**Plzf** as a Candidate Gene Predisposing the Spontaneously Hypertensive Rat to Hypertension, Left Ventricular Hypertrophy, and Interstitial Fibrosis

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**BACKGROUND**
The spontaneously hypertensive rat (SHR) is the most widely used model of essential hypertension and is susceptible to left ventricular hypertrophy (LVH) and myocardial fibrosis. Recently, a quantitative trait locus (QTL) that influences heart interstitial fibrosis was mapped to chromosome 8. Our aim was to dissect the genetic basis of this QTL(s) predisposing SHR to hypertension, LVH, and interstitial fibrosis.

**METHODS**
Hemodynamic and histomorphometric analyses were performed in genetically defined SHR.PD-chr.8 minimal congenic strain (PDS subline) rats.

**RESULTS**
The differential segment, genetically isolated within the PDS subline, spans 788 kb and contains 7 genes, including the promyelocytic leukemia zinc finger (**Plzf**) gene that has been implicated in hypertrophy and cardiac fibrosis. Mutant **Plzf** allele contains a 2,964-bp deletion in intron 2. The PDS congenic strain, when compared with the SHR, showed significantly reduced systolic blood pressure by approximately 15 mm Hg ($P = 0.002$), amelioration of LVH (0.23 ± 0.02 vs. 0.39 ± 0.02 g/100 g body weight; $P < 0.00001$), and reduced interstitial fibrosis (17,478 ± 1,035 vs. 41,530 ± 3,499 μm$^2$; $P < 0.0001$). The extent of amelioration of LVH and interstitial fibrosis was disproportionate to blood pressure decrease in congenic rats, suggesting an important role for genetic factors. Cardiac expression of **Plzf** was significantly reduced in prehypertensive (8 and 21 days) congenic animals compared with controls.

**CONCLUSIONS**
These results provide compelling evidence of a significant role for genetic factors in regulating blood pressure, LVH, and cardiac fibrosis and identify mutant **Plzf** as a prominent candidate gene.

**Keywords:** blood pressure; hypertension; left ventricular hypertrophy; myocardial interstitial fibrosis; quantitative trait locus; spontaneously hypertensive rat; **Plzf** (promyelocytic leukemia zinc finger) gene.

doi:10.1093/ajh/hpt156

Arterial hypertension is a major independent risk factor for adverse cardiovascular events. Hypertension predisposes to left ventricular hypertrophy (LVH), which further increases cardiovascular morbidity and mortality. The heart of hypertensive patients undergoes a process of structural and functional remodeling, which involves all tissue components. In addition to cardiomyocyte hypertrophy, this process consists of increased interstitial and perivascular deposition of collagen and remodeling of intramural arterioles. These structural changes are thought to be the anatomical counterpart of the functional abnormalities that affect the left ventricle and coronary circulation in patients with hypertension.

Myocardial fibrosis is a multifactorial pathological change that involves the remodeling of the extracellular matrix and its cellular components, that is, fibroblast/myofibroblast, inflammatory cells, and others. This results in an adverse remodeling of ventricles leading to abnormalities in heart function, especially regarding the impairment of diastolic relaxation and filling without systolic dysfunction; currently, about 50% of patients with heart failure suffer diastolic heart failure.

In addition to hypertension, other hemodynamic factors are likely to contribute to the development of myocardial fibrosis in human hypertension. First, myocardial fibrosis has been found not only in the left ventricle but also in the right ventricle and the interventricular septum in postmortem studies of human hearts with hypertensive heart disease. Second, recent studies have shown that the ability of
antihypertensive treatment to reduce fibrosis in hypertensive patients with biopsy-proven myocardial fibrosis can be independent of its antihypertensive efficacy.8,9

The spontaneously hypertensive rat (SHR) represents an excellent model of LVH and cardiac fibrosis. Multiple cardiac mass regulatory quantitative trait loci (QTL) have been mapped in the SHR and related strains (Rat Genome Database). Recently, the first 2 blood pressure–independent QTLs associated with LVH were identified at the molecular level as mutated Ogn (osteoglycin) and Endog (endonuclease G) genes on chromosomes 17 and 3, respectively.10,11 These findings further support the possibility that LVH is not just secondary to hypertension and that genetic predisposition may play an important role in hypertrophic response. In our previous studies, we identified a QTL on chromosome 8, which was associated with predisposition to hypertension and LVH, genetically isolated within the SHR–Lx congenic strain.12 In addition, a new QTL associated with cardiac interstitial fibrosis has been mapped recently in the BXH/ HXB recombinant inbred strains on chromosome 8 with the peak of QTL linkage in the vicinity of the promyelocytic leukemia zinc finger (Plzf) gene.13 The Plzf gene is a prominent candidate gene for QTLs associated with both cardiac hypertrophy and fibrosis.14–17 In our study, we genetically defined the differential chromosome segment isolated within the SHR.PD-chr.8 minimal congenic subline and used gene sequencing and expression experiments, as well as histomorphometric and hemodynamic analyses, to identify Plzf as a quantitative trait gene associated with predisposition to hypertension, LVH, and interstitial fibrosis.

METHODS

Animals

We used male rats from the SHR.PD-chr.8 minimal congenic strain (PD5 hereafter)18 and SHR/Ola controls. The rats were housed in an air-conditioned animal facility and allowed free access to a standard diet and water. Surgical procedures (see Blood pressure measurement section below) were performed under halothane (Narcamon) and xylasine (Rometar) anesthesia with dosing according to the manufacturer's recommendations. To obtain tissue, the rats were killed by halothane overdosing. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the ethics committees of the Institute of Physiology, Academy of Sciences of the Czech Republic, and the First Faculty of Medicine, Charles University, Prague.

Mapping of the congenic segment in the PDS subline

We isolated genomic DNA from the tail by phenol–chloroform extraction and ethanol precipitation, subjected it to amplification by standard polymerase chain reaction (PCR) technique, and analyzed the products on native polyacrylamide gel electrophoresis (PAGE) stained with ethidium bromide. Primers for PCR correspond to established microsatellite markers in the PD5 differential segment: D8Rat42, D8Got72, D8Rat94, and D8Arb23. We also searched for additional microsatellites by Pompous19 or used preformatted Tandem Repeat Finder20 data available through the University of California–Santa Cruz Genome Browser at http://www.genome.ucsc.edu. We designed primers flanking microsatellites using Primer3;21 the amplicons were tested for length polymorphism. Primers for polymorphic microsatellites are listed in Supplementary Table 1. We also used single nucleotide polymorphisms (SNPs) identified during sequencing for genotyping (Supplementary Table 1).

Blood pressure measurement

Arterial blood pressures were measured continuously using radiotelemetry in paired experiments between conscious, unrestrained 10-week-old male rats from PD5 subline (n = 7) and SHR controls (n = 7). All rats were allowed to recover for at least 7 days after surgical implantation of radiotelemetry transducers before the start of blood pressure recordings. Pulsatile pressures were recorded in 5-second bursts every 10 minutes throughout the day and night, and 24-hour averages for systolic arterial blood pressure were calculated for each rat. After measuring blood pressure for 2 weeks, all rats were given 1% NaCl for drinking instead of tap water for an additional week (days 15–21) of blood pressure measurements to test for the effects of the PD5 locus on salt sensitivity of blood pressure regulation. The results for each rat in the same group were then averaged to obtain the group means.

Histomorphometric analysis of the heart

We performed histological and histomorphometric studies on hearts isolated from 30-week-old males fed a standard diet and provided with tap water ad libitum (no salt loading) from PD5 subline (n = 6) and SHR controls (n = 6). After fixation, short axis heart slices were cut and processed for paraffin embedding. Multiple 4-μm-thick sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin for microscopic evaluation of morphology. For histomorphometric analysis of myocyte hypertrophy, high-resolution images of short-axis myocytes were acquired at 40× magnification with a digitalized computer camera (MOTICAM 2300, MOTIC BA 300, Motic China Group Co. Ltd, Xiamen, People's Republic of China). Images were stored as JPG files and analyzed using computerized software (IMAGE J 1.43, National Institutes of Health, Bethesda, MD) by an experienced cardiovascular pathologist. The nucleus and sarcoplasma area were manually traced and measured, and their ratio was then derived in order to prevent myofiber stretching artifacts or imperfect alignment of the myocyte. Additional sections were stained with Sirius red for assessment of cardiac fibrosis. Slides were observed under light microscopy. For histomorphometric analysis, high-resolution images of 5 random fields (40× magnification Sirius red stain, roughly 90% of the section area) were used to evaluate interstitial fibrosis.

Sequencing

Tail genomic DNA was amplified by PCR with specific primers (Supplementary Table 1). PCR fragments
were analyzed by electrophoresis and sequenced directly using PCR primers and the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequencing products were analyzed using the ABI PRISM 310 genetic analyzer (Applied Biosystems).

**Taqman quantitative real-time polymerase chain reaction**

RNA was isolated from left ventricles or from whole kidneys using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and reverse transcribed by SuperScript III (Invitrogen, Carlsbad, CA) using oligo-dT primers. Taqman probes were purchased from Applied Biosystems. The following sets were used: Plzf, Htr3a, Htr3b, Usp28, and Drd2.

Genetic isolation of the QTL for cardiac interstitial fibrosis in the P5 minimal congenic strain

The chromosome 8 QTL associated with interstitial fibrosis, recently mapped by Mancini et al., was confirmed by analysis of the P5 minimal congenic strain. The extent of interstitial fibrosis in congenic animals is reduced compared with SHR (17,478 ± 1,035 vs. 41,530 ± 3,499 μm²; P < 0.0001; Figure 2a,b). Moreover, the observed difference (approximately 2-fold reduction in the SHR.PD5 subline compared with SHR) corresponds well with that observed in recombinant inbred (RI) strains (approximately 2-fold reduction in strains carrying the BN-Lx protective allele).

Cardiac hypertrophy is reduced in P5D congenic rats

Heart weights were significantly smaller in the P5D congenic animals compared with SHR (Figure 2c). Furthermore, histological examination revealed that the heart size reduction is accompanied by reduction of cardiomyocyte volume by more than one-third (0.06194 ± 0.00110 vs. 0.03892 ± 0.0006570 μm³ in SHR vs. P5D; P < 0.0001), clearly demonstrating amelioration of hypertrophy (Figure 2d). In addition, no significant correlations were observed between blood pressure and interstitial fibrosis or LVH in the RI strains (data not shown), which suggests that these traits are largely independent of blood pressure.

Blood pressure is decreased in P5D congenic animals

Radiotelemetric measurements showed a significant decrease in systolic blood pressure (Figure 3) in the P5D congenic subline when compared with the SHR, while diastolic blood pressure was also lower but not significantly

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different (data not shown). Blood pressure decrease did not depend on salt intake, as salt loading increased blood pressure proportionally in both groups (Figure 3).

**Plzf expression is downregulated in the heart of PD5 congenic rats**

The *Plzf* contains the intronic deletion that is a prominent candidate for LVH and interstitial fibrosis and also leads to a decrease in limb bud *Plzf* expression with subsequent polydactyly. We therefore measured cardiac *Plzf* expression in prehypertensive juvenile rats (aged 8 and 21 days) by Taqman RT-PCR and found significantly reduced *Plzf* mRNA levels in PD5 congenic animals compared with controls (Figure 4a). In contrast, renal *Plzf* expression did not show significant differences between SHR and PD5 rats (Figure 4b). In addition, we did not observe differences in cardiac expression of p85α and (pro)renin receptor, 2 possible targets of *Plzf* in the heart (data not shown). We also did not see differential expression of the other genes present within the congenic segment, with the exception of elevated *Tmprss5* mRNA in the heart of the 8-day-old rats only (data not shown). *Htr3a, Htr3b,* and *Drd2* mRNAs were barely detectable in both organs.

**DISCUSSION**

In this study, we used the PD5 minimal congenic strain to identify genetic determinants predisposing to hypertension, LVH, and interstitial fibrosis. Cardiac hypertrophy and fibrosis are usually considered to be secondary responses of the ventricle to injuries such as pressure overload caused by hypertension. On the other hand, it has been demonstrated that genetic factors could also play a prominent role in predisposition to cardiac hypertrophy and fibrosis that are independent of blood pressure. In the PD5 strain, we observed a significant decrease in interstitial fibrosis and left ventricular hypertrophy that was disproportionate to a relatively minor reduction in systolic blood pressure (systolic blood pressure in the PD5 subline was lower by 10%, while left ventricular mass was reduced by 41% and fibrosis area by 58%). In addition, in the RI strains, telemetrically measured systolic blood pressure decreased in both groups. Blood pressure decrease did not depend on salt intake, as salt loading increased blood pressure proportionally in both groups (Figure 3).
Figure 2. Interstitial fibrosis and left ventricular mass reduction in PD5 congenic line. (a) Interstitial fibrosis is clearly discernible in a section stained with Sirius red in the spontaneously hypertensive rat (SHR) and much less pronounced in PD5. (b) Quantification of interstitial fibrosis shows significant reduction of the fibrosis area in the congenics compared with SHR (Student t test $P < 0.0001$). (c) Heart weights of SHR and PD5 rats. Significant reduction of myocardial mass in congenic rats compared with SHR (Student t test $P = 3.84 \times 10^{-6}$). (d) Cardiomyocyte size reduction matches the weight reduction (Student test $P < 0.0001$).

Figure 3. Radiotelemetric blood pressure measurement in the spontaneously hypertensive rat (SHR; squares) and congenic PD5 (diamonds). Significant systolic blood pressure reduction is observed in congenic strain compared to SHR (repeated measures analysis of variance $P = 0.002$).
pressure did not correlate with interstitial fibrosis or LV mass (data not shown). These findings suggest that predisposition to interstitial fibrosis and cardiac hypertrophy may be determined predominantly by genetic factors and is not a secondary effect of blood pressure variability. Alternatively, the larger difference in LVH and fibrosis could be due to the cumulative effect of blood pressure difference over a long time period. Continuous monitoring of blood pressure for 30 weeks will be necessary to address this question. It is possible that the pleiotropic effects of the Plzf gene are responsible for all phenotypes, reduced blood pressure, and amelioration of LVH and interstitial fibrosis. The lower expression of the Plzf protective allele that contains the 3-kb deletion in intron 2 suggests that the deleted conserved noncoding sequence might be an enhancer. The deletion originated in the inbred strain PD/Cub (normotensive strain with hypertriglyceridemia and insulin resistance) from which a segment of chromosome 8, including the mutant PD allele, was transferred onto the BN/Cub and SHR/Ola genetic backgrounds. To the best of our knowledge, this intronic deletion in the Plzf gene is unique to the PD/Cub strain and is not present in any other normotensive or hypertensive rat strain.

Plzf acts as a transcriptional repressor binding DNA by its C-terminal zinc finger domain, while the N-terminal BTB/POZ domain is essential for homodimerization and transcriptional repression. Several lines of evidence point to possible involvement of Plzf in the pathogenesis of hypertension, cardiac hypertrophy, and cardiac fibrosis. First, in renal epithelial cells, Plzf is a part of the negative feedback regulation of mineralocorticoid action. Aldosterone induces Plzf, which in turn suppresses expression of beta- and gamma-epithelial sodium channel (ENaC) subunits, thus limiting sodium reabsorption. Also, direct interaction of the Plzf with AT1, angiotensin receptor induces expression of the phosphatidylinositol-3 kinase p85α subunit (p85α PI3K), which promotes protein synthesis. This pathway might explain missing cardiac hypertrophic response in mice deficient in AT1. More recently, Plzf knockout mice were found to be resistant to angiotensin-induced cardiac hypertrophy and fibrosis. Finally, direct interaction of Plzf with the (pro)renin receptor leads to increased expression of p85α PI3K, the same target as in the AT1 cascade. The (P)RR seems to play a key role in cardiovascular end-organ damage. This was suggested by the use of a decoy peptide that corresponded to the handle region of prorenin, which competitively inhibits prorenin binding to its receptor. This peptide ameliorated the development of cardiac fibrosis in SHR rats placed on a high-salt diet. Moreover, renin stimulation has proliferative and antiapoptotic effects on rat cardiomyocytes that are completely dependent on Plzf function. Therefore, this pathway might be connected with predisposition to cardiac hypertrophy and/or fibrosis associated with hypertension. However, we did not observe differential expression of Plzf in the kidney, suggesting that the aldosterone-ENaC pathway is not disturbed. Furthermore, prorenin receptor expression and p85α expression in juvenile hearts of PD5 and SHR males were not significantly different. These findings suggest that other pathways or developmental regulation of these genes could be responsible for the observed phenotypes. Possible pathways that have yet to be investigated are Ras and Wnt, which are positively regulated by Plzf in Caenorhabditis elegans.

In addition to the Plzf, the differential segment of the PD5 subline harbors an additional 6 genes. Htr3a and Htr3b are subunits of the 5-HT3 receptor, a subtype serotonin receptor that is expressed predominantly in the central nervous system. Inhibitors of 5-HT3 are potent antiemetic drugs. Usp28 (ubiquitin-specific peptidase 28) codes for a deubiquitinating enzyme that is implicated in tumor development, either directly through stabilization of oncogene Myc or indirectly through angiogenesis and metastasis. Zw10 (Zeste White 10) codes for a kinetochore that is an associated protein necessary for proper chromosome segregation during mitosis. Tmprss5, also called spinesin, is a serine transmembrane protease of unknown function that is expressed mainly by astrocytes of the spinal cord. Drd2 codes for the D2 subtype of the dopamine receptor. Its mutations are associated with myoclonic dystonia and schizophrenia. In addition, Drd2 knockout mice show hypertension that is dependent on reactive oxygen species. However, the fact that the Drd2 coding sequence has been recombined off in the PD5 subline, as well as the absence of expression difference between SHR and PD5, makes its involvement in the case presented here less likely.

In conclusion, we identified mutant Plzf as a prominent candidate gene in the development of hypertension,
LVH, and interstitial fibrosis in SHR. Targeting of Plzf on SHIR background (e.g., with zinc finger nucleases (ZFN) or transcription activator-like effector nucleases (TALEN) technology) will be most instrumental in establishing a causal role of mutant Plzf conclusively. Current findings might increase our understanding of the pathogenesis of hypertension and its cardiac complications—hypertrophy and fibrosis—factors that ultimately lead to cardiac failure.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

ACKNOWLEDGMENTS

This work was supported by Czech Science Foundation grants P301/10/0756 to E.L. and P301/12/0696 to M.P. and by grant LL1204 within the ERC CZ program (European Research Council grant provided by the Ministry of Education, Youths and Sports of the Czech Republic) to M.P., research project of Charles University in Prague, First Faculty of Medicine PRAUK-P25/LF1/2 and European Union Framework 7 project EURTRANS (HEALTH-F4-2010-241504).

DISCLOSURE

The authors declared no conflict of interest.

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