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Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A

(gene targeting/echocardiography/cardiac dilatation/interstitial fibrosis/aortic dissection)

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ABSTRACT **Natriuretic peptides, produced in the heart, bind to the natriuretic peptide receptor A (NPRA) and cause vasodilation and natriuresis important in the regulation of blood pressure. We here report that mice lacking a functional *Npr1* gene coding for NPRA have elevated blood pressures and hearts exhibiting marked hypertrophy with interstitial fibrosis resembling that seen in human hypertensive heart disease. Echocardiographic evaluation of the mice demonstrated a compensated state of systemic hypertension in which cardiac hypertrophy and dilatation are evident but with no reduction in ventricular performance. Nevertheless, sudden death, with morphologic evidence indicative in some animals of congestive heart failure and in others of aortic dissection, occurred in all 15 male mice lacking *Npr1* before 6 months of age, and in one of 16 females in our study. Thus complete absence of NPRA causes hypertension in mice and leads to cardiac hypertrophy and, particularly in males, lethal vascular events similar to those seen in untreated human hypertensive patients.**

The precursor of atrial natriuretic peptide (ANP) is produced and stored mainly in the right atrium of the heart. ANP formed from this precursor is released in response to atrial stretch (1, 2). Once in the circulation, ANP binds to the natriuretic peptide receptor A (NPRA, also known as guanylate cyclase A or GC-A) mainly in the kidney, vascular tissue, and adrenal gland. This binding induces an increase in intracellular cGMP and initiates natriuresis, diuresis, and vasodilation, all of which contribute to lowering blood pressure (3–5). "B-type" natriuretic peptide, a structurally related peptide mainly formed in the cardiac ventricles, also acts through NPRA and has effects similar to ANP. Several studies have suggested a relationship between ANP and blood pressure. For example, the plasma ANP levels in children of two normotensive parents are higher than in children of one normotensive and one hypertensive parent, especially at high levels of salt intake (6). The response of circulating ANP to salt loading is also deficient in the spontaneously hypertensive rat (7). Furthermore, genetic manipulations affecting ANP or NPRA synthesis lead to alterations in the blood pressures of mice (8–10).

The role of the ANP-NPRA system in cardiac pathophysiology is not well understood. Although expression of the genes encoding ANP and "B-type" natriuretic peptide is markedly increased in patients with hypertrophic or failing hearts, it is unclear if this system is activated to play a protective role by reducing the detrimental effects of high blood pressure, or if it is simply a consequence of the hypertrophic changes occurring in the heart. To study the role of NPRA in the regulation of blood pressure and in the cardiovascular response to sustained hypertension we have made mice completely lacking this receptor. Our results demonstrate that NPRA deficiency in mice leads to elevated blood pressures and, particularly in males, to cardiac hypertrophy and sudden death.

MATERIALS AND METHODS

Gene Targeting. Three electroporations of embryonic stem (ES) cells (E14TG2a) were carried out as described (11). The targeting construct contained 5′ and 3′ fragments of mouse strain 129 *Npr1* (the gene coding for NPRA) genomic DNA and a 2.4-kb insert composed of a 1.1-kb fragment from pMC1neoPolyA (Stratagene) placed in an orientation opposite to the transcription of *Npr1* and an irrelevant 1.3-kb *Hin*dIII fragment from approximately 6 kb downstream of exon 22 of the *Npr1* gene. The 5′ region of homology was a 6.5-kb *Sal*I–*Eag*I fragment from upstream of exon 1. The 3′ region of homology was a 1.4-kb *Pst*I–*Hin*dIII fragment of DNA that includes most of exons 2–4. Correctly targeted ES cell clones were identified by the presence of a 5.5-kb hybridizing band in addition to a 12-kb endogenous band in Southern blots of ES cell DNA digested with *Bam*HI and hybridized to a PCR-derived probe for intron 4. Animals were derived from the correctly targeted ES cells as previously described (12) and handled under protocols approved by the institutional animal care and use committees.

Western Blot Analysis. Whole kidneys from *Npr1* −/− or wild type (+/+) mice were homogenized in a polytron in ice-cold buffer containing 20 mM Na *N*-Tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid/10 mM mannitol, pH 7.4/30 μg/ml phenylmethylsulfonyl fluoride/2 μg/ml leupeptin/16 μg/ml benzamidine. After an initial centrifugation at 800 × *g* for 10 min, soluble and membrane fractions were separated by centrifugation at 413,000 × *g* for 1 hr at 4°C. The particulate fractions were resuspended in the same buffer containing 1% Triton X-100. Proteins (60 μg) in soluble and membrane fractions were fractionated in 7.5% SDS-polyacrylamide gels, transferred to Immobilon-P, and analyzed by Western blotting as described (13). Antiserum 281 directed against the rat NPRA extracellular domain was generated by immunizing rabbits with polyhistidine-tagged NPRA (residues 242–493) and was purified after bacterial expression. No NPRA-immunoreactive proteins were present in the soluble fractions.

Photoaffinity Labeling of ANP Receptor. Plasma membranes (200 μg) were incubated with ^{125}I-4-azidobenzoyl ANP in 250 μl of a reaction mixture containing 50 mM $MgCl_2$, 0.5

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Abbreviations: ANP, atrial natriuretic peptide; NPRA, natriuretic peptide receptor A; *Npr1*, mouse gene coding for NPRA.

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mg/ml bacitracin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 mM *N*-ethylmaleimide, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 60 min at 25°C in the dark as previously described (14, 15). The reaction mixture then was photolyzed on ice for 30 min with a 250-watt General Electric sun lamp at about 10 cm. After photolysis, samples were washed twice in reaction buffer without bacitracin and subjected to SDS/PAGE. The receptor bands were localized by autoradiography.

Blood Pressure Measurements. Blood pressures were measured in conscious young adult male or female mice ranging from 3 to 4 months of age by a noninvasive computerized tail-cuff method (16) by an individual blinded to the genotypes of the animals. After 7 days of training, the measurement was made for each animal as the mean of at least six subsequent sessions on each of 5 days. Blood pressures first were measured in animals after 2 weeks on low salt chow (0.05% NaCl, TD94025, Teklad, Madison WI). Animals then were placed on an intermediate salt diet (2% NaCl, TD96037, Teklad) for an additional 2 weeks and retrained and remeasured. Finally, the animals were placed on a high-salt diet (8% NaCl, TD96038, Teklad) and again trained, and blood pressures were measured. Statistical analysis was performed by using multiple ANOVAs, with salt, gender, and genotype as variables.

Transthoracic Echocardiography and Hemodynamic Evaluation. Mice (3–4 months old) were anesthetized with ketamine (100 mg/kg) and xylazine (2.5 mg/kg) intraperitoneally, the chest was shaved, and echocardiograms were obtained by using an echocardiograph (Apogee CX, Interspec-ATL, Bothell, WA). The transducer (9 Mhz) was applied by using a gel-filled standoff to obtain two-dimensional guided M-mode tracings of a cross section of the left ventricular minor axis at the tips of the papillary muscles. The details of these methods and their reliability have been described (17). Several days after echocardiographic evaluation, mice were reanesthetized, and a 1.4 French high-fidelity micromanometer catheter (Millar Instruments, Houston, TX) was inserted into the right carotid artery. After bilateral vagotomy (to limit counter regulatory autonomic reflexes), the micromanometer was advanced retrograde into the left ventricle, and hemodynamic measurements were recorded at baseline and 5 min after the infusion of 1 ml of 0.7% saline. Continuous high-fidelity left ventricle pressure was recorded simultaneously on an eight-channel chart recorder and in digitized form at 2,000 Hz for later analysis. Experiments were then terminated, hearts were rapidly excised, and individual chambers were separated, weighed, and frozen in liquid N_2.

Plasma Renin Concentration. Arterial blood samples from *Npr1* $-/-$ and age-matched *Npr1* $+/+$ litter mates under CO_2 anesthesia were rapidly withdrawn into ice-cold microcentrifuge tubes containing EDTA, by using noncoated glass pipettes, and immediately centrifuged to isolate plasma. Plasma renin concentrations were determined by a radioimmunoassay for angiotensin I as described (18).

RESULTS AND DISCUSSION

Elevated Blood Pressures in Mice Lacking Natriuretic Peptide Receptor A. We used gene targeting in strain 129 embryonic stem cells to replace exon 1 and intron 1 of the gene (19) coding for NPRA (*Npr1* in mice) with a DNA fragment containing a neomycin selectable marker (Fig. 1*A*). This replacement disrupts the gene and removes 237 amino acids from the N-terminal region of the ligand-binding domain, generating a nonfunctional allele. Male chimeras generated from the targeted embryonic stem cells were mated to C57BL/6 (B6) females and transmitted the inactivated *Npr1* gene to their F_1 offspring. The 129/B6 F_1 hybrids that were *Npr1* $+/-$ were intercrossed to obtain *Npr1* $-/-$, $+/-$, and $+/+$ F_2 generation animals, which were used for all of the experiments reported here. At weaning, the three genotypes were represented in normal Mendelian proportions. Kidney membrane preparations from animals homozygous for the *Npr1* mutation ($-/-$) show no NPRA protein (135 kDa) by Western blot analysis (Fig. 1*B*). Receptor-binding analysis performed on lung membrane extracts (Fig. 1*C*) confirms the absence of functional NPRA in the $-/-$ animals. In contrast, the amount of the natriuretic peptide receptor C remained unchanged; this receptor binds natriuretic peptides but has no guanylate cyclase activity and is thought to function in clearance of the peptides (3).

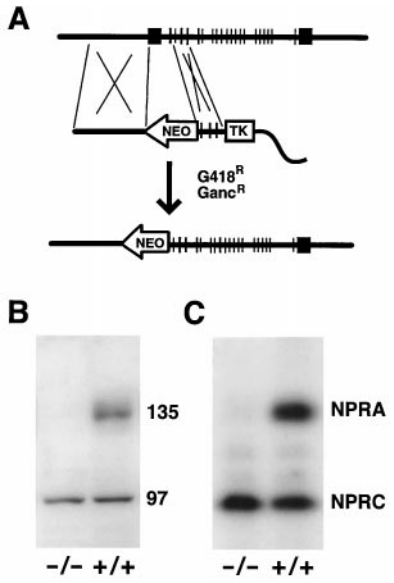

FIG. 1. Targeted disruption of the *Npr1* gene. (*A*) The top line shows the structure of the *Npr1* gene containing 22 exons spanning approximately 18 kb. The targeting construct (middle line) was designed to replace exon 1 through intron 1 with a neomycin resistance gene (NEO). TK indicates the Herpes simplex thymidine kinase gene, which with NEO allowed selection in a medium containing ganciclovir (Ganc) and G418. The cloning vector is represented by a wavy line. The bottom line shows the targeted locus. (*B*) A Western blot, using an antibody against the extracellular domain of rat NPRA, of the membrane fraction of kidney extracts prepared from *Npr1* $-/-$ (homozygous mutant) and $+/+$ (wild type) mice. The 135-kDa NPRA protein, present in the wild-type mice but absent in the mutants, is indicated, as is an unidentified cross-reacting protein of 97 kDa that is equally present in mice of both genotypes. (*C*) An autoradiogram showing the absence or presence of the 135-kDa NPRA after photoaffinity labeling with 4-azidobenzoyl [^{125}I]-ANP in plasma membrane preparations of lung tissue from *Npr1* $-/-$ and wild-type ($+/+$) mice. The positions of the 135-kDa NPRA and 70-kDa natriuretic peptide receptor C radiolabeled bands are indicated.

The effects of *Npr1* genotype on tail cuff blood pressures were assessed by using *Npr1* $+/+$ and $-/-$ animals with three concentrations of NaCl in their chow: 0.05% (low), 2% (intermediate), or 8% (high). The mean blood pressures of the *Npr1* $-/-$ animals of either sex and with all three salt intakes were markedly higher than the pressures of *Npr1* $+/+$ animals (Fig. 2*A*). Homozygous mutants had an average increase in blood pressure of 16 mmHg with the effect of genotype being highly significant ($P < 0.0001$, by ANOVA). The blood pressures of males of either genotype and on all three diets were higher than the corresponding pressures for females (average difference 10 mmHg) again with the difference being highly significant ($P < 0.0001$). There were indications that the effect of *Npr1* genotype is greater in males than in females, but the interaction of genotype and gender did not reach significance ($P = 0.1$). No significant effects of diet on the blood pressures of mice of either sex and of either *Npr1* genotype were observed, although the mean blood pressures of the *Npr1* $-/-$ males on high salt (158 mmHg) were the highest of

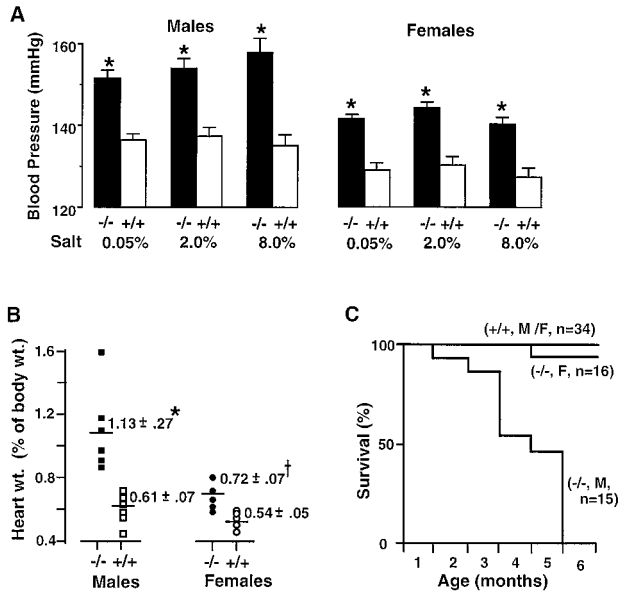

FIG. 2. Blood pressures, heart weights, and survivals of mutant and wild-type mice. (*A*) Blood pressures as a function of *Npr1* genotype and dietary salt. Mean blood pressures measured by a noninvasive computerized tail-cuff method in conscious young adult mice aged 95–115 days are shown for male ($n = 12$ per genotype) and female ($n = 20$ per genotype) *Npr1* $-/-$ (solid bars) and $+/+$ (open bars) animals on a low (0.05% NaCl), intermediate (2% NaCl), or high (8% NaCl) salt diet. SEMs are indicated above the bars. *, $P < 0.001$ vs. wild-type controls by ANOVA. (*B*) Heart weights as a percent of total body weight of 3-month-old mice. Individual values are represented as squares (males) and circles (females), and mean values are indicated by horizontal lines. *, $P < 0.001$ and †, $P < 0.01$ vs. wild-type controls by two-tailed *t* test. (*C*) The percentage survival of male (M) and female (F) *Npr1* $-/-$ animals and wild-type ($+/+$) controls as a function of age.

any group. Overall, these data demonstrate the importance of NPRA in regulating blood pressure in both males and females. The plasma renin concentration in our *Npr1* $-/-$ mice was less than one-third normal (28 ± 5%; $P < 0.01$ by Student's *t* test; $n = 4$), so that these animals provide a model of genetically induced hypertension associated with low plasma renin concentration.

Cardiac Hypertrophy in the Mice Lacking NPRA. In humans, severe untreated hypertension typically progresses through stages, including cardiac hypertrophy, left ventricular dilatation, and an increased incidence of sudden death (20, 21). The first (compensated) stage is an adaptive, hypertrophic response of the heart muscle to the increased load. Although this response is appropriate, it often progresses to later (decompensated) stages that can have severe consequences, as indicated by the identification of left ventricular hypertrophy as an independent risk factor for heart failure and cardiac death associated with hypertension (22–24). We found that our female mice lacking NPRA have an elevated heart-to-body weight ratio averaging 133% of wild type ($P < 0.01$) by 3 months of age (Fig. 2*B*). A similar ratio of approximately 140% of wild-type controls has been reported in animals lacking ANP (8). Heart weights this much greater than controls could be considered pathological in humans although they are within the maximum limits seen in healthy athletes (up to approximately 170% of the normal average) (22). Hearts from the *Npr1* $-/-$ male mice were considerably more enlarged than in the females, with heart-to-body weight ratios averaging 185% that of wild-type males ($P < 0.001$). Cardiac hypertrophy of this degree is indisputably pathological in humans and often is accompanied by ventricular dilatation.

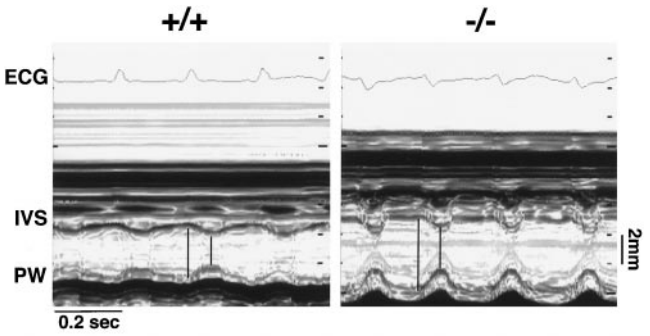

FIG. 3. M-mode echocardiographic tracings from an *Npr1* $+/+$ and an *Npr1* $-/-$ mouse. ECG, electrocardiogram; IVS, interventricular septal wall; PW, posterior wall. The longer and shorter vertical bars illustrate the left ventricular end diastolic and end systolic dimensions, respectively. The ECG is used for timing purposes and, because it is not calibrated, cannot be used to assess conduction patterns.

Echocardiographic and Hemodynamic Evaluation Shows the *Npr1* $-/-$ Hearts Are Fully Functional. To investigate ventricular dilation, we used echocardiography (illustrated in Fig. 3) to compare the *in vivo* cardiac functions of three male and one female *Npr1* $-/-$ mice between 4 and 5 months of age with those of comparable wild-type mice. The resulting echocardiographic data are shown in Table 1 together with the weights of the individual heart chambers of the same animals. Without exception, all of the *Npr1* $-/-$ animals of both sexes proved to have dilated ventricles, with a mean of more than 125% normal left ventricular end diastolic dimension ($P < 0.00005$) and end systolic dimensions ($P < 0.005$). The mutant mice used for the echocardiography had heart-to-body weight ratios significantly greater than wild type (160% wild type), and also left auricle-to-body weight ratios (250%), right auricle (220%), right ventricle (160%), and left ventricle (150%), with all *P* values being less than 0.005. However, despite the hypertrophy and dilatation of the mutant hearts, they were still capable of normal left ventricular performance under the conditions of the echocardiography, as judged (Table 1) by the fractional shortening of the left ventricular walls (% fractional shortening, FS) and rate of wall shortening (velocity of circumferential fiber shortening, Vcf).

Table 1. Echocardiographic and chamber weight findings in *Npr1*-deficient mice

	Npr1 $+/+$ ($n = 10$)	*Npr1* $-/-$ ($n = 4$)
BW (g)	28.19 ± 0.69	29.62 ± 1.37
LVEDD (mm)	3.82 ± 0.08	4.89 ± 10†
LVESD (mm)	2.43 ± 0.11	3.06 ± 0.08*
PWth (mm)	0.66 ± 0.04	0.67 ± 0.03
IVSth (mm)	0.65 ± 0.04	0.66 ± 0.02
FS (%)	36.6 ± 1.9	37.3 ± 1.10
HR (bpm)	273 ± 21	253 ± 26
Vcf (circ/s)	5.19 ± 0.34	5.15 ± 0.27
HW/BW (mg/g)	4.55 ± 0.15	7.13 ± 32†
LV/BW (mg/g)	3.30 ± 0.11	4.89 ± 0.28†
RV/BW (mg/g)	1.00 ± 0.04	1.64 ± 0.16*
LA/BW (mg/g)	0.13 ± 0.01	0.32 ± 0.03†
RA/BW (mg/g)	0.13 ± 0.01	0.28 ± 0.01†

Data expressed as mean ± SE. BW, body weight; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; IVSth, interventricular septal wall thickness; PWth, posterior wall thickness; HR, heart rate; FS, fractional shortening; Vcf, velocity of circumferential fiber shortening; HW, heart weight; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium.

*P , 0.005.

†$P < 0.00005$ $-/-$ compared to $+/+$, Student's *t* test.

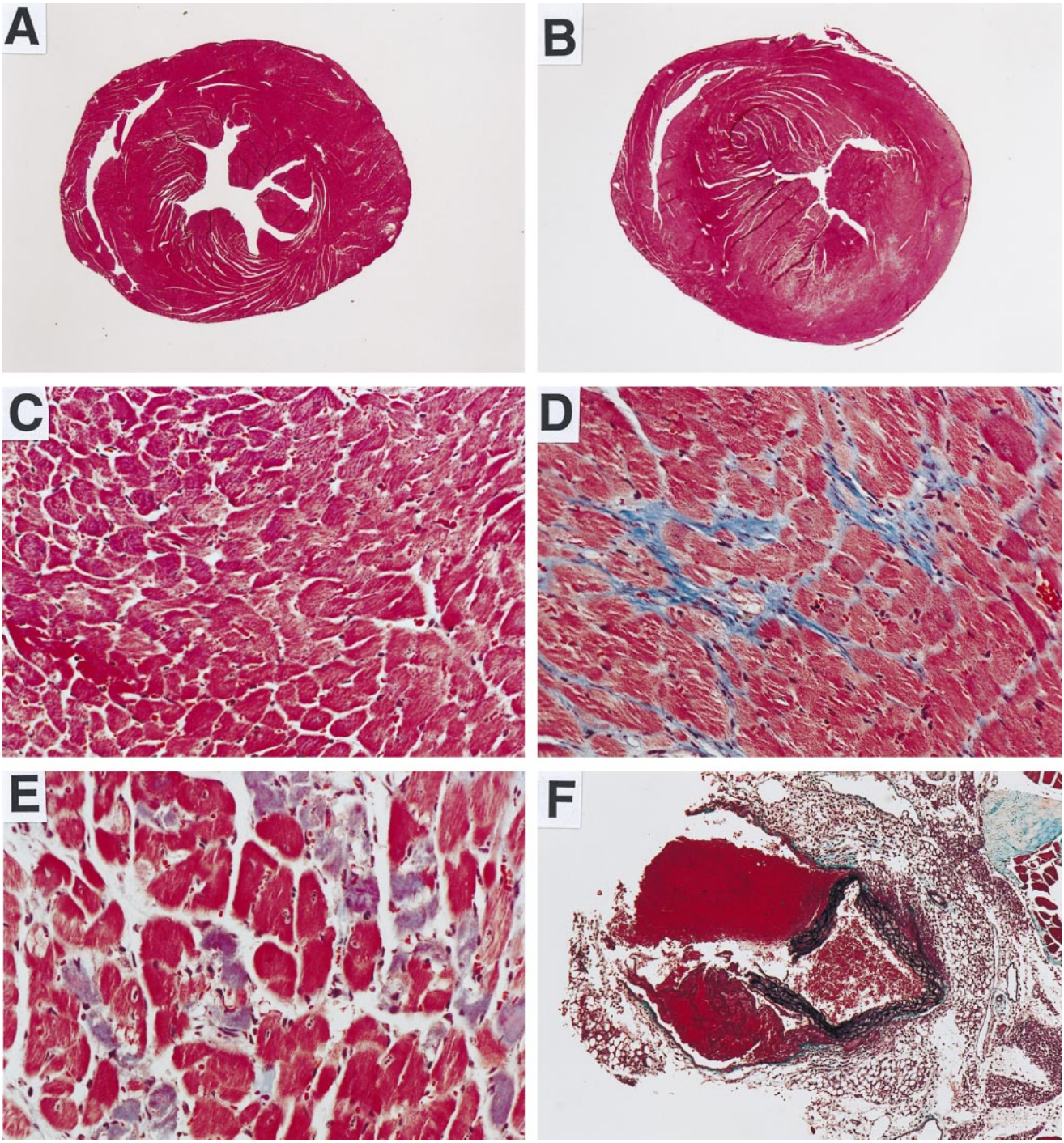

FIG. 4. Histological analysis of tissues from *Npr1* −/− male mice and wild-type controls. (*A–E*) Heart tissues from euthanized animals stained with Masson's trichrome. Hearts were fixed without diastole arrest and with no intracardiac pressure, consequently the *in vivo* dilatation of the *Npr1* −/− hearts was not preserved. (*A*) ×9 and (*C*) ×175 magnification of transverse sections of the heart from a 3.5-month-old wild-type male. (*B*) ×9 and (*D*) ×175 magnification of sections from a 3.5-month-old *Npr1* −/− male. Fibrotic regions stain blue. (*E*) ×175 magnification of a section of a 4-month-old *Npr1* −/− male showing dying myocytes (blue). (*F*) ×45 magnification of the aortic dissection from a deceased 3.5-month-old *Npr1* −/− male, stained with trichrome-elastin stain to show elastic and connective tissue. The platelet-fibrin strands in the clot in the lower left quadrant show that some bleeding had occurred before death of the mouse.

A subsequent hemodynamic evaluation of the same *Npr1* −/− mice before and after challenge with an intravenous injection of 1 ml of normal saline demonstrated that they had elevated left ventricular systolic pressures under both basal and volume-loaded conditions (171 ± 21 mmHg and 213 ± 21 mmHg, respectively) compared with wild-type mice (116 ± 6 mmHg and 159 ± 4 mmHg, both P values < 0.005) consistent with the finding of elevated tail cuff blood pressure noted above. Their unchallenged left ventricular end diastolic pressure (7.5 ± 1.5 mmHg) did not differ from wild type (7.2 ± 1.0 mmHg), and the left ventricular end diastolic pressure in the −/− mice after volume load was 16 ± 4 mmHg compared with 22 ± 2 mmHg in +/+ mice. Thus, the *Npr1* −/− mice appear able to handle an increase in filling pressure as well as wild-type animals. This ability is confirmed by our finding that the maximum change in left ventricular pressure, dP/dt_{max} (a measure of ventricular contractility), in the mutants (14.2 ± 3.4 × 10^3 mmHg/sec) was in fact greater than that in the

wild-type animals ($8.2 \pm 0.6 \times 10^3$ mmHg/sec). Overall, the heart weight, and echocardiographic and hemodynamic data of these 4- to 5-month-old mutants are consistent with a compensated state of systemic hypertension accompanied by marked cardiac hypertrophy and ventricular enlargement with no evidence of myocardial dysfunction.

Sudden Death in the *Npr1* −/− Male Mice. Extensive hypertrophy in humans often is associated with local ischemia in the hypertrophic myocardium that leads to death of some myocytes and to patchy fibrosis. To assess if pathological changes were occurring in the hearts of the *Npr1* −/− animals, we sacrificed four mutant males (3 to 5 months of age), five mutant females (6 months of age), and equal numbers of age-matched wild-type animals. Fig. 4 *A* and *B* compare ventricular cross sections from a wild-type control and a male *Npr1* −/− mutant and illustrate a marked concentric hypertrophy in the mutant mice. Histological (Fig. 4 *C* and *D*) and morphometric comparisons show that this hypertrophy is paralleled by an increase in the myocyte cross-sectional area which in the male mutants averages more than 200% normal (213% ± 32%). Perivascular fibrosis (Fig. 4*D*) was seen in all four *Npr1* −/− males examined, although the extent of this scarring was somewhat variable. In addition, we observed patchy areas of incipient myocyte death in the ventricles of some of the mutant males (Fig. 4*E*). The degree of cardiac hypertrophy and fibrosis was lower in female mutants than in males, with fibrotic foci observed in all four males but in only one of the five females examined. Histological evaluations of the lungs and kidneys of the *Npr1* −/− mice were unremarkable.

Differences in the degree of pathological changes in the male and female *Npr1* −/− mice are further emphasized by comparing their survivals. Thus, as illustrated in Fig. 3*C*, all 15 male but only one of 16 female mutants died before 6 months of age. No wild-type mice died. Initially we had noticed that seven of seven male *Npr1* −/− mice housed with litter mates died between 7 and 8 weeks of age, whereas one that was housed alone survived. At this point all −/− males were housed alone except during some ongoing behavioral tests. However, beginning at about 9 weeks, deaths still occurred. The lethal incidents may be precipitated by stress. For example, one animal died on an exercise wheel after prolonged voluntary exercise at night; others died after being placed with other males, or while on the high-salt diet. Necropsy was performed on as many of the animals as possible within 24 hr of death. Of eight animals examined, four had congested hearts and lungs; there was no evidence of gross abnormalities in other organs such as the kidneys. Three animals had aortic dissections (Fig. 4*F*). One animal died with external bleeding, the source of which was not discernible; this animal also had a pulmonary artery luminal inclusion likely to have resulted from pulmonary embolism. Evidence of death from cerebral hemorrhage was not observed in any of the eight animals.

Exactly what precipitates the lethal events remains to be determined. However, cardiac output and basal stroke work calculations from the echocardiography and hemodynamic data suggest that the hearts of the mutants are working at least twice as hard as those of the wild-type animals. Thus, even under nonstressed conditions, the mutant myocardium has much greater oxygen demands than normal. A relatively small increase in demand therefore may stress the mutant hearts to the point of failure. Death also may be precipitated by sudden surges of blood pressure during conditions of stress, as suggested by the three cases of aortic dissection. It is important, therefore, to explore the possibility that genotype- and gender-related differences in blood pressures are enhanced when the *Npr1* −/− animals are stressed or during the night when mice are normally active.

***Npr1*−/− Mice Provide a Unique Model for Hypertensive Heart Disease.** The occurrence of marked hypertrophy and sudden cardiovascular death in our *Npr1* −/− mutants in the absence of clear evidence of cardiac dysfunction is one of their most intriguing aspects and makes them unique. Although NPRA-deficient mice with a similar degree of hypertension have been reported by Lopez *et al.* (10), the hearts, kidneys, and vasculature of their mutants (carrying a simple insertion of a neomycin-resistance gene into exon 4 of the *Npr1* gene) were normal when examined by histological methods at less than 5 months of age; no increase in postnatal mortality was reported. Likewise, sudden death from cardiovascular events was not observed in other mice with similar hypertension induced by, for example, a genetic increase in angiotensinogen (18) or by a genetic absence of endothelial nitric oxide synthase (25, 26). In this context it is noteworthy that the steady-state levels of ANP and its mRNA increase more than 20-fold in the mouse ventricular myocardium in response to pressure overload caused by banding the thoracic aorta (27). Possibly a normal natriuretic peptide system acts locally to moderate the hypertrophy induced by hypertension.

In conclusion, we have observed in the *Npr1* −/− mice many of the features of hypertensive heart disease seen in untreated human patients, including an initial compensated cardiac hypertrophy, chamber enlargement, and myocyte death, followed by lethal incidents possibly precipitated by stress. The relevance of decreases in the function of NPRA specifically to human patients must await further work. Meanwhile the *Npr1* −/− mice described here provide a genetically well-defined animal model likely to be of considerable value in attempts to understand and moderate the progression of a lethal form of hypertension.

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