ORIGINAL RESEARCH

Nuclear Activation Function 2 Estrogen Receptor α Attenuates Arterial and Renal Alterations Due to Aging and Hypertension in Female Mice

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***Background*—**The cardiovascular protective effects of estrogens in premenopausal women depend mainly on estrogen receptor α (ERα). ERα activates nuclear gene transcription regulation and membrane-initiated signaling. The latter plays a key role in estrogen-dependent activation of endothelial NO synthase. The goal of the present work was to determine the respective roles of the 2 ERα activities in endothelial function and cardiac and kidney damage in young and old female mice with hypertension, which is a major risk factor in postmenopausal women.

***Methods and Results*—**Five- and 18-month-old female mice lacking either ERα ($ER\alpha^{-/-}$), the nuclear activating function AF2 of ERα (AF2°), or membrane-located ERα (C451A) were treated with angiotensin II (0.5 mg/kg per day) for 1 month. Systolic blood pressure, left ventricle weight, vascular reactivity, and kidney function were then assessed. Angiotensin II increased systolic blood pressure, ventricle weight, and vascular contractility in $ER\alpha^{-/-}$ and AF2° mice more than in wild-type and C451A mice, independent of age. In both the aorta and mesenteric resistance arteries, angiotensin II and aging reduced endothelium-dependent relaxation in all groups, but this effect was more pronounced in $ER\alpha^{-/-}$ and AF2° than in the wild-type and C451A mice. Kidney inflammation and oxidative stress, as well as blood urea and creatinine levels, were also more pronounced in old hypertensive $ER\alpha^{-/-}$ and AF2° than in old hypertensive wild-type and C451A mice.

***Conclusions*—**The nuclear ERα-AF2 dependent function attenuates angiotensin II–dependent hypertension and protects target organs in aging mice, whereas membrane ERα signaling does not seem to play a role. **(*J Am Heart Assoc*. 2020;9:e013895. DOI: 10.1161/JAHA.119.013895.)**

Key Words: aging • endothelium • estrogen • hypertension • kidney

Epidemiological studies have shown that fewer women than men are affected by cardiovascular disorders.[1,2] Estrogens provide protection against atherosclerosis, prevent neointimal proliferation, and accelerate reendothelialization of injured arteries.[1,3] Estrogens acutely stimulate endothelial nitric oxide (NO) production, and conversely, the decline in ovarian function leading to estrogen deprivation, as encountered with age, is associated with reduced endothelium-dependent vasodilatation, explained at least in part by decreased endothelial NO production.[4,5]

These vascular protective actions are mainly mediated by the estrogen receptor (ER)α, although ERβ or G protein–coupled estrogen receptor 1 have been proposed to play a role in some models and species.[6–9] These effects of ERα can be attributed to 2 main mechanisms of action: membrane (rapid) and nuclear (genomic) actions. The membrane-initiated steroid signaling of ERα in response to estrogens mainly leads to the acute production of NO and to the acceleration of endothelial migration and healing.[10] It was initially believed to play a prominent role in vascular protection.[11,12] Nevertheless, the nuclear ERα signaling, in particular, through its activation function AF2, has been more recently shown to play a key role in the protection

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Clinical Perspective

What Is New?

- The nuclear activating function 2 of estrogen receptor α, and not its membrane-associated signaling, protected both 5- and 18-month-old female mice from hypertension and the associated hypertrophy and hypercontractility.
- Furthermore, activating function 2–estrogen receptor α protected 18-month-old mice from endothelial and kidney dysfunction induced by hypertension.

What Are the Clinical Implications?

- Postmenopausal women are more exposed to cardio- and cerebrovascular disorders, especially when exposed to risks factors such as hypertension.
- Activating function 2–estrogen receptor α might be a novel therapeutic target against hypertension and its consequences in postmenopausal women.
- The present work highlights a new protective role of estrogens during aging through activating function 2–estrogen receptor α activation.

conferred by ERα stimulation, at least in young female mice.[13] However, these actions have so far been always studied in young animals, although aging is the first risk factor for cardiovascular diseases. The prevalence of hypertension is particularly high among women over 60 years of age. Indeed, the first decade after menopause is accompanied by an increase in blood pressure and has been associated with a higher risk of cardiovascular disorders.[2]

In the present work we investigated the role of ERα subfunctions on the effects of vascular aging in female mice. During the past decade we developed models to determine the phenotype resulting from the full inactivation of ERα (ERα$^{-/-}$ mice), as well as from the selective inactivation of the nuclear actions of ERα through the deletion of the activating function 2 (ERα-AF2° mice),[14] or from the selective inactivation of the membrane-initiated steroid signaling actions of ERα through the point mutation of ERα at the palmitoylable Cys451 (ERα-C451A mice).[10] Here, we report the consequences of such inactivation of the nuclear or membrane ERα actions on the function and structure of the cardiovascular system in 5- and 18-month-old female mice, submitted or not submitted to a chronic infusion of angiotensin II.[13] The impact of both aging and angiotensin II–dependent hypertension was then assessed on these different mutant mice models by measuring arterial blood pressure and vascular functions. The kidney was studied because it is a major target organ affected by aging and hypertension.[15,16]

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Animals

We used 5- and 18-month-old female mice lacking the gene encoding for ERα (ERα$^{-/-}$),[17] mice lacking the nuclear activation function AF2 (AF2° mice),[14] and mice in which the codon for palmitoylable Cys451 of ERα was mutated into alanine (C451A mice).[10] Littermate +/+ mice were used in each group as controls (wild type [WT]). The mice were randomly divided into 4 groups: young or old and hypertensive or normotensive. The aim of the present study was to investigate the protective role of the receptor ERα in female mice in order to decipher the mechanism of this protection in premenopausal women. Consequently, male mice were not used in the present work.

Blood Pressure Measurement

As previously described,[18] systolic blood pressure was measured in conscious mice using a noninvasive and fully automated and computerized tail-cuff method (photoplethysmograph BP-2000 Blood Pressure Analysis System; Visitech Systems, Apex, NC). Mice were randomly assigned to the control (sham) or hypertensive group. They were then subjected to a sham operation or were implanted subcutaneously with osmotic minipumps (model 2004, 0.25 μL/h, 28 days; Alzet, Cupertino, CA) releasing angiotensin II (0.5 mg/kg per day; Bachem, Bubendorf, Switzerland; no. 4006473, solubilized in NaCl 0.9%) for 4 weeks to induce hypertension.[19] Systolic blood pressure was measured in the fourth week. Surgery was performed under isoflurane (2%) as anesthesia. Analgesia was obtained with buprenorphine (Temgesic; 0.1 mg/kg, subcutaneously) before and after surgery.

After 28 days the mice were euthanized using a CO_2 chamber, and the mesentery was quickly removed and placed in ice-cold physiological salt solution. Several segments of mesenteric resistance arteries were isolated for functional and biochemical studies.

The experiments performed in the present study complied with European Community standards for the care and use of laboratory animals and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol was approved by the regional ethics committee (*Comité d'éthique en Expérimentation Animale des Pays de la Loire*, authorization # CEEA PdL 2012.141). Exclusion criteria were specified in the protocol submitted to the ethical

committee (excessive weight loss and obvious health problems). No animal was excluded from the study after being assigned into a specific group.

Pharmacological Profile of the Isolated Aorta and Mesenteric Resistance Artery

Segments of the thoracic aorta and of the second-order mesenteric arteries were mounted in a wire myograph (Danish Myo Technology, Aarhus, Denmark) as previously described.[20] Briefly, 2 tungsten (for the mesenteric artery) or steel (for the aorta) wires were inserted into 2-mm-long arterial segments; 1 was fixed to a force transducer and 1 to a micrometer. The arterial segments were bathed in a physiological salt solution of the following composition: 130 mmol/L NaCl; 15 mmol/L $NaHCO_3$; 3.7 mmol/L KCl; 1.2 mmol/L KH_2PO_4; 1.2 mmol/L $MgSO_4$; 11 mmol/L glucose; 1.6 mmol/L $CaCl_2$; 5 mmol/L HEPES, pH 7.4; 160 mm Hg P_{O_2}; 37 mm Hg P_{CO_2}. Wall tension was applied as described previously.[21] Arterial segment viability was tested using a potassium-rich solution (KCl, 80 mmol/L). Endothelial function was then tested using acetylcholine (10^{-6} mol/L) after precontraction with phenylephrine (10^{-6} mol/L). After washout, a cumulative concentration-response curve to acetylcholine (10^{-9} to 10^{-5} mol/L) and then to sodium nitroprusside (10^{-9} to 10^{-5} mol/L) was performed.

Western Blotting Analysis

As previously described, proteins were extracted from the kidney[22] and from the aorta.[23] Electrophoresis was performed with 4% to 15% acrylamide Criterion gels (Bio-Rad, Hercules, CA). Protein transfer onto nitrocellulose membranes (Bio-Rad) was performed with the Trans-Blot Turbo Transfer System (Bio-Rad). Blots were blocked with Tris-buffered saline–Tween-BSA 5% and then incubated with the following primary antibodies: anti-eNOS (endothelial nitric oxide synthase; BD Biosciences, Franklin Lakes, NJ; #610297), anti–phosphorylated eNOS (pS1177, BD Biosciences, #612393), anti-CD45 (Abcam, Cambridge, UK; ab10558), anti-NRF1 (Cell Signaling Technology, Danvers, MA; 46743s), anti-UCP1 (Cell Signaling Technology; #14670), anti-OPA1 (BD Biosciences; #612606), anti-FIS-1 (Santa Cruz Biotechnology, Dallas, TX; sc98900), anti-p47 (BD Biosciences; #610355), anti-gp91 (BD Biosciences; #611414) anti-COX2 (BD Biosciences; #610204), and anti–β-actin (Sigma, St. Louis, MO; A5316). Following incubation with a goat antirabbit IgG (H+L) or a goat antimouse IgG (H+L) secondary antibody, and the horseradish peroxidase conjugate (Thermo Fisher Scientific, Waltham, MA), the reaction was developed by enhanced chemiluminescence (Bio-Rad) according to the manufacturer's instructions, and the signal was visualized by chemiluminescence.

Statistical Analyses

Data were expressed as mean±standard error of the mean (SEM). Two-factor ANOVA was used for the concentration-response curves followed by a Bonferroni post hoc test. For all other comparisons the Kruskal-Wallis (more than 2 groups) or Mann-Whitney tests (when comparing 2 groups) were used. The numbers of experiments performed imply that nonparametric tests should be used, and the tests used were chosen according to previous recommendation.[24] A value of $P<0.05$ was considered to denote statistical significance.

Results

Angiotensin II–Dependent Hypertension and Cardiac Hypertrophy

Angiotensin II infusion induced an increase in systolic blood pressure in all 5-month-old (Figure 1A) and 18-month-old (Figure 1B) female mice. However, the rise in blood pressure was more pronounced in ER$\alpha^{-/-}$ and AF2° mice compared with the corresponding WT mice (Figure 1A and 1B).

Left ventricle hypertrophy was only significant in young WT mice and not in old WT mice treated with angiotensin (Figure 1C and 1D). In contrast, both 5- and 18-month-old ER$\alpha^{-/-}$ and AF2° mice receiving angiotensin II developed significant left ventricle hypertrophy. Finally, chronic angiotensin II administration did not induce significant left ventricle hypertrophy in 5- and 18-month-old C451A mice.

Aorta Hypercontractility Associated With Hypertension

In addition to left ventricle hypertrophy, angiotensin II–mediated hypertension also induces aorta hypertrophy[13] and, consequently, aorta hypercontractility. Accordingly, we observed greater contractility in response to phenylephrine in angiotensin II–treated mice than in untreated animals (Figure 1E and 1F). In line with the moderate increase in blood pressure found in WT and C451A mice, the increased phenylephrine-mediated contraction observed in the aorta (Figure 1E and 1F) was moderate and not statistically significant in angiotensin II–infused WT and C451A mice compared with control untreated mice. This was observed in both 5- and 18-month-old mice.

In contrast, phenylephrine-mediated contraction (Figure 1E and 1F) was significantly increased in angiotensin II–infused ER$\alpha^{-/-}$ and AF2° mice compared with the corresponding WT mice. This hypercontractility was observed in both 5- and 18-month-old ER$\alpha^{-/-}$ and AF2° mice.

These data indicate that ER$\alpha^{-/-}$ and AF2° mice are more susceptible to the consequences of hypertension in response to angiotensin II than WT and C451A mice.

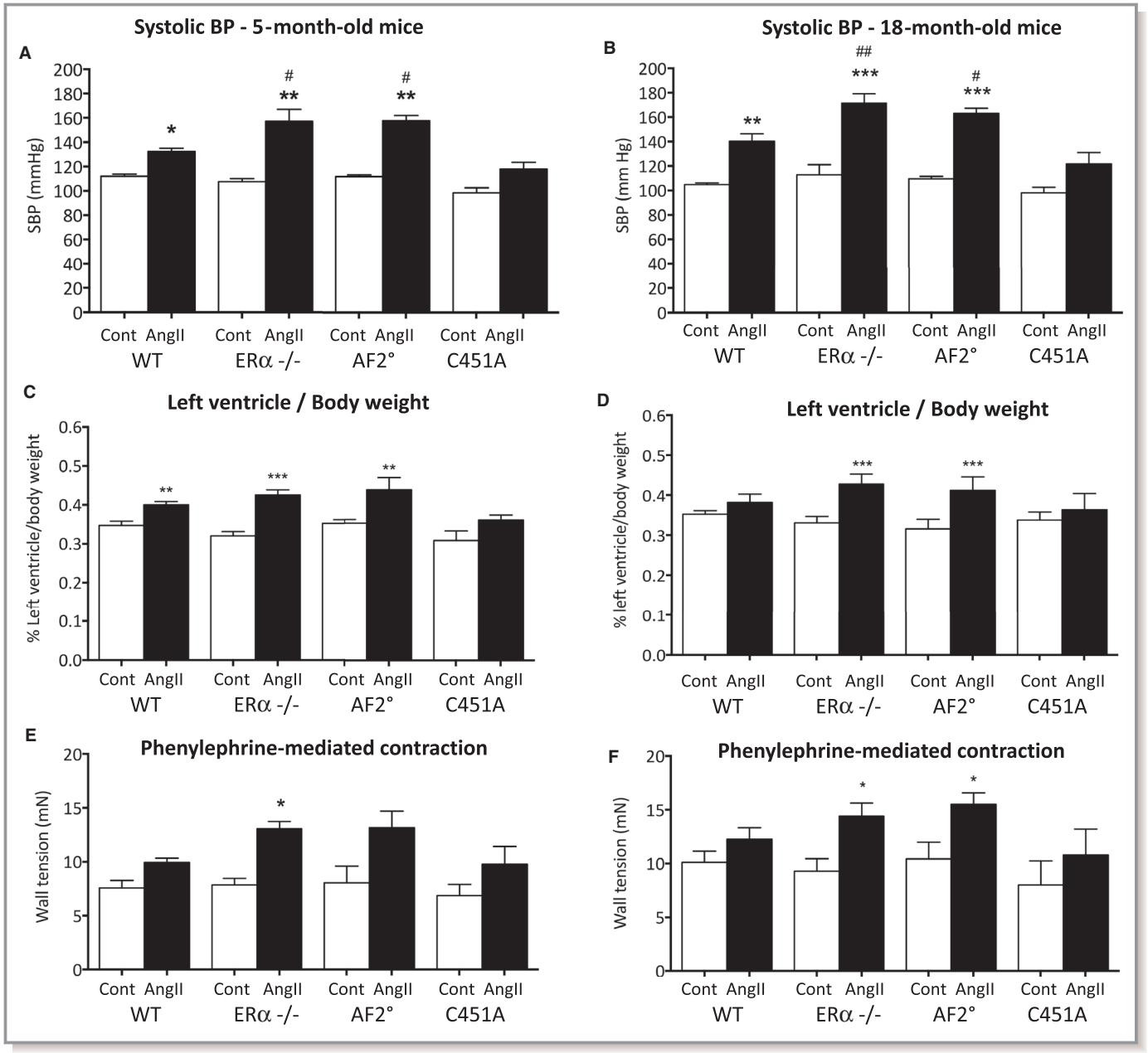

Figure 1. Systolic blood pressure, left ventricle weight, and aortic contractility. Systolic blood pressure (**A** and **B**), left ventricle/body weight ratio (**C** and **D**), and phenylephrine (1 μmol/L)-mediated contraction (**E** and **F**) were measured in 5- and 18-month-old wild-type (WT), ERα$^{-/-}$, AF2°, and C451A mice treated with (AngII) or without (Cont) angiotensin II. Values are presented as mean±SEM (n=8 mice per group). **P*<0.05; ***P*<0.01; ****P*<0.001: AngII vs Cont (Kruskal-Wallis test). $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01: ERα$^{-/-}$, AF2°, or C451A vs WT (Kruskal-Wallis test). BP indicates blood pressure; SBP, systolic BP.

Endothelium-Dependent Relaxation in the Mouse Aorta

Acetylcholine induced a concentration-dependent relaxation in the mouse aorta (Figure 2A through 2D).

In 5-month-old ERα$^{-/-}$ and AF2° mice, acetylcholine-dependent relaxation was significantly lower in hypertensive than in normotensive animals. Nevertheless, this difference was not significant in WT and C451A mice (Figure 2B versus 2A, statistics in the inset next to Figure 2B). In 18-month-old AF2° mice, acetylcholine-dependent relaxation was significantly lower in hypertensive than in normotensive animals, although this difference was not significant in WT, ERα$^{-/-}$, and C451A mice (Figure 2D versus 2C, statistics in the inset next to Figure 2D).

In normotensive 5-month-old animals, acetylcholine-dependent relaxation was lower in C451A, ERα$^{-/-}$, and AF2° mice than in WT mice, although this was significant only in AF2°

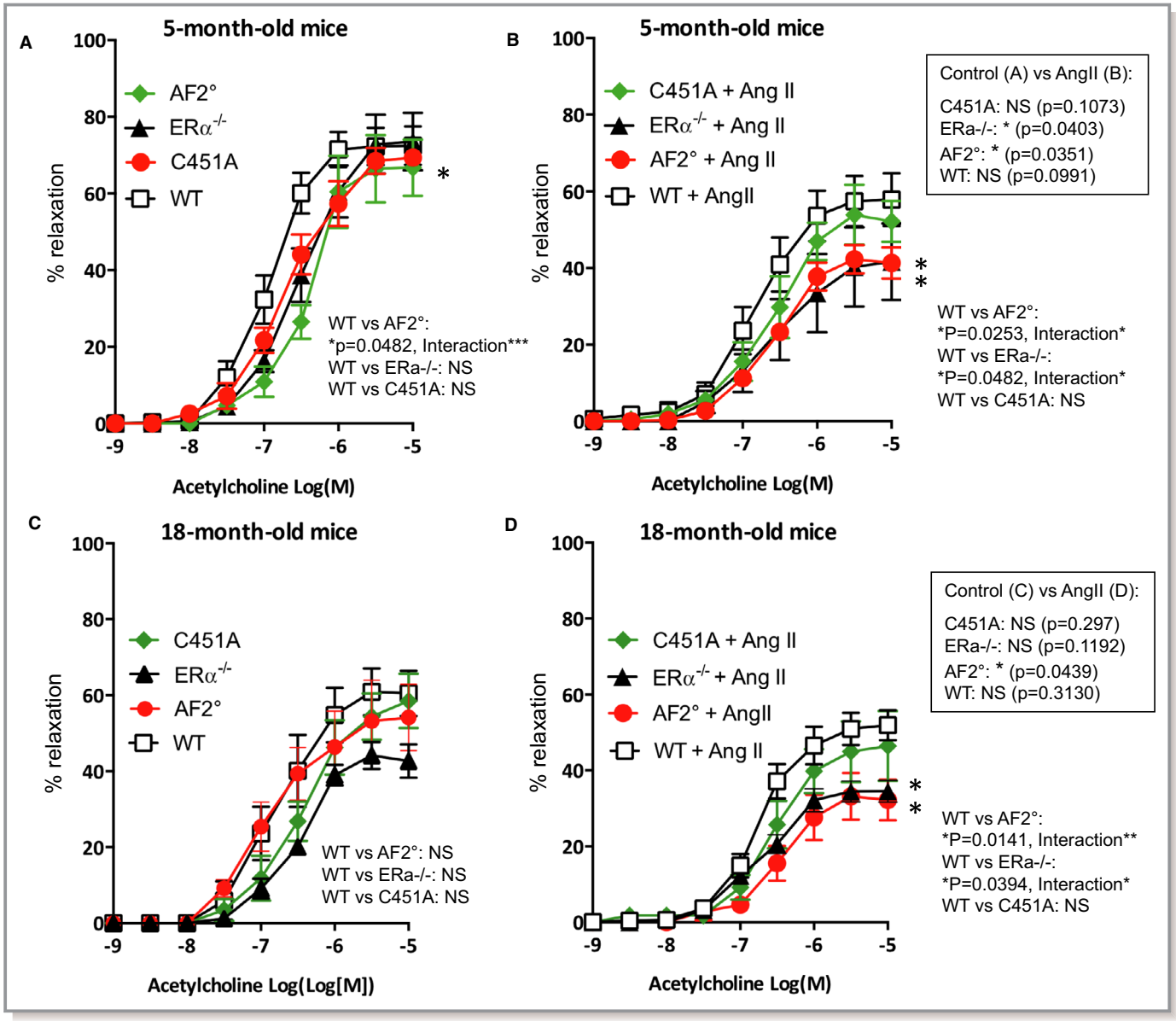

Figure 2. Endothelium-dependent relaxation in the aorta. Acetylcholine-mediated relaxation was measured in the thoracic aorta isolated from 5-month-old (**A** and **B**) and 18-month-old (**C** and **D**) WT, ERα$^{-/-}$, AF2°, and C451A mice treated with (**A** and **C**) or without (Ang II; **B** and **D**) angiotensin II. Values are presented as mean±SEM (n=8 mice per group). *P<0.05: ERα$^{-/-}$, AF2° or C451A vs WT and effect of angiotensin II infusion (2-way ANOVA for repeated measurements and Bonferroni test). The insets show the comparison between angiotensin II–treated groups and the corresponding untreated group (**B** vs **A** or **D** vs **C**).

mice (Figure 2A). In hypertensive 5-month-old mice, acetylcholine-dependent relaxation was significantly lower in ERα$^{-/-}$ and AF2° mice than in WT mice. This effect was not significant in C451A mice (Figure 2B).

In normotensive 18-month-old mice, no significant difference was observed between C451A, ERα$^{-/-}$, or AF2° mice and WT mice (Figure 2C).

In hypertensive 18-month-old mice, acetylcholine-dependent relaxation was significantly lower in ERα$^{-/-}$ and AF2° mice compared with WT animals, whereas there was no significant difference between C451A and WT mice (Figure 2D).

Endothelium-independent relaxation induced by sodium nitroprusside in the aorta was not significantly affected by chronic angiotensin II, aging, or the different genotypes (Figure 3).

The protein expression levels of eNOS (Figure 4A and Figure S1) and the ratio of the expression levels of phosphorylated eNOS versus those of total eNOS (Figure 4B and Figure S1), which represents eNOS activation, were not significantly affected by aging, hypertension, and ERα inactivation in the 3 groups (ERα$^{-/-}$, AF2°, and C451A).

In contrast, gp91 expression levels (Figure 4C and Figure S1) were significantly higher in aortas isolated from old

hypertensive ERα$^{-/-}$ and AF2° mice than in the corresponding WT mice.

Thus, in addition to aorta hypercontractility, the absence of AF2-ERα induced a reduction in endothelium-mediated relaxation in young and old hypertensive mice. This effect was less pronounced in normotensive mice. These results suggest a protective effect of AF2 against aging-associated hypertension.

Endothelium-Dependent Relaxation in Mesenteric Resistance Arteries

Because hypertension is also associated with endothelial disorders in resistance arteries, endothelium (acetylcholine)-dependent relaxation was measured in isolated mesenteric resistance arteries. Acetylcholine induced a concentration-dependent relaxation of the female mouse mesenteric artery (Figure 5A through 5D).

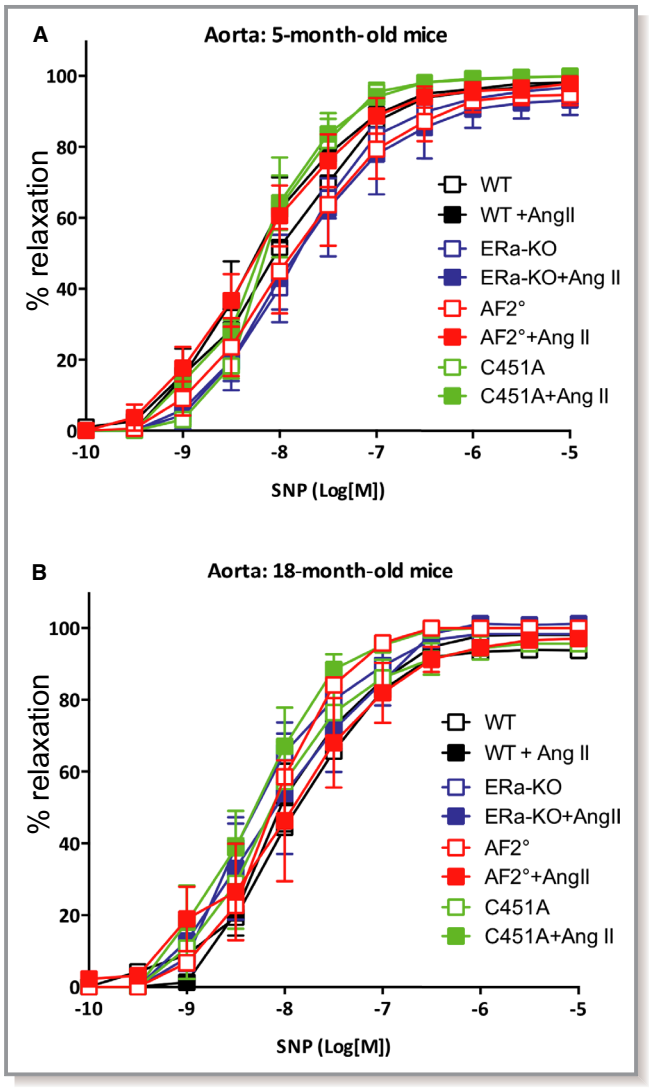

Figure 3. Endothelium-independent relaxation in the aorta. Sodium nitroprusside (SNP)-mediated relaxation was measured in segments of the aorta isolated from 5-month-old (**A**) and 18-month-old (**B**) ERα$^{-/-}$ (ERα KO), AF2°, and C451A mice treated with or without angiotensin II. Values are presented as mean±SEM (n=8 mice per group). NS (non-significant): ERα$^{-/-}$, AF2° or C451A vs WT. NS: effect of AngII. NS: effect of aging. AngII indicates angiotensin II; KO, knockout; NS, nonsignificant; WT, wild type.

In normotensive 5-month-old animals, acetylcholine-dependent relaxation was not significantly lower in C451A, AF2°, or ERα$^{-/-}$ than in WT animals (Figure 5A).

In 5-month-old angiotensin II–treated mice, acetylcholine-mediated relaxation was significantly lower in ERα$^{-/-}$ and AF2° mice compared with WT mice. There was no significant difference between C451A and WT mice (Figure 5B).

Although acetylcholine-mediated relaxation tended to be lower in hypertensive 5-month-old mice than in normotensive 5-month-old animals, this did not reach significance (right inset of Figure 5B).

In 18-month-old normotensive animals, acetylcholine-mediated relaxation was significantly lower in ERα$^{-/-}$ and AF2° than in WT mice (Figure 5C). No significant difference was found between C451A and WT mice (Figure 5C).

Similarly, in 18-month-old mice treated with angiotensin II, acetylcholine-dependent relaxation was significantly lower in ERα$^{-/-}$ and AF2° mice than in WT mice, whereas there was no significant difference between C451A and WT mice (Figure 5D).

In 18-month-old ERα$^{-/-}$ and AF2° mice, acetylcholine-dependent relaxation was significantly lower in hypertensive than in normotensive animals. This was not significant in WT and C451A mice (Figure 5D versus 5C, statistics in the inset next to Figure 5D).

Endothelium-independent relaxation in the mesenteric artery induced by sodium nitroprusside was not significantly affected by chronic administration of angiotensin II, aging, or the different genotypes (Figure 6A and 6B).

Thus, in both young and old mice, endothelium-dependent relaxation was reduced by the absence of AF2-ERα, suggesting a protective role of the nuclear function of ERα in resistance arteries.

Kidney Protein Expression

Because the kidney is a major target of hypertension, we measured markers of inflammation and oxidative stress in samples of kidney homogenates from ERα$^{-/-}$, AF2°, C451A, and WT female mice (Figure 7).

The protein expression levels of eNOS (Figure 7A and Figure S2) were not significantly different between KO (ERα$^{-/-}$, AF2°, and C451A) and WT mice within each group of age or treatment. Similarly, the phosphorylated eNOS/total eNOS ratio, which represents eNOS activation, was not affected by aging, hypertension, or ERα activation in the 3 groups (Figure 7B and Figure S2).

The COX2 expression levels were significantly increased in 18-months-old angiotensin II-treated ERα$^{-/-}$ mice than in

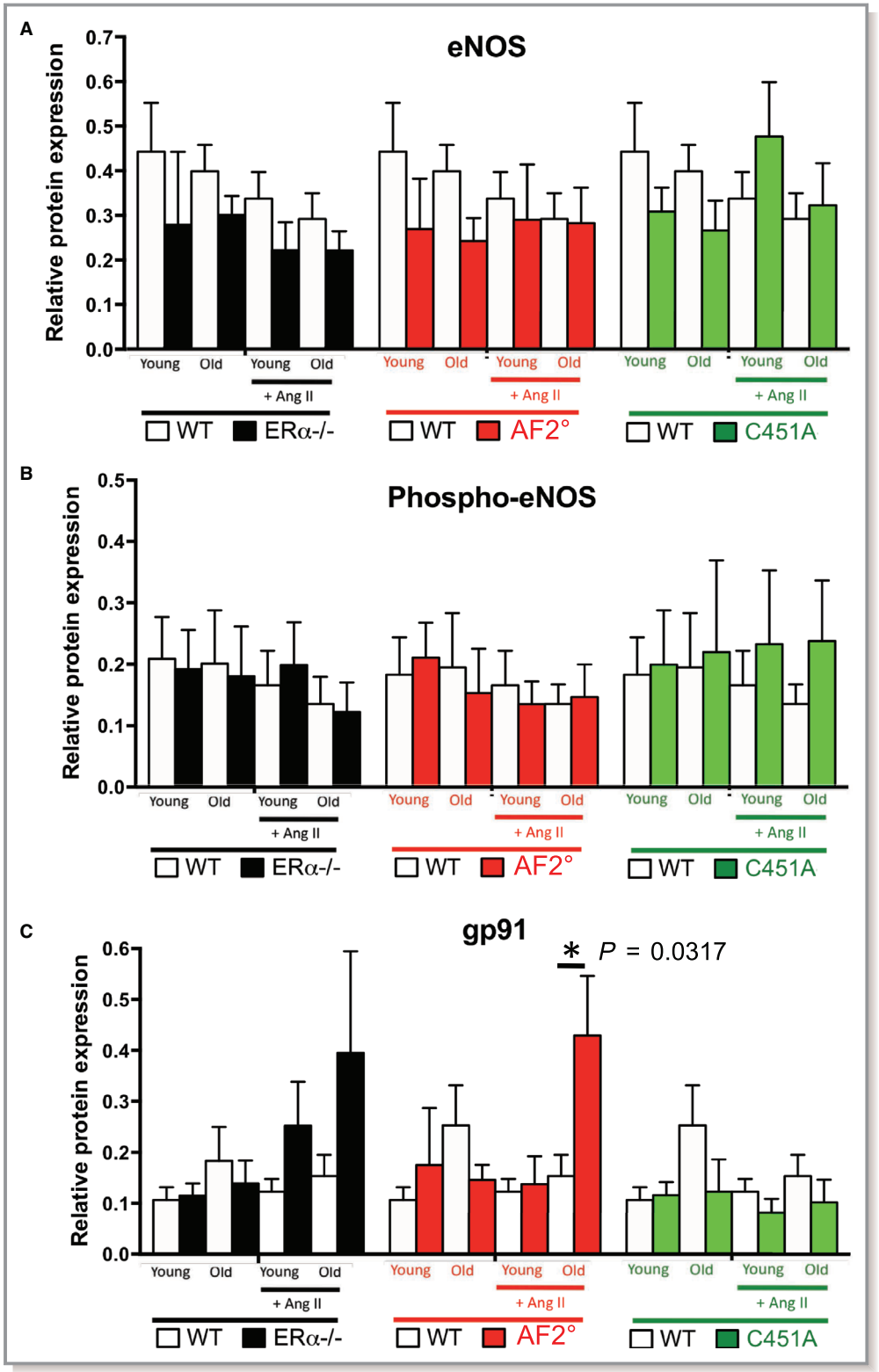

Figure 4. NO synthesis and gp91 expression levels in the aorta. Protein expression levels of eNOS (**A**), phosphorylated eNOS/total eNOS ratio (**B**), and gp91 expression levels (**C**) were measured in aortas isolated from 5-month-old (young) and 18-month-old (old) ERα$^{-/-}$, AF2°, and C451A mice, and their corresponding wild-type (WT) littermates (pooled WT mice), treated with or without angiotensin II (Ang II). Values are presented as mean±SEM (n=8 mice per group). For each age and treatment group, the WT groups (white bars) are the same for all 3 knockout (KO) mouse groups (ERα$^{-/-}$, AF2°, C451A). Western blots are shown in Figure S1. *P<0.05: ERα$^{-/-}$ and WT vs AF2°; Mann-Whitney test.

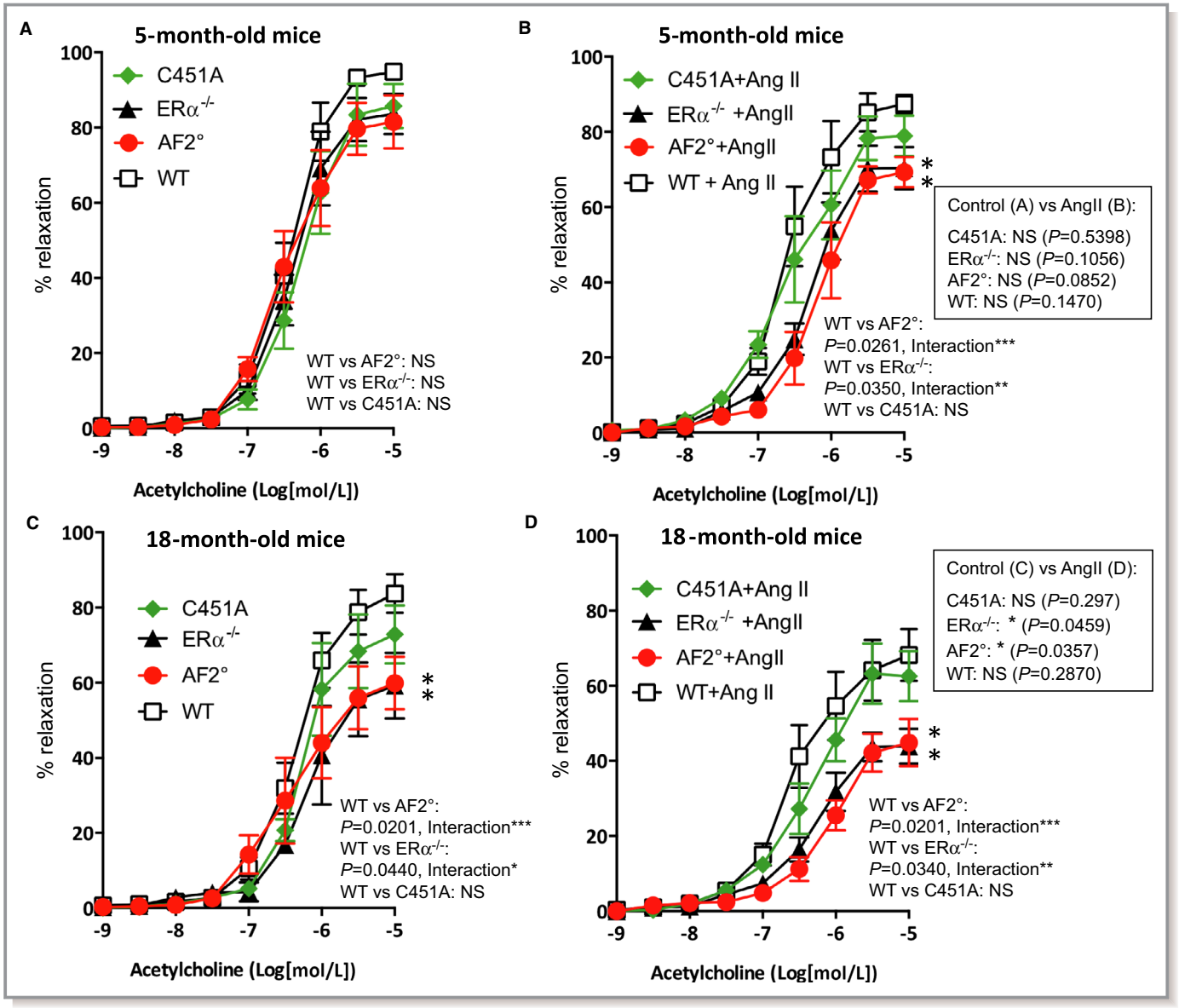

Figure 5. Endothelium-dependent relaxation in the mesenteric artery. Acetylcholine-mediated relaxation was measured in segments of mesenteric arteries isolated from 5-months-old (**A** and **B**) and 18-months-old (**C** and **D**) WT, ERα$^{-/-}$, AF2°, and C451A mice treated with (**B** and **D**) or without (**A** and **C**) angiotensin II (Ang II). Values are presented as mean±SEM (n=8 mice per group). *P<0.05: ERα$^{-/-}$, AF2°, or C451A vs WT and effect of angiotensin II infusion (2-way ANOVA for repeated measurements and Bonferroni's test). The inserts show the comparison between angiotensin II-treated groups and the corresponding untreated group (**B** vs **A** or **D** vs **C**). WT indicates wild type.

the corresponding angiotensin II-treated WT littermates (Figure 8A and Figure S3). No other significant effect of age, genotype, or angiotensin II administration was observed.

CD45 expression, a marker of leukocyte infiltration, followed the same pattern of expression as COX2. CD45 was significantly increased in 18-months-old ERα$^{-/-}$ and AF2° mice treated with angiotensin II compared with the corresponding WT mice (Figure 8B and Figure S3). This was not observed in C451A mice; p47 (Figure 8C and Figure S3) and gp91 (Figure 8D and Figure S3) expression levels followed a similar pattern.

The protein expression levels of the nuclear respiratory factor 1 (NRF1) were significantly higher in angiotensin II-treated 5- and 18-months-old ERα$^{-/-}$ and AF2° mice than the corresponding untreated mice (Figure 9A and Figure S3). No other effect of genotype, age, or treatment was observed. A similar pattern was found with the uncoupling protein-1 UCP1 (Figure 9B and Figure S3). In contrast, OPA1 and FIS-1 expression levels were similar in all groups (Figure 9C, 9D and Figure S3).

In summary, an inflammatory and oxidative profile was observed in old hypertensive animals mutated for the nuclear

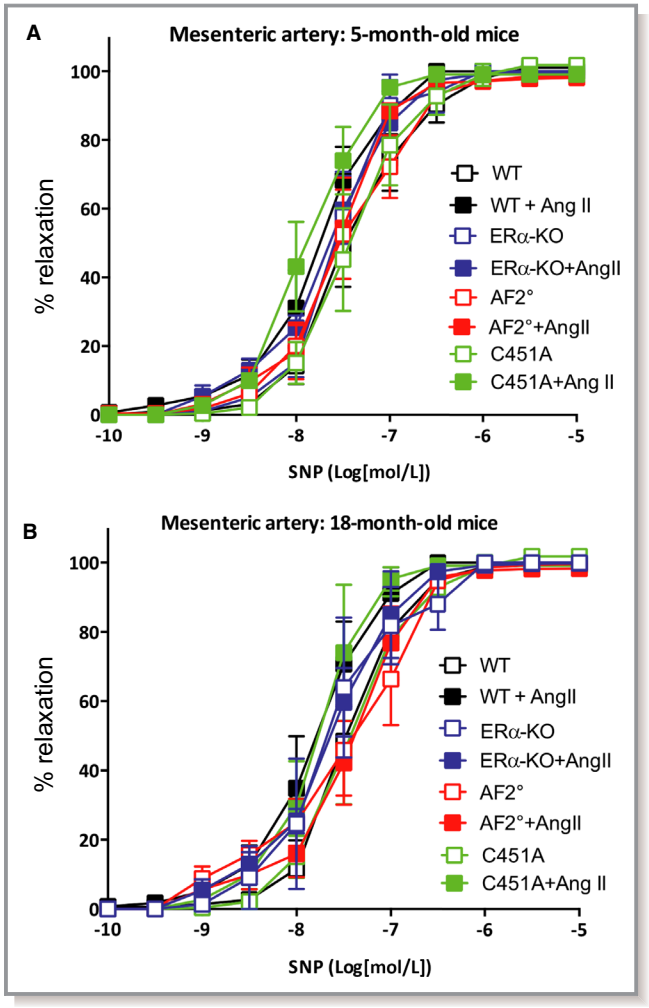

Figure 6. Endothelium-independent relaxation in the mesenteric artery. Sodium nitroprusside (SNP)-mediated relaxation was measured in segments of the mesenteric artery isolated from 5-month-old (**A**) and 18-month-old (**B**) ERα$^{-/-}$ (ERα KO), AF2°, and C451A mice treated with or without angiotensin II (Ang II). Values are presented as mean±SEM (n=8 mice per group). NS: ERα$^{-/-}$, AF2°, or C451A vs WT. NS; effect of AngII and effect of aging. KO indicates knockout; NS, nonsignificant; WT, wild type.

function of ERα and not for the membrane action of ERα. This suggests a protective role of nuclear (AF2) ERα in old hypertensive mice.

Blood Urea and Creatinine

In order to evaluate kidney function, blood urea and creatinine were measured in the different groups.

In 5-months-old mice, blood urea was not affected by the different ERα alterations or by the angiotensin II treatment (Figure 10A). However, in 18-months-old mice, blood urea was significantly elevated in angiotensin II-treated ERα$^{-/-}$ and AF2° mice compared with the corresponding untreated mice (Figure 10B).

Similarly, in 5-months-old mice, blood creatinine was not affected by the genotype or by angiotensin II (Figure 10C). Again, in 18-month-old mice, blood creatinine was significantly increased in ERα$^{-/-}$ and AF2° mice compared with WT animals (Figure 10D). Of note, no significant difference between angiotensin II-treated mice and the corresponding control group was observed.

Altogether, these data demonstrate a significant increase of urea and creatinine in old hypertensive mice mutated for ERα and, in particular, for the loss of function of nuclear ERα (AF2° mice).

Mice Body and Uterus Weight

Mice body weight was significantly greater in ERα$^{-/-}$, AF2°, and C451A than in the corresponding WT littermate mice, although this level reached significance in young normotensive ERα$^{-/-}$ and C451A animals only (Figure 11A and 11B).

Uterus weight was significantly reduced in ERα$^{-/-}$ and AF2° mice compared with the corresponding WT mice, whereas it was not different between C451A mice and their WT littermates. Furthermore, it was not significantly affected by age or by angiotensin II treatment (Figure 11C and 11D).

Discussion

This study first revealed that ERα largely prevents the deleterious consequences of aging and of angiotensin II-mediated hypertension, as well as their combination, in the female mouse. Indeed, angiotensin II-induced hypertension was more pronounced in female mice with a total absence of ERα (ERα$^{-/-}$) compared with age-matched littermate WT female mice. Consistently, such increased hypertension was accompanied by greater left ventricle hypertrophy and aortic contractility in addition to an altered endothelium-mediated relaxation of both large (aorta) and small (mesenteric resistance artery) blood vessels. ERα deficiency also exacerbated the renal abnormalities elicited by aging- and angiotensin II-induced hypertension, including an inflammatory and oxidative phenotype and a decreased kidney function. Second, we provide novel information about the role of membrane and nuclear ERα in aged mice exposed or not exposed to hypertension, shedding light on the complex relationships between estrogens and cardiovascular risk factors, which may have potential medical implications.

Angiotensin II-Induced Hypertension Associated With Hypertrophy and Hypercontractility

AF2° mice showed a similar increase in systolic blood pressure in response to a 1-month-long treatment with

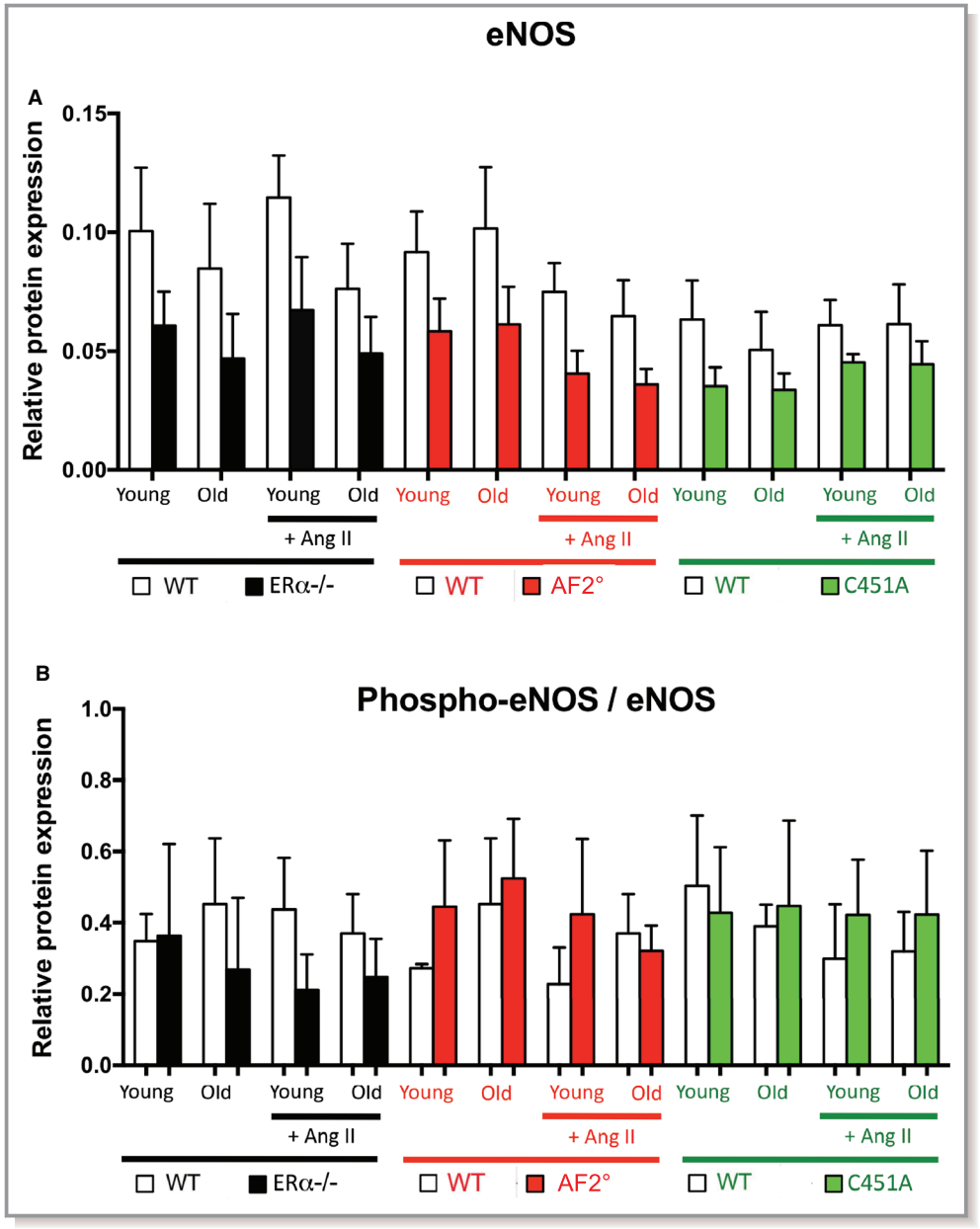

Figure 7. Protein expression levels of total and phosphorylated eNOS in the kidney. Protein expression level of eNOS (**A**) and the phosphorylated eNOS/total eNOS ratio (**B**) were measured in kidneys isolated from 5-month-old (young) and 18-month-old (old) ERα$^{-/-}$, AF2°, and C451A mice, and their corresponding WT littermates, treated with or without angiotensin II (Ang II). Values are presented as mean±SEM (n=8 mice per group). Western blots are shown in Figure S2. NS: ERα$^{-/-}$, young vs old, AngII vs control and WT vs KO (ERα$^{-/-}$, AF2°, or C451A); ordinary 2-way ANOVA and Bonferroni multiple comparisons test. NS: ERα$^{-/-}$, WT vs KO; Mann-Whitney test. KO indicates knockout; NS, nonsignificant; WT, wild type.

angiotensin II and a similar remodeling as ERα$^{-/-}$ mice. By contrast, C451A mice (deficient in membrane ERα signaling) exhibited a blood pressure similar to that of WT mice. Thus, the present work extends our previous conclusions from young mice[13] to aged mice. The absence of the nuclear AF2 transactivating function, but not the absence of the membrane-associated function (membrane-initiated steroid signaling), was also associated with a greater left ventricle mass and vascular contractility and with a larger reduction in endothelium-mediated relaxation in the aorta and small resistance arteries both in young and old mice. This reduction in endothelium-mediated relaxation was more pronounced in resistance arteries isolated from old mice. Beside their major role in reproduction, endogenous estrogens have a protective role against cardiovascular diseases.[2,25,26] Nevertheless, in the absence of hormonal treatment, this protection is

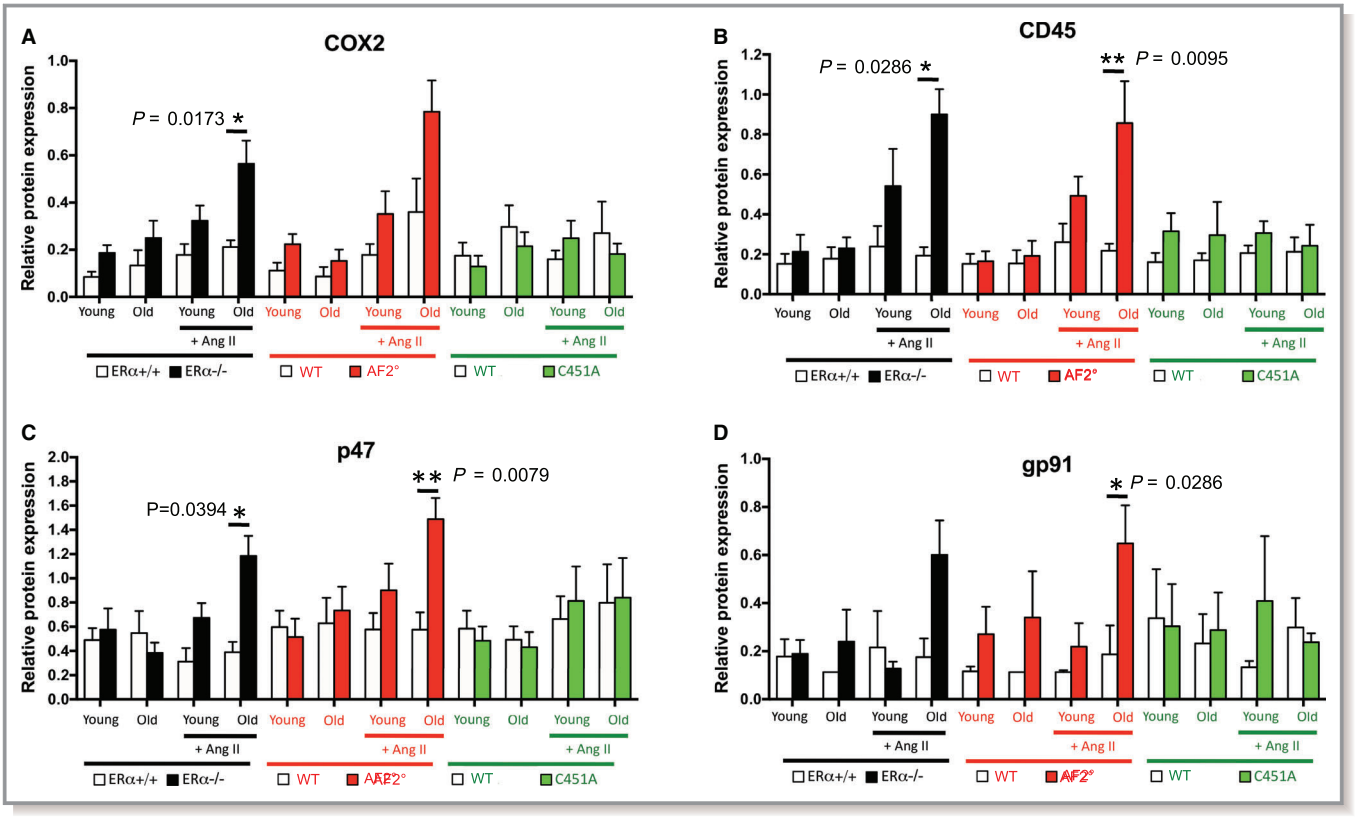

Figure 8. Kidney protein expression levels of COX2 and CD45. Protein expression levels of COX2 (**A**), CD45 (**B**), p47 (**C**), and gp91 (**D**) were measured in kidneys isolated from 5-month-old (young) and 18-month-old (old) ERα$^{-/-}$, AF2°, and C451A mice, and their corresponding WT littermates, treated with or without angiotensin II (Ang II). Values are presented as mean±SEM (n=8 mice per group). Western blots are shown in Figure S3. NS: ERα$^{-/-}$, young vs old, AngII vs control, and WT vs KO (ERα$^{-/-}$, AF2°, or C451A); ordinary 2-way ANOVA and Bonferroni multiple comparisons test. *P<0.05; **P<0.01: ERα$^{-/-}$, WT vs KO; Mann-Whitney test. KO indicates knockout; NS, nonsignificant; WT, wild type.

progressively lost after menopause, and risk factors, such as hypertension, that become more frequent with age could contribute to this decline.[2,27] ERα and its nuclear action appear thus to provide a global protection against the effect of aging and hypertension, not only in arteries but also in the kidney.

Heart and aorta remodeling is a consequence of hypertension.[28,29] We observed that the extent of cardiac hypertrophy correlated to the levels of blood pressure both in young and old female mice, in agreement with previous works, which however, were mainly performed in male animals.[28] Nevertheless, the left ventricle hypertrophy found in young WT female mice was not significant in old female mice, and in both young and old WT mice it was lower than in ERα$^{-/-}$ and AF2° mice. However, in spontaneously hypertensive rats, hypertension increases similarly in male and female mice with age, whereas hypertrophy remains lower in the females.[29] This is in agreement with the present work showing that female mice are protected against the effect of hypertension by the nuclear (AF2) action of ERα. Similarly, the hypercontractility observed in the aorta in association with hypertension was correlated to the level of blood pressure irrespective of age. These observations are consistent with previous works showing that estrogens reduce blood pressure and the effect of angiotensin II in young female mice through ERα activation.[30,31]

The measurement of blood pressure in mice using plethysmography (tail-cuff) can be considered as a limitation, as this technique is less accurate than telemetry. Nevertheless, the number of mice included in the present study should be taken into account (16 groups), as well as the duration of the experiments, because both 5- and 18-months-old mice were used. Although the tail-cuff method is less accurate for baseline or central blood pressure measurements, there is also some concern about the impact of the surgery necessary for the implantation of the probe and catheter used for telemetry[32] in addition to the surgery required for the implantation of the minipump delivering angiotensin II. Indeed, as female mice are usually smaller than male mice, the addition of the probe to the minipump represents a weight and a volume that are difficult to manage in the mouse mesentery. Nevertheless, the rise in pressure induced by angiotensin II has been shown to be the same whether measured by plethysmography or telemetry.[32] Finally, the

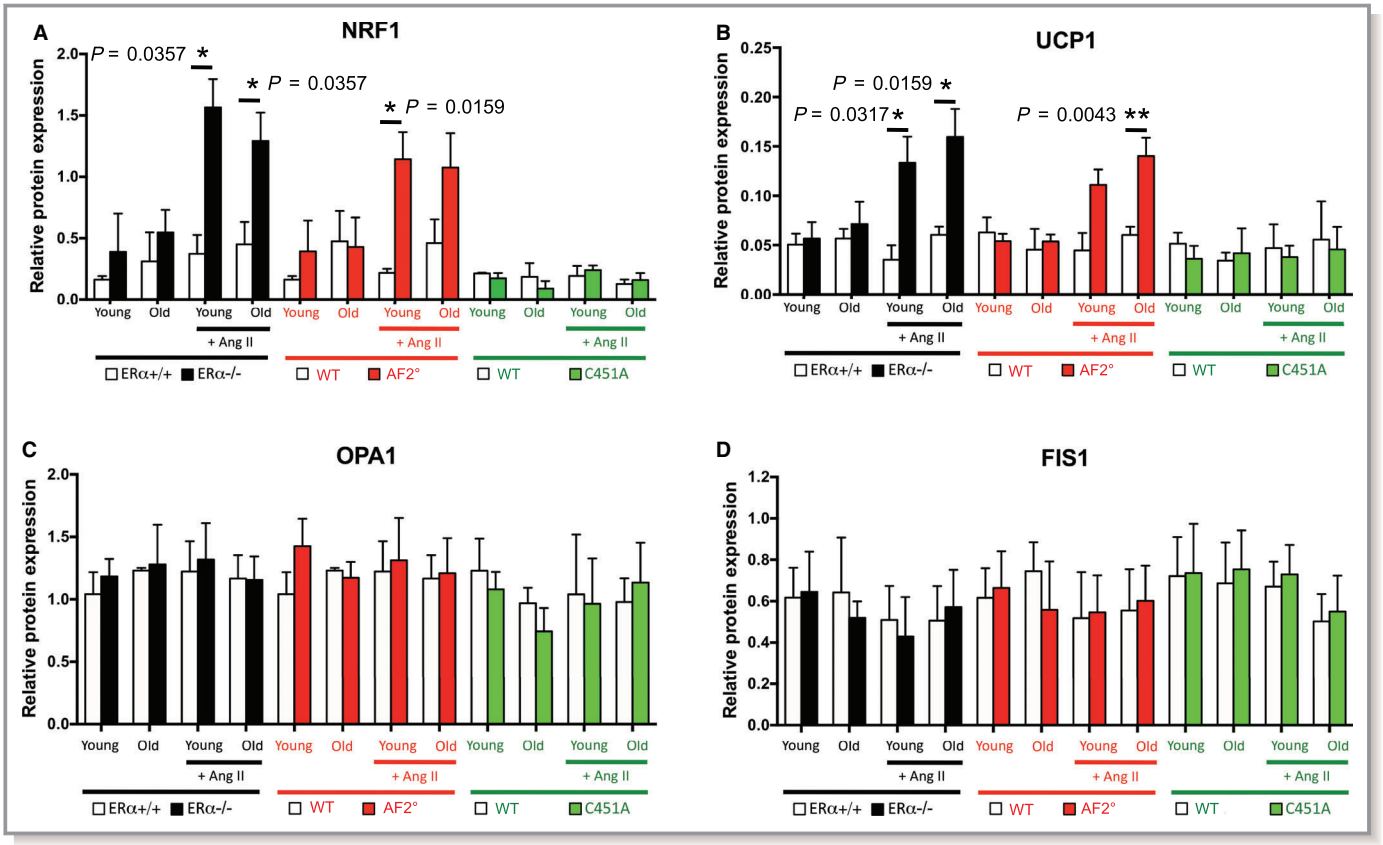

Figure 9. Kidney protein expression levels of NRF1, UCP1, OPA1, and FIS1. Protein expression levels of NRF1 (**A**), UCP1 (**B**), OPA1 (**C**), and FIS1 (**D**) were measured in kidneys isolated from 5-month-old (young) and 18-month-old (old) ERα$^{-/-}$, AF2°, and C451A mice, and their corresponding WT littermates, treated with or without angiotensin II (Ang II). Values are presented as mean±SEM (n=8 mice per group). Western blots are shown in Figure S3. NS: ERα$^{-/-}$, young vs old, AngII vs control and WT vs KO (ERα$^{-/-}$, AF2°, or C451A); ordinary 2-way ANOVA and Bonferroni multiple comparisons test. *P<0.05; **P<0.01: ERα$^{-/-}$, WT vs KO; Mann-Whitney test. KO indicates knockout; NS, nonsignificant; WT, wild type.

level of hypertension was also confirmed in the present work by the assessment of left ventricle hypertrophy and aorta contractility. Indeed, both were well correlated with the level of hypertension.

Membrane-Initiated Steroid Signaling Is Not Involved in ERα Protection Against Hypertension

Although the membrane ERα function is required for the accelerating effect of estrogens on postinjury reendothelialization, endothelial cell migration, and endothelium-dependent vasodilation,[10] it does not mediate the protective effects of estrogens against hypertension, as shown by our previous work.[13] In contrast, we showed the indispensable role of the nuclear signaling of ERα through its AF2 transactivating function for the protection against the effects of hypertension.[13,14] AF2 activation is also necessary for the protective effect of estrogens during flow-mediated remodeling,[13] a process required for postischemic revascularization that is fully dependent on estrogens and ERα.[5,33] Thus, AF2-ERα exerts a protective role through the reduction of the effects of hypertension (present study) and the acceleration of postischemic revascularization.[5,33]

Endothelium-Mediated Relaxation is More Sensitive to Aging

Although vascular hypercontractility was not significantly higher in old compared with young mice, endothelium-mediated relaxation was reduced more in resistance arteries from old hypertensive mice lacking AF2 or ERα than in old WT and C451A mice. This is of importance as resistance arteries are involved in the initiation and progression of hypertension.[34,35] As no major impact on the endothelium-independent response to the NO donor SNP was observed, it is likely that the protective effect of the nuclear AF2 function of ERα is mainly due to the maintenance of endothelium-dependent relaxation in old hypertensive female mice. Endothelium dysfunction is the hallmark of vascular disorders.[36,37] The vascular endothelium is progressively altered with age, and

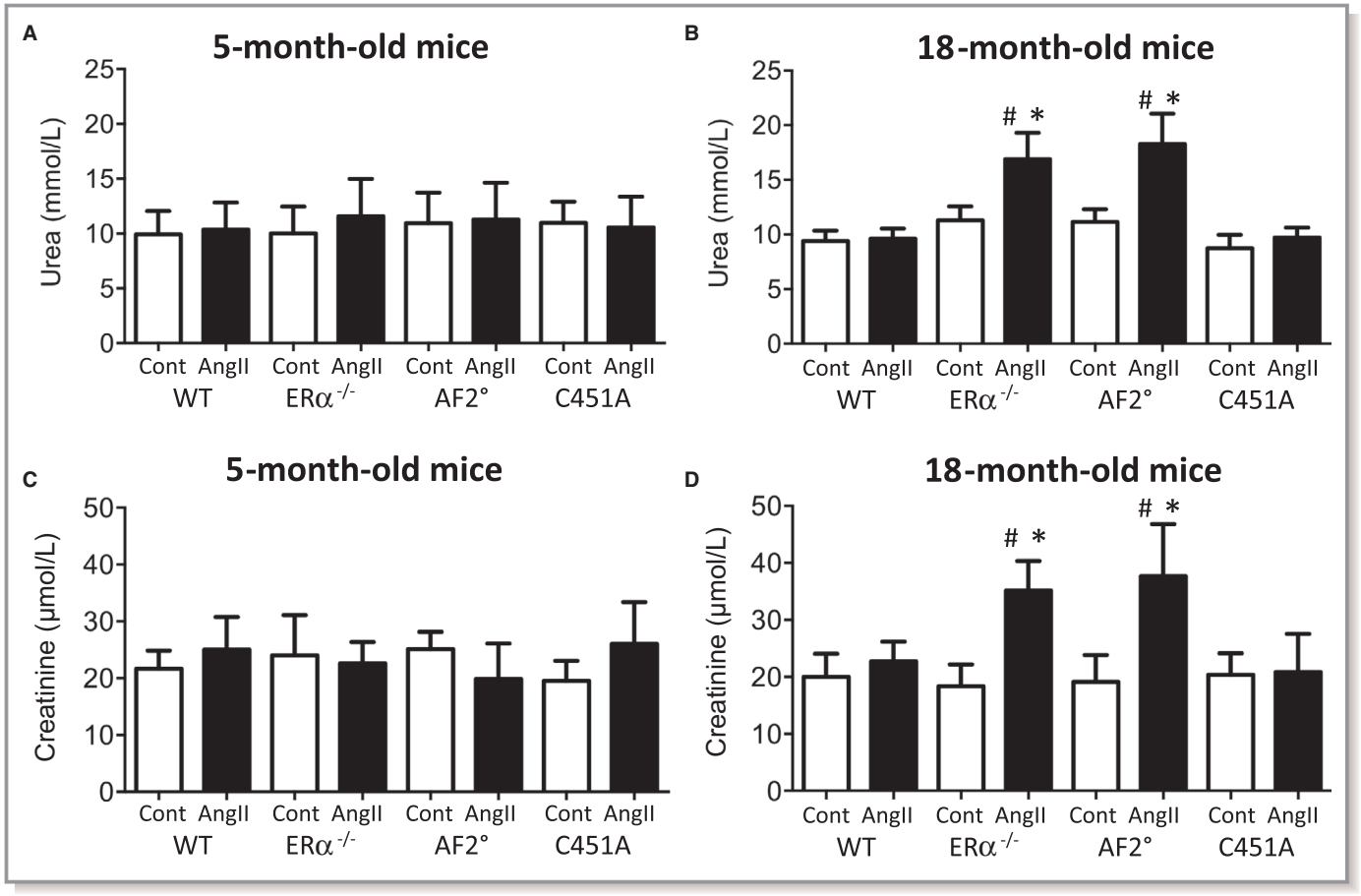

Figure 10. Blood urea and creatinine. Blood urea (**A** and **B**) and creatinine (**C** and **D**) were measured in 5-month-old (**A** and **C**) and 18-month-old (**B** and **D**) ERα$^{-/-}$, AF2°, and C451A mice, and their corresponding WT littermates, treated without (Cont) or with angiotensin II (AngII). Values are presented as mean±SEM (n=8 mice per group). *P<0.05: AngII vs Cont (Kruskal-Wallis test). #P<0.05: ERα$^{-/-}$, AF2°, or C451A vs WT (Kruskal-Wallis test). Cont indicates control; WT, wild type.

endothelial dysfunction is accelerated by most of the other known risk factors, such as hypertension.[38] Similarly, outward vascular remodeling, which is governed by the endothelium and its capacity to produce NO,[39,40] is altered very early during aging in male rats[41,42] but much later in female rats.[43] Interestingly, this remodeling depends also on AF2-ERα.[13]

Endothelium-dependent relaxation was reduced in both the aorta and mesenteric resistance arteries from old hypertensive mice lacking AF2 or ERα. This was not associated with a significant decrease in eNOS expression levels or in eNOS activation (phosphorylated-eNOS/total eNOS ratio). Although an increased eNOS expression level has initially been proposed as a mechanism for the increase in endothelial function in response to high chronic estrogen exposure,[44] subsequent studies have shown that it was the consequence of an activation of eNOS without increase in eNOS expression level.[45,46] The effect of the absence of ERα on eNOS expression is less known. In agreement with the present study, a previous work has reported a moderate but not significant decrease in eNOS expression level in the aorta and coronary arteries from ERα$^{-/-}$.[47] This study has also shown that eNOS expression level was decreased in ovariectomized ERα$^{-/-}$ and WT mice and that this level was restored by a treatment with estradiol.

In contrast, a significant increase in gp91 expression levels in the aorta was observed in the same groups. These observations suggest that the reduction in NO-dependent relaxation found in old hypertensive AF2-ERα$^{-/-}$ mice could result to a great extent from an increased production of reactive oxygen species that would scavenge NO and, thus, decrease its bioavailability. This is in agreement with the results of a previous study showing that estrogens do not directly increase eNOS expression levels in endothelial cells but, instead, inhibit superoxide anion production and, thus, increase the release of bioactive NO.[48] This assumption is also in agreement with our observation in the kidney, as discussed below, showing that excessive oxidative stress also occurs in old hypertensive AF2-ERα$^{-/-}$ mice.

Endothelial dysfunction plays a central role in the development of end-organ damage, and especially in kidney disorders.[16,49] Thus, we investigated markers of oxidative

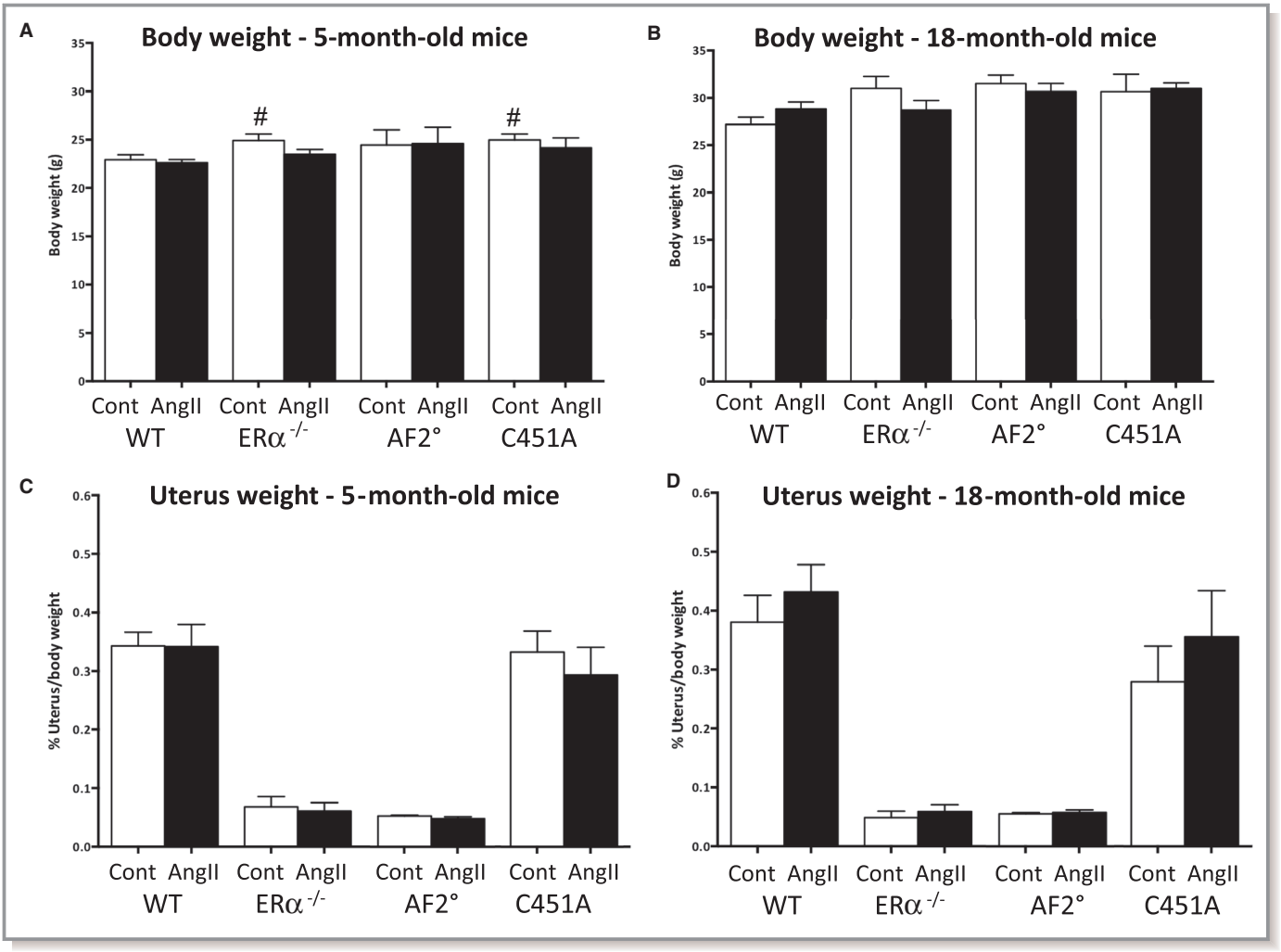

Figure 11. Mouse body and uterus weight. Mouse body (**A** and **B**) and uterus (**C** and **D**) weight were measured in 5-month-old (**A** and **C**) and 18-month-old (**B** and **D**) ERα$^{-/-}$, AF2°, C451A, and WT mice treated with or without (Cont) angiotensin II (AngII). Values are presented as mean±SEM (n=8 mice per group). NS: AngII vs Cont (Kruskal-Wallis test). $^{\#}P<0.05$: ERα$^{-/-}$, AF2°, or C451A vs WT (Kruskal-Wallis test). Cont indicates control; NS, nonsignificant; WT, wild type.

stress and inflammation in the kidney of young and old WT and knockout mice.

Pathophysiological Implications

In addition to the reduction in endothelium-mediated relaxation observed in old AF2° and ERα$^{-/-}$ mice, we observed higher levels of markers of inflammation (CD45 and COX2) and oxidative stress (the indicators of mitochondrial oxidative metabolism NRF1 and UCP-1 and the NADPH-oxidase subunits p47 and gp91) in kidneys isolated from old mice lacking AF2 or ERα than from old WT and C451A mice. UCP-1 is a proton channel expressed in mitochondria.[50] NRF1 activates mitochondrial biogenesis and functions.[51] Here, the increase in NRF1 and UCP-1 was observed in both young and old hypertensive mice completely lacking ERα or only the AF2 function. Thus, in the present study, this effect was independent of aging. Our finding is in agreement with that of a previous work showing mitochondrial oxidative dysregulation affecting NRF1 in particular in chronic kidney disease patients.[51] Although mitochondria biogenesis seems affected by the absence of AF2-ERα, mitochondria dynamics was probably not modified as OPA1 and FIS-1 expression were modified. OPA1 and FIS-1 are involved in mitochondria fusion and fission, respectively.[52] Hence, the protective effect of estrogens against angiotensin II–mediated hypertension, previously reported in young female mice,[31,53] appears to involve a reduction in renal oxidative metabolism[54] dependent on AF2-ERα signaling. This effect seems to be independent of age, and it is controlled by AF2-ERα.

Whereas the increases in NRF1 and UCP-1 were observed in both young and old hypertensive mice, increases in p47 and gp91 expression levels were found only in old hypertensive AF2° and ERα$^{-/-}$ mice. Thus, the kidney dysfunction found in

the current study requires both a rise in mitochondria oxidative metabolism and an increase in reactive oxygen species production by NADPH oxidase. This is in agreement with the results of a previous study showing that oxidative stress and p22 expression increase in the rat kidney with age and hypertension.[55] Furthermore, estradiol protection of the female kidney involves a reduction in renal superoxide production.[56]

CD45 and COX2 expression increased preferentially in old hypertensive female mice lacking ERα or only ERα-AF2. These data suggest higher leukocyte infiltration and kidney inflammation in old hypertensive female mice. Accordingly, we found elevated urea and creatinine levels in old female mice treated with angiotensin II that may result from kidney damage caused by inflammation when the protective effect of nuclear ERα is lost.

Interestingly, the role of AF2-ERα is highlighted in the context of low circulating levels of estrogens in old mice, rendering observations made in aged female mice comparable to those made in male mice. Indeed, although female mice do not undergo menopause, a senescent reproductive profile is associated with a progressive decrease in estradiol levels in aging mice.[57] Nevertheless, the persistence of significant estradiol levels in aged mice is proven in the present study by the maintenance of a normal uterus weight in 18-month-old female mice. The present study is in agreement with previous work investigating kidney damage in high-fat diet–induced hypertension in 6-month-old Dahl salt-sensitive rats, showing that only male rats exhibited hypertension-associated renal inflammation and injury.[58] Indeed, sex differences in cardiovascular and kidney diseases are now wellestablished,[59,60] reinforcing the importance of investigating the effect of hypertension on kidney damage in old female mice. Kidney protection in hypertensive female compared with male rats is due, at least in part, to lower blood pressure levels associated with lower T-cell infiltration.[61,62] Nonetheless, despite a similar rise in blood pressure, hypertension-associated kidney damage remains lower in female than in male mice.[63,64]

Although eNOS expression levels tended to be lower in the kidney of mice lacking ERα or only ERα-AF2 than in WT and C451A mice, no significant decrease was observed in old and hypertensive mice despite the endothelial dysfunction seen in the aorta and in mesenteric resistance arteries, which would have suggested an endothelial dysfunction extended to other vascular territories such as the kidney. Indeed, as in the aorta, kidney eNOS expression levels, as well as the phosphorylation of eNOS, were not affected by age or hypertension in female mice and were not reduced by the absence of total ERα or AF2-ERα. This is in agreement with results from previous studies showing that the circulating levels of estrogen are what determines eNOS expression levels, which are reduced in ovariectomized mice lacking ERα[47] or in the rat.[33] Nevertheless, the absence of ERα does not necessarily affect eNOS expression levels, although it reduces estradiol-mediated NO production.[10,65]

Thus, these finding suggest that the protective effect of ERα-AF2 involves a reduction in oxidative stress rather than an activation of the NO pathway. This is in agreement with the observations of Arnal et al showing that estradiol reduces oxidative stress leading to better NO bioavailability in endothelial cells.[48]

Conclusions

Selective activation of membrane ERα was initially seen as an attractive option to provide cardiovascular protection without inducing uterine or breast cancer.[12] Nevertheless, we have recently demonstrated an unexpected prominent role of nuclear ERα in the vasculoprotective effects of estrogens.[13] Thus, it seems that nuclear, but not membrane, ERα is a key factor of arterial protection, especially in aging, as suggested by the present study.

The major concern with estrogen treatment at menopause is the increase in breast cancer risk. Several groups have shown that membrane ERα participates in the epithelial-mesenchymal transition of cancer cells, suggesting that activation of membrane ERα could be deleterious.[66-68]

The present work delineates the benefits that could result from the selective activation of nuclear ERα because it appears that the resulting arterial protection is also efficient in aged animals. Altogether, we provide evidence that strategies based on nuclear ERα activation are conceptually attractive, as they exert cardiovascular protective effects not only in young but also in old and hypertensive female mice. Thus, this approach should now be considered to alleviate the burden of cardiovascular diseases in women after menopause.

It should be emphasized that tamoxifen is a very efficient specific estrogen-receptor modulator to prevent breast cancer recurrence, and it also protects bones and arteries.[69] Although it is known that its antagonistic action on AF2-ERα accounts for protective effects in breast cancer, its agonistic effect on AF1-ERα clearly accounts for the bone and artery protection, underlining another level of complexity in the nuclear actions of ERα. The lack of membrane ERα action on the endothelium[70] could contribute to protection. Recently, it was found that oral administration of the fetal estrogen estetrol did not increase the levels of circulating hepatic-derived coagulation factors, preventing venous thromboembolism.[71] We found that estetrol activates the nuclear, but not the membrane, ERα action.[65] Altogether, we can postulate that therapeutic targeting of nuclear ERα activation (1) is conceptually attractive and pharmacologically feasible, (2) is protective not only in young but also in old and hypertensive female mice, and (3) should now be considered more

seriously because cardiovascular diseases in women are becoming more frequent.

Finally, the present work also suggests the possibility of targeting ERα in men. This should be limited to the functions involving only the membrane-located ERα. Membrane-initiated signaling induced by the stimulation of ERα is limited to acute estrogen-mediated vasodilatation and to reendothelialization following injury.[10,13] However, because no such pharmacological tool with enough specificity and bioavailability exists yet, the therapeutic targeting of membrane ERα in men remains hypothetical.

In conclusion, the AF2-dependent genomic function of ERα is necessary for its protective effect in hypertension during aging, and its targeting could be a novel therapeutic tool to reduce hypertension and its cardiovascular consequences in aging women.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

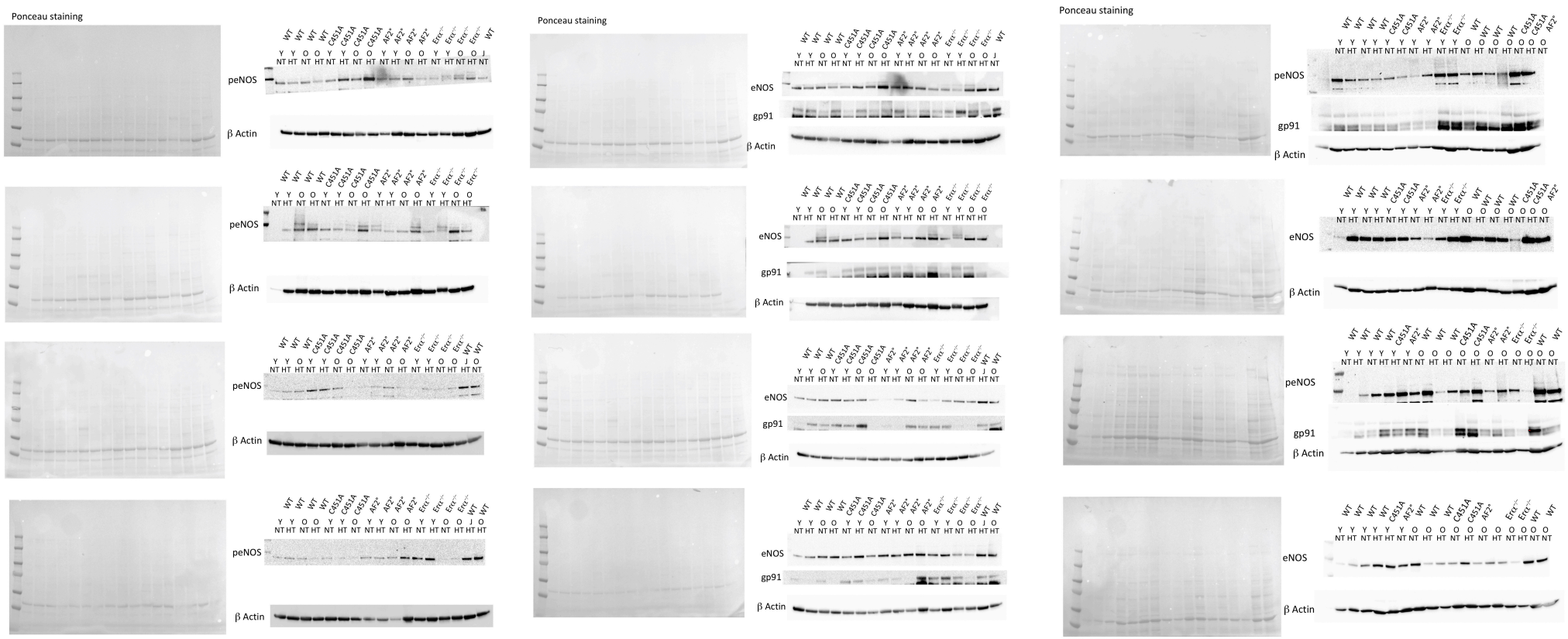

Figure S1. Full blots for gp91, eNOS and ph-eNOS measured in the thoracic aorta corresponding to the bargraphs shown in figure 4.

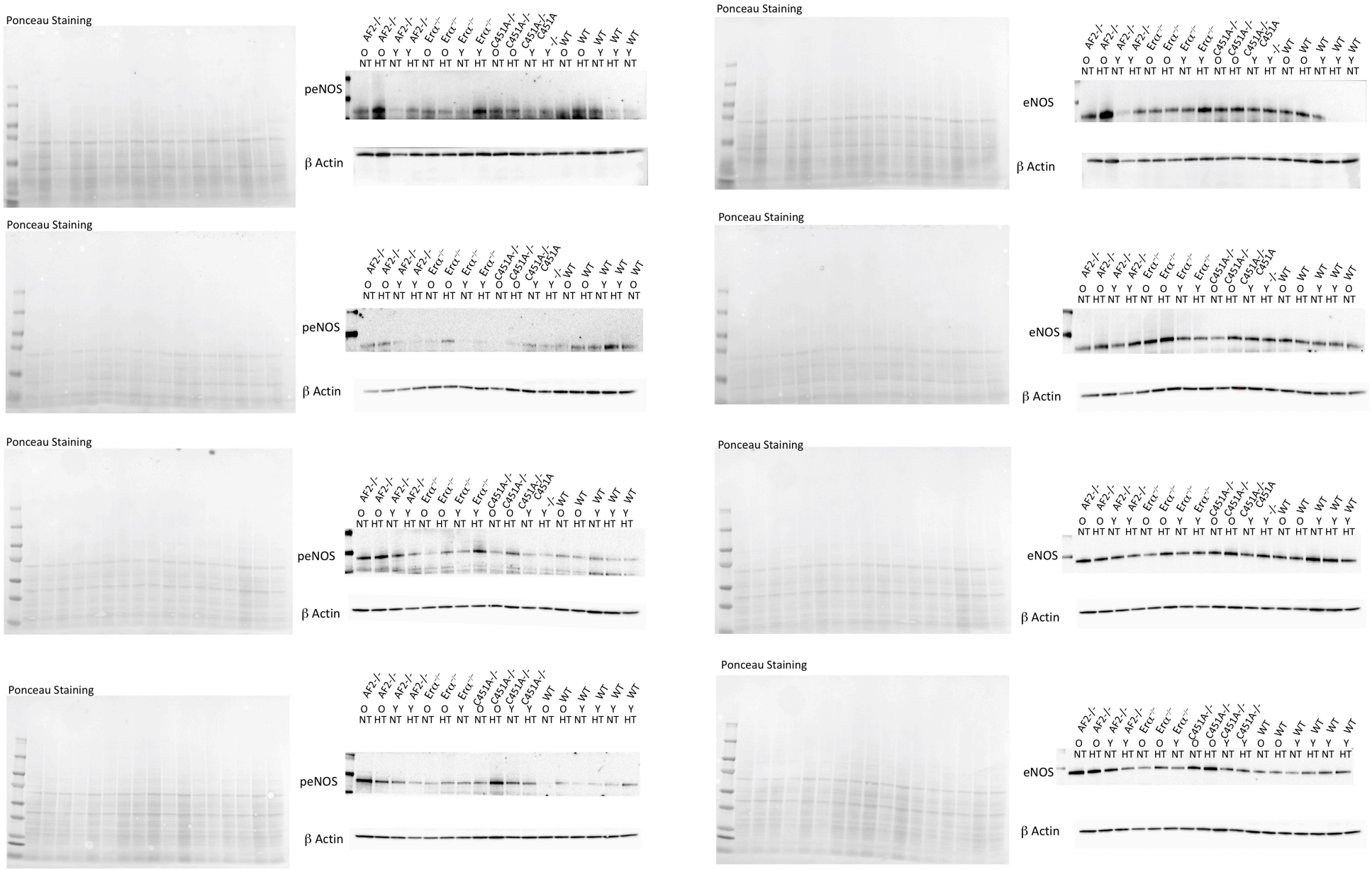

Figure S2. Full blots for eNOS and phosphorylated-eNOS: second set of measurements in the Kidney and corresponding to the bargraphs shown in figures 7.

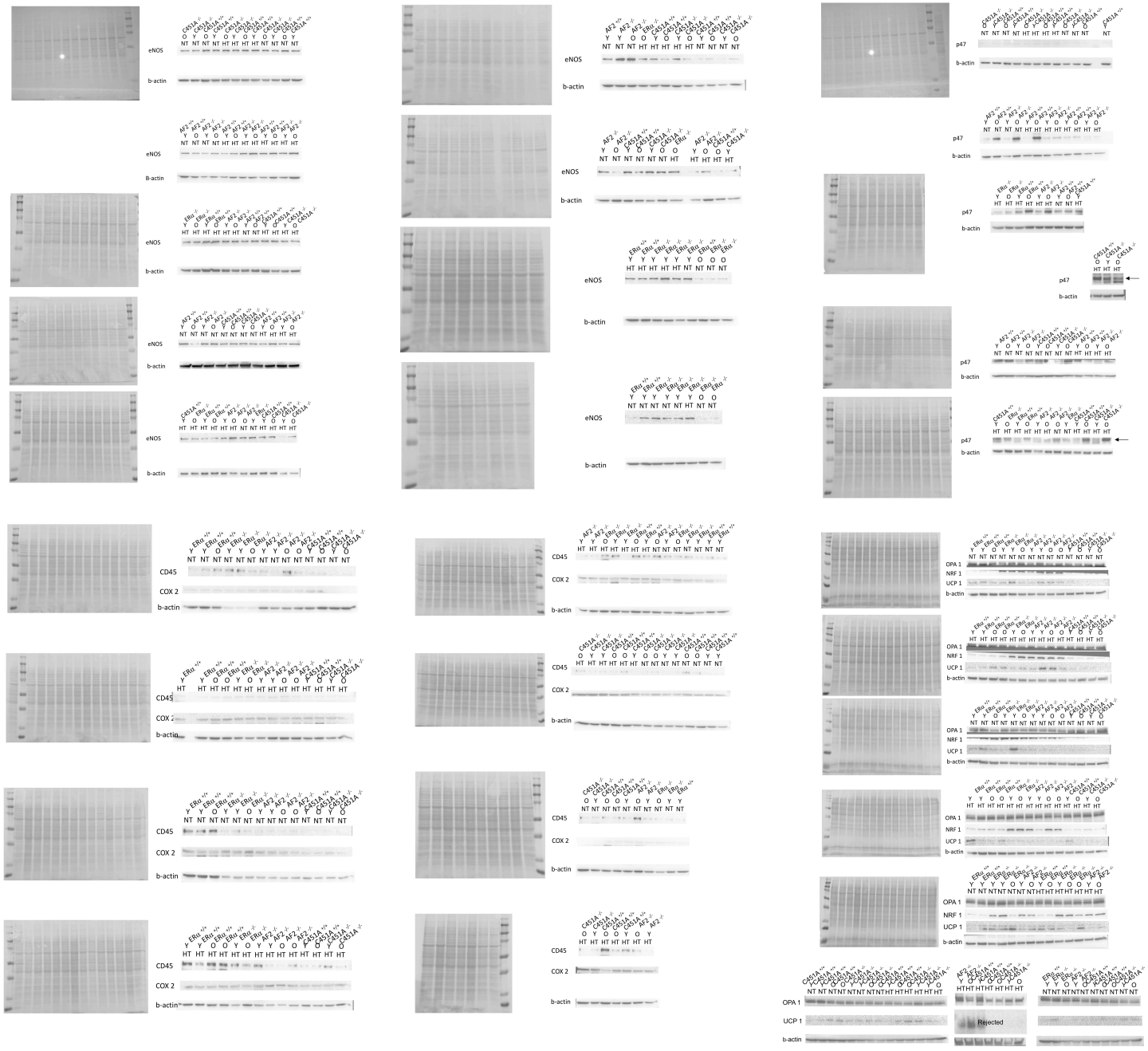

Figure S3. Full blots for eNOS, CD45, COX2, p47, gp91, UCP-1, NRF1, OPA1 and FIS-1 measured in the Kidney and corresponding to the bargraphs shown in figures 7, 8 and 9.