kinase

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Running Title: 20-HETE and IKK in androgen-dependent hypertension

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MATERIALS AND METHODS

Animal studies. All of the experimental protocols were performed following an Institutional Animal Care and Use Committee–approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sprague–Dawley male rats (8-9-week-old; Charles Rivers, Wilmington, MA) were administered DHT (56 mg/kg/day, ip) or its vehicle (20% benzyl alcohol in corn oil, ip) for up to 17 days. The indicated dose of DHT have been previously shown to induce hypertension in normotensive rats.[1] In some experiments, rats were given the CYP4A selective inhibitor *N*-hydroxy-*N*'-(4-butyl-2-methylphenyl)-formamidine [2] (HET0016; 10 mg/kg/day in 10% w/v lecithin in saline, ip), or the 20-HETE antagonist, 20-6,15-HEDE [3] (10 mg/kg/day in 5% ethanol in saline, ip) either concomitantly or after 7 days of DHT treatment. In another set of experiments, rats were given the IKK inhibitor Parthenolide (1 mg/kg/day in DMSO, ip).[4] For blood pressure measurements, rats were acclimated for seven days prior to the start of experiments. Systolic BP was determined by the tail cuff method (Kent Scientific). In brief, rats were placed on a far infrared heating pad for 7-10 minutes. Systolic blood pressure measurements were recorded after five cycles of acclimatization. At the end of experiments, rats were anesthetized with phenobarbital (50 mg/kg body wt, ip), and laparotomy was performed. The kidneys were removed, and renal interlobar arteries were microdissected for biochemical and functional studies.

Measurements of 20-HETE. Renal interlobar arteries were isolated from rats and incubated in oxygenated Krebs biocarbonate buffer, pH7.4, with 1mM NADPH for 1 hour at 37^{o}C with gentle shaking. 20-HETE was extracted and quantified by LC/MS/MS (Applied Biosystems, Foster City, CA) as previously described.[5]

Vascular function. Interlobar arteries (~230 µm, internal diameter) were cut into rings (~2 mm) andmounted on wires in the chambers of a multivessel myograph (JP Trading, Aarhus, Denmark) filled with Krebs' buffer (37°C) gassed with 95% O_2/5% CO_2. After 30 to 60 min of equilibration, the vessels were set to an internal circumference equivalent to 90% of that which they would have in vitro when relaxed under a transmural pressure of 80 mm Hg. Isometric tension was monitored continuously before and after experimental interventions. A cumulative concentration-response curve to phenylephrine (10^{-9} to $5x10^{-5}$ mol/L) was constructed in the presence and absence of 20-6,15-HEDE (1 µmol/L). Phenylephrine-induced increase of isometric tension was expressed as the percentage of the increase in tension produced by the maximum contraction achieved. To determine acetylcholine-mediated vasorelaxation, vessels were rinsed with Krebs three times and incubated for 1 h followed by a final wash. After washing with Krebs buffer, vessels were preconstricted with phenylephrine and a cumulative concentration-response curve to acetylcholine (10^{-9} to $5x10^{-5}$ mol/L) was constructed in the presence and absence of 20-6,15-HEDE (1 µmol/L).

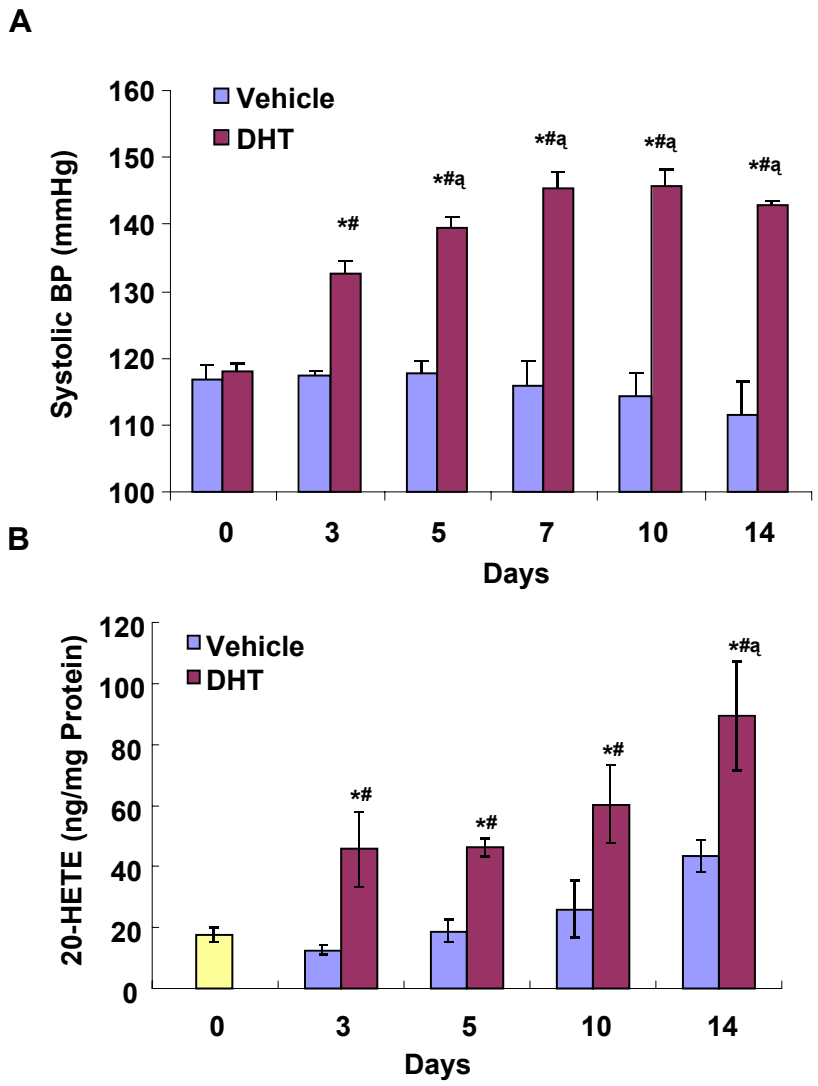
Western blot analysis. Western blot analysis of renal interlobar arterial segments were performed as previously described (27, 33 jenn AJP) using primary antibodies against phosphorylated IκBα (Cell Signaling, Beverly, MA), IκBα, VCAM (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma, St. Louis, MO).

NF-κB activation. Nuclear and cytosolic proteins were extracted from microdissected renal interlobar arteries using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. Nuclear protein concentration was measured by the Bradford protein assay (Bio-Rad,

Hercules, CA). NF-κB activation was determined using the NF-κB p65 transcription factor assay (Cayman Chemical Company, USA.) according to the manufacturer's instructions.

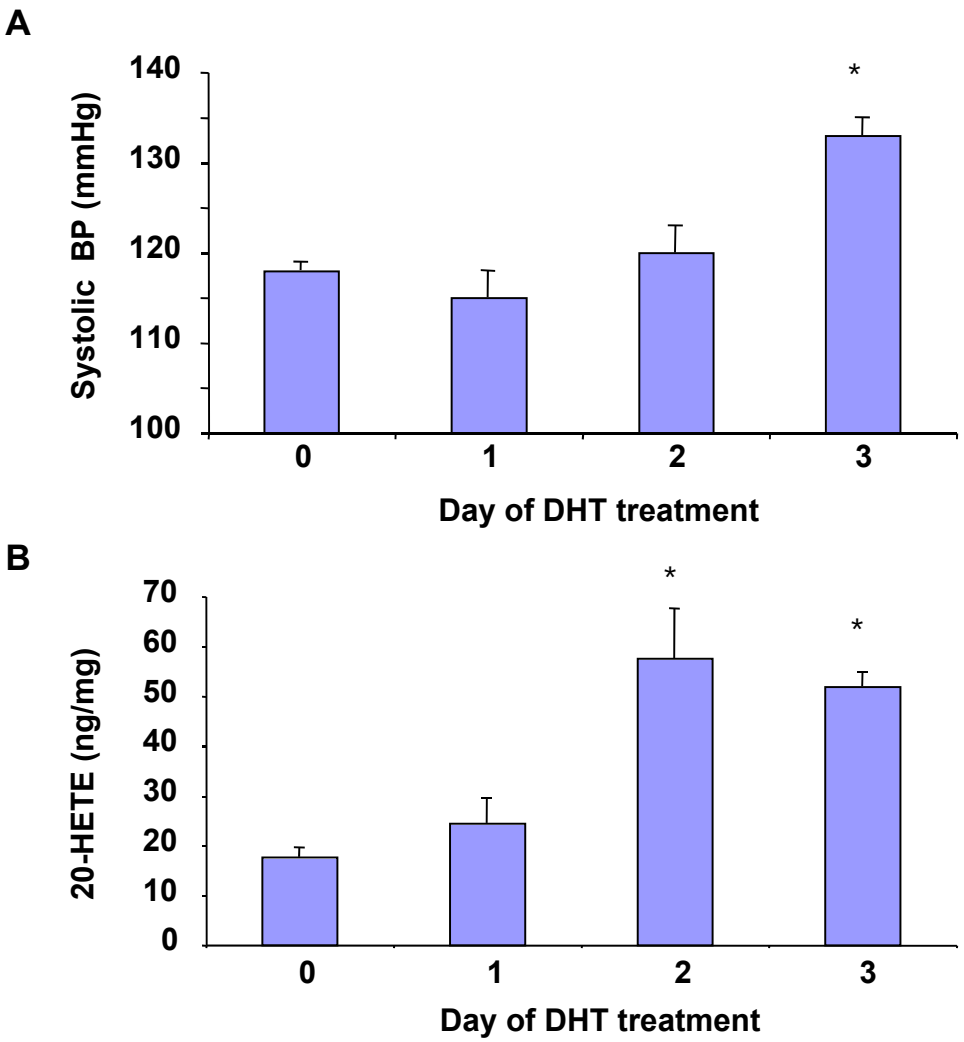
Statistics. The data are presented as mean ± standard error (SE). Statistical significance ($p<0.05$) between the experimental groups was determined by the Fisher method of analysis for multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single factor analysis of variance (ANOVA; Dunnett's Multiple Comparison Test) for multiple groups or unpaired *t*-test for two groups.

Figure 1



<u>Figure S1:</u> Time course of increases in blood pressure and vascular 20-HETE in DHT-treated rats. (A) Systolic blood pressure **(**N=10-16). **(B)** 20-HETE levels in renal interlobar arteries (N=6-12). Results are mean±SE, *p<0.05 vs vehicle treated rats; $^{\#}$p<0.05 vs day 0; ‡p<0.05 vs DHT after 3 days; †p<0.05 vs DHT after 5 days.

Figure 2



<u>Figure S2:</u> Initial time course of blood pressure increase and vascular 20-HETE in DHT-treated rats. (A) Systolic blood pressure **(**N=6-12). **(B)** 20-HETE levels in renal interlobar arteries (N=6). Results are mean±SE; *p<0.05 vs day 0.

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