MATERIALS AND METHODS

Generation of Cyp4a12 transgenic mice. A 1,624 bp cDNA containing the Cyp4a12 coding sequence, as well as 6 and 91 bp upstream and downstream of its ATG and TAA initiation and termination codons was cloned in to the EcoRV and BamH1 sites of the pTRE2 vector, downstream from a tetracycline responsive and PminCMV promoter sequences. Amplification and sequence analysis for cloning integrity were followed by removal of vector sequences by Xho1/Ase1 digestion, and purification of the linearized Cyp4a12 transcriptional unit by gel electrophoresis. Similarly, a pUHrT62-1 vector (a gift from Dr. Hermann Bujard, Universitaet, Hidelberg, Germany) containing the tetracycline reversed transactivator (rtTA) under the control of a CMV promoter and a VP16 activator domain was digested with XhoI, HindIII and PvuI to remove vector sequences, and purified by gel electrophoresis. The mixed Cyp4a12 p-TRE2 and rtTA2 linearized constructs were used for pronuclear injection into B6D2 (a cross between female C57BL/6J and male DBA/2J) mouse embryos, and their transfer to recipient mice. Founders were identified by PCR analysis of tail genomic DNAs using exon-exon selective primers

Cyp4a12 and rtTA specific primers. Cyp4a12**-** 5'-CCAACTCTTCCTCATCCT-3'; 5'-GTTGAGGACCTGAACGA-TCT-3'. Diagnostic Fragments: Cyp4a12 Transgene: 178 bp; Cyp4a12 gene: 611 bp; rtTA (M2) -5' -GGACAAGAGCAAAGTCATAAACGG-3'; 5'-GGCATAGAATCGGTGGTA. Diagnostic Fragment: 472 bp.

Analysis of Cyp4a12 transgene copy number. Relative transgene copy numbers ~~was~~ were determined by Tag-man Quantitative real time PCR (Q-PCR)(AB Applied Biosystems,) using the $2^{-\Delta\Delta Ct}$ comparative Ct method [1, 2] and the following ~~combinations~~ ~~of~~ PCR primers and gene/transgene/rtTA specific probes: Cyp4a12 Transgene: 5'-ACCCTGACTA-CATGAAGCTGATTCT-3';5'-TGAAACCATGTCTGTCCATCCA-3'. *Probe: 5'-TCCCTGGATT-GGGCGTG-3'***.** WT non-transgenic mice (normalization control): 5' -TCTTGGGAC-AATGGACATCATGAAT-3'; 5'-AGTTCCTTCCTCTGGCTGGTA-3'*Probe: CCCAGCATGT-GATTTC-3'*. rtTA (M2): 5'-GGCCTGACGACAAGGAAACTC-3'; 5'-TGTTCTTCACGTGC-CAGTACAG-3'Probe: 5'-CTCAACTCCCAGCTTTT-3'. Cyp4a12 tg/rtTA hemizygous mice were mated until reproducible and constant Cyp4a12 and rtTA trangene copy number = homozygous at the different Cyp4a12tg locus.

Animal experimentation. All experiments were performed following an Institutional Animal Care and Use Committee–approved protocol in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Male and female *Cyp4a14(-/-)* [3] (129/Sv background) were used between 8-14 weeks of age. Cyp4a12tg mice were administered doxycycline (DOX, 1mg/ml) in their drinking water for 15-60 days. In some experiments, mice were administered the 20-HETE antagonists, 20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid (20-HEDE) or *N*-(20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoyl)glycine (20-HEDGE) (10 mg/kg of body weight per day in 5% ethanol in saline, i.p.). This dose was chosen based on studies [4, 5] demonstrating that the increase in vascular reactivity and/or the decrease in relaxation to acetylcholine seen in models of 20-HETE dependent hypertension are reversed by in vivo administration of these antagonists. The effect of in vivo administration of these antagonists was similar to that of ex vivo administration of the same antagonists. Moreover, adding back 20-HETE to the organ bath reversed the effect of the antagonists (administered in vivo or ex vivo). Androgen-driven hypertension was produced in *C57BL/6* mice that were implanted with pellets containing placebo or 5α-dihydrotestosterone (DHT; 5 mg/kg/day for 14 days; Innovative research of America). Briefly, a small subcutaneous pocket was created between the scapula and the pellet was inserted away from the incision site. For blood pressure

measurements, mice were acclimatized to the tail-cuff measurement for seven days prior to the start of experiments. In brief, mice were placed on a far infrared heating pad for 7-10 minutes. Systolic blood pressure measurements (CODA non-invasive blood pressure system, Kent Scientific, Torrington CT) were recorded after five cycles of acclimatization. At the end of experiments, mice were anesthetized with phenobarbital (50 mg/kg body wt) and laparotomy was performed. The kidneys were removed and renal preglomerular microvessels (PGMV) or mesenteric arteries were microdissected for biochemical and functional analyses.

Measurements of 20-HETE. Renal microsomal 20-HETE synthesis from arachidonic acid was measured as previously described.[6] PGMV were isolated from mice and incubated in oxygenated Krebs bicarbonate buffer, pH 7.4, with 1 mM NADPH for 1 hour at 37^{o}C with gentle shaking. Deuterated-20-HETE was added as an internal standard and 20-HETE was extracted and quantified by LC/MS/MS (Applied Biosystems, Foster City, CA) as previously described.[7]

Vascular function. Renal interlobar arteries (~100 μm diameter) were cut into ring segments (2 mm) and mounted on wires in the chambers of a multi-vessel 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 $5x10^{-5}$ M) was constructed. Isometric tension is expressed as the percentage of the increase in tension produced by maximum contraction achieved. Acetylcholine (10^{-9} to $5x10^{-5}$ M)-mediated vasorelaxation was measured in interlobar arteries preconstricted with phenylephrine (1-2 x 10^{-6} M). Myogenic responses were measured in freshly isolated segments from the 3rd branch of mesenteric arteries (1–2 mm length) mounted between two micropipettes in the chamber (1 ml) of a pressure myograph (Living System Instrumentation; Burlington, VT) filled with Krebs buffer gassed with 95% O_2/5% CO_2, which was exchanged at a rate of 1 ml/min. The intraluminal pressure-internal diameter relationship was studied as previously described.[8] After equilibration at 80 mmHg, the pressure was decreased to 0 mmHg followed by 20-mmHg step increases until it reached 100 mmHg. The pressure was maintained for 5–10 min at each pressure step so that the vessels could reach a steady-state diameter. Before an experiment was concluded, the vascular preparation was superfused with calcium-free Krebs buffer containing 1 mmol/l EGTA, and the pressure-diameter relationship was examined again to obtain the passive diameter of the vessels at each level of intraluminal pressure. The internal diameter during superfusion of the arterioles with calcium-containing buffer (absolute diameter) and with calcium-free buffer (passive diameter) is expressed in micrometers. The normalized diameter refers to the absolute diameter expressed as a percentage of the passive diameter.

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.

RESULTS

Figures 1A and 1B show a RT-PCR quantification of relative copy numbers for tetracycline rtTA-reverses transactivator (M2) alone (line 13), as well as for the combined Cyp4a12 and rtTA-M2 transgenes (Cyp4a12tg) (lines 22, 89 and 96).

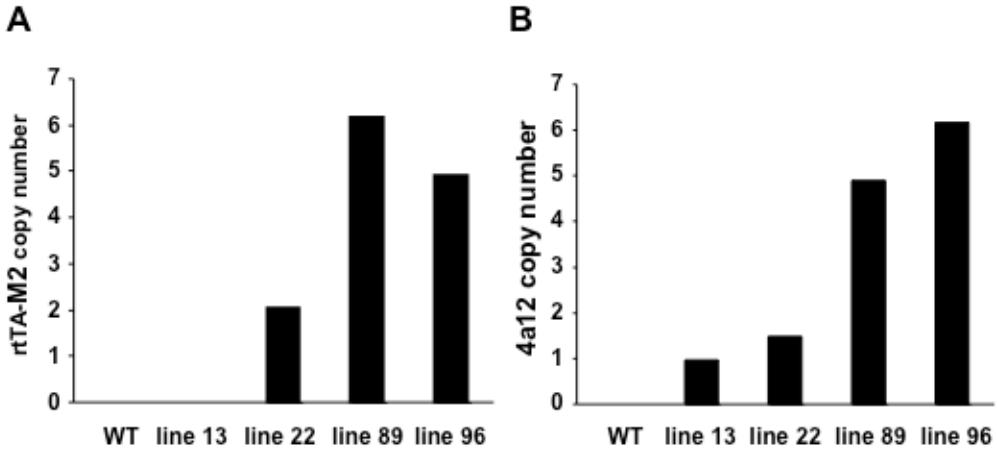

Figure 1: Copy number of rtTA-M2 (A) and Cyp4a12 (B) in four different lines of Cyp4a12tg mice. Relative copy numbers were determined by Q-PCR. Line 13 mice express the rtTA-M2 transgene (M2) only, while mice of lines 22, 89 and 96 express both the rtTA-M2 and the Cyp4a12 transgenes.

REFERENCES

1. Schmittgen TD, Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101-1108, 2008
2. Yuan JS, Reed A, Chen F, Stewart CN, Jr.: Statistical analysis of real-time PCR data. *BMC Bioinformatics* 7:85, 2006
3. Holla VR, Adas F, Imig JD, Zhao X, Price E, Jr., Olsen N, Kovacs WJ, Magnuson MA, Keeney DS, Breyer MD, Falck JR, Waterman MR, Capdevila JH: Alterations in the regulation of androgen-sensitive Cyp 4a monooxygenases cause hypertension. *Proc Natl Acad Sci U S A* 98:5211-5216, 2001
4. Sodhi K, Wu CC, Cheng J, Gotlinger K, Inoue K, Goli M, Falck JR, Abraham NG, Schwartzman ML: CYP4A2-Induced Hypertension Is 20-Hydroxyeicosatetraenoic Acid- and Angiotensin II-Dependent. *Hypertension* 56:871-878, 2010
5. Wu CC, Cheng J, Zhang FF, Gotlinger KH, Kelkar M, Zhang Y, Jat JL, Falck JR, Schwartzman ML: Androgen-Dependent Hypertension Is Mediated by 20-Hydroxy-5,8,11,14-Eicosatetraenoic Acid-Induced Vascular Dysfunction: Role of Inhibitor of {kappa}B Kinase. *Hypertension* 57:788-794, 2011
6. Nakagawa K, Marji JS, Schwartzman ML, Waterman MR, Capdevila JH: Androgen-mediated induction of the kidney arachidonate hydroxylases is associated with the development of hypertension. *Am J Physiol Regul Integr Comp Physiol* 284:R1055-R1062, 2003

7. Inoue K, Sodhi K, Puri N, Gotlinger KH, Cao J, Rezzani R, Falck JR, Abraham NG, Laniado-Schwartzman M: Endothelial-specific CYP4A2 overexpression leads to renal injury and hypertension via increased production of 20-HETE. *Am J Physiol Renal Physiol* 297:F875-884, 2009
8. Zhang F, Wang MH, Wang JS, Zand B, Gopal VR, Falck JR, Laniado-Schwartzman M, Nasjletti A: Transfection of CYP4A1 cDNA decreases diameter and increases responsiveness of gracilis muscle arterioles to constrictor stimuli. *Am J Physiol Heart Circ Physiol* 287:H1089-H1095, 2004