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Peroxisome Proliferator Ameliorates Endocardial Endothelial and Muscarinic Dysfunction in Spontaneously Hypertensive Rats

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ABSTRACT

Spontaneously hypertensive rats (SHR) develop hypertension (HT) at the age of 2–6 weeks. Endocardial endothelial (EE) dysfunction, autonomic suppression, left ventricle hypertrophy (LVH), and fibrosis are hallmarks of HT. The mechanism of EE dysfunction, LVH, and fibrosis in SHR is largely unknown. It is known, however, that the levels of peroxisome proliferator-activated receptor γ (PPARγ) are negatively correlated with EE function, LVH, and HT. PPARγ ameliorates EE dysfunction and LVH, in part, by increasing endothelial nitric oxide (eNO) and muscarinic activity. Male SHR and normotensive Wistar rats (NWR) at 1 week of age were administered 8 μg/ml ciglitazone (CZ), a PPARγ agonist, in drinking water. The rats were grouped as follows: NWR, NWR+CZ, SHR, SHR+CZ, at 2 and 6 weeks (*n* = 6 in each group). The levels of PPARγ were low in the nuclear extracts of the left ventricle (LV) in SHR, but increased in CZ-treated rats, measured by western analysis. The contractile response to norepinephrine in cardiac rings prepared from the above groups of rats, measured in tissue myobath and normalized by tissue weight, demonstrated no difference in the maximum response to norepinephrine in any group. However, the EC_{50} was significantly lower in SHR at 2 weeks (SHR2wk) than in any other groups, and CZ normalized this decrease. The response to acetylcholine demonstrated no difference in EC_{50}; however, the maximum response was attenuated in SHR2wk, and substantially increased in SHR6wk as compared with age-matched NWR, suggesting that early in HT eNO dysfunction in SHR2wk leads to depression of autonomic muscarinic cholinergic receptor in SHR6wk. The PPARγ agonist ameliorated both the early eNO dysfunction and late autonomic suppression in HT. The response to nitroprusside demonstrated no change in EC_{50}; however, the maximum response was attenuated only in SHR6wk. There were significant fibrosis, LVH, and increased LV pressure in SHR6wk compared with any other group, and CZ regressed LVH and LV pressure. Results suggest that early in HT, except for eNO dysfunction, other contractile responses are preserved; however, at 6 weeks there is significant nonendothelial cell dysfunction, and treatment with CZ reverses this nonendothelial dysfunction as well. *Antioxid. Redox Signal.* 6, 367–374.

INTRODUCTION

ALTHOUGH AUTONOMIC DYSFUNCTION is associated with left ventricle (LV) hypertrophy (LVH) and hypertension (HT), there is no report linking autonomic control and interstitial fibrosis. In addition, little is known about the role of endocardial endothelium (EE) in cardiac relaxation, muscarinic regulation, and extracellular matrix remodeling. Spontaneously hypertensive rat (SHR) has been shown to resemble humans in the development of HT (38). SHR develops hypertensive

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phenotype between 2 and 6 weeks of age (42). In histological studies, an evolution of changes take place in the morphology of both the aorta and peripheral arteries in SHR (18). The evidence suggests that the injury caused by HT leads to endothelium damage, promoting medial thickening, fibrosis, and intimal lesions (18). Previous studies from our laboratory have demonstrated EE dysfunction and myocytic cell degeneration in SHR (24, 26, 27). Others have shown a decrease in endothelial nitric oxide (eNO) concentration associated with LVH in SHR (22). In addition, there is high level of homocysteine in SHR (23, 24), and homocysteine induces cardiac hypertrophy and fibrosis (25). There is negative correlation between high homocysteine and peroxisome proliferator-activated receptor (PPAR) expression (4, 16). Decreased levels of PPAR are associated with LVH in transgenic mice (2). PPAR agonists reduce cardiac hypertrophy and HT (11, 31, 44). Although Diep and Schiffrin (8) demonstrated increased expression of PPARα and γ in resistance arteries, the levels were low or showed no change in heart and muscle in SHR at 6 and 16 weeks (8). The hypothesis is that the degeneration of eNO instigates suppression of muscarinic receptors. The induction of PPARγ decreases LVH, in part, by ameliorating muscarinic depression.

MATERIALS AND METHODS

Animals

SHR and normotensive Wistar rats (NWR) at the age of 8–12 weeks were obtained from Charles River Laboratories. Rats were bred at the animal care facility of the University of Mississippi Medical Center. After birth, male and female were separated. All male SHR develop HT at ~6 weeks. The animal protocol was reviewed and approved by the Animal Care and Use Committee according to the guidelines of the National Institutes of Health.

Test groups

Because SHR develops hypertension at 2–6 weeks (42), 1-week-old male SHR and NWR were administered ciglitazone (CZ; Calbiochem Corp.) at 8 μg/ml in drinking water. Although 1-week-old rats are in the lactation period, the rats drink ~20 ml of water/day; therefore, 2 mg/kg/day CZ was ingested. In a 70-kg man, 100 mg/day PPAR agonist has a potent effect (4). The rats were grouped as follows: (a) NWR; (b) NWR+CZ; (c) SHR; and (d) SHR+CZ. To determine CZ effect in the absence of HT, CZ was administered to NWR. The rats were studied at 2 weeks and 6 weeks; $n = 6$ was used in each group. All rats were fed standard rat chow. To determine whether the administration of CZ impairs a rat's ability to drink or eat, the food and water were measured every other day, and no difference in water and food intake was found.

LV pressure

Rats were anesthetized with Inactin (100 mg/kg of body weight) to limit the effect on the cardiovascular system (5, 26). Cardiac catheterization of anesthetized rats was performed via the right common carotid artery. A PE-50 catheter was connected with Digi-Med's Blood Pressure Analyzer, interfaced with a PC. After the mean arterial pressure was recorded, the catheter was slowly advanced into the LV; LV function was recorded with the Digi-Med's Heart Performance Analyzer and data were analyzed with a PC. After blood pressure measurement, the heart was arrested in diastole by the injection of 0.2 ml/100 g of body weight of a 20% KCl solution. The heart was rapidly excised, placed in cold physiological salt solution, and perfused at LV end-diastolic pressure.

Cardiac rings

The determination of cardiac muscle function in isolated papillary muscle preparation does not demonstrate what happens in the entire transmyocardial wall. To determine cardiac function, Langendorff preparation has been used. However, this does not differentiate the specific contribution of the regional ischemia, hypertrophy, stunning, and/or hibernation of myocytes in myocardial wall. Rather it gives global contractile response to cardiotonic agents. Furthermore, it does not separate the effects of LV from right ventricle (RV). We have compared the data obtained from cardiac rings prepared from hypertensive rats (27) and found similar pressure–volume curves as obtained from the Langendorff preparation. In addition, the cardiac ring preparation separates the effect of LV from RV. To determine the specific regional differences in contractile function, the rings can be prepared to include or to exclude the homogeneous or inhomogeneous regions of the transmural myocardial wall (7, 25, 27, 40). In brief, cardiac rings were prepared by making a 1-mm horizontal cross-section of the excised heart. LV and RV rings were prepared as follows: (a) removing the RV wall leaves the septum and LV walls intact, thus making an LV ring; (b) removing the LV wall leaves the septum and RV walls intact, thus making an RV ring. Once the cardiac rings were prepared, they were placed on ice in cold modified Ringer's buffer. To minimize the effect of time on contractility of cardiac rings, the contraction experiment was carried out within 40 min after removal of the heart, during which time there is minimal injury (40).

Buffer and drugs

All tissue was placed in a bath of modified Ringer's buffer without calcium chloride. The Ringer's buffer contained the following (in m*M*); NaCl 131.5, KCl 1.0, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 23.5, and glucose 11.2, buffered to a pH of 7.4. Responsiveness to norepinephrine (NE), acetylcholine (ACH), and nitroprusside (NP) was tested by adding these drugs to the tissue bath. Drugs were prepared to a concentration of 1 *M* and then diluted to give two concentrations of the drugs equal to 10^{-3} and 10^{-6} *M*.

Experimental protocol

To determine endothelial-dependent endocardial function, cardiac rings were mounted on an isometric tissue myobath between two stainless steel hooks. One hook was connected to an isometric force transducer (World Precision Instruments, Sarasota, FL, U.S.A.), and the other was attached to a mi-

crometer. Responsiveness to ACH, NP, and NE was tested on the rings as they were mounted on the transducers. The cardiac rings were first stretched and then allowed to stabilize to resting tension. At this tension, NE was added in increasing doses to generate maximum contraction. At maximum tension, the response to ACH or NP was measured. The signal of the transducer was digitized and analyzed on-line by using Cardiovascular Function Measurement (World Precision International) software. To minimize the variation due to cardiac muscle orientation, the rings were rotated 90° and contraction was measured. The average of two was recorded. After the data were collected, they were analyzed using Microsoft Excel. All tensions were normalized to tissue weight (grams per gram). The percent contraction was calculated based on maximum contraction. The percent relaxation was estimated with respect to maximum contraction to NE.

PPAR analysis

Nuclear extracts from LV were prepared as described (39). The levels of PPARγ were measured by western analysis. The 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed under reducing conditions. The protein was transferred to nitrocellulose membrane. Nonspecific binding sites were blocked by 5% fat-free milk. PPARγ was probed using anti-PPARγ (Cayman Corp.). Secondary alkaline phosphatase-conjugated IgG was used as detection system. Bands were scanned by a Bio-Rad GS-700 densitometer and normalized with actin.

Histological analysis

Cross-sections of intact perfused hearts were placed in 10% formalin fixative and prepared for histological analysis. The sections were stained with hematoxylin and eosin, and Masson's trichrome stains. The sections were analyzed under light microscopy. All light micrographs were analyzed at 40× magnification.

Myocyte cross-section area

LV diameter and wall thickness were determined by a digital micrometer. Myocyte lengths were measured by a digital light micrograph at 40× magnification. The images were captured using a Spot digital camera and analyzed using the software Image-Pro Plus. Randomly selected myocytes were measured and the data analyzed using Microsoft Excel.

Collagen content

LV tissue collagen levels were measured biochemically using hydroxyproline as standard. Hydroxyproline from identical amounts of LV tissue was extracted as described (26).

Statistical analysis

Values are given as means ± SD from $n = 6$ in each group. Differences between groups were evaluated by using ANOVA, followed by the Bonferroni post hoc test (37), focusing on the effects of SHR [NWR at 2 weeks (NWR2wk) to SHR at 2 weeks (SHR2wk), indicated by **] and treatment (SHR6wk+CZ treatment compared with NWR6wk+CZ treatment, indicated by *). $p < 0.05$ was considered significant.

RESULTS

PPAR

The levels of PPARγ were abrogated in SHR at 2 and 6 weeks. Early treatment with CZ enhanced the expression of PPARγ. The levels were unchanged in NWR treatment with or without CZ (Fig. 1).

Cardiac contraction and relaxation

NE induced dose-dependentcontraction. ACH induced dose-dependent relaxation in NE-mediated contraction in cardiac rings prepared from SHR at 6 weeks (Fig. 2).

Response to NE

There were no changes in the maximum contractile response to NE in SHR or NWR. However, the EC_{50} was significantly lower in SHR2wk than in NWR or SHR6wk. These results suggest up-regulation of NE receptor at 2 weeks. Treatment with CZ ameliorated the up-regulation of NE receptors in SHR (Fig. 3).

Response to ACH

Although there were no differences in the EC_{50} to ACH response in SHR and NWR, the maximum response to ACH was attenuated in SHR2wk as compared with NWR. However, at 6 weeks there was a robust increase in ACH sensitivity in SHR as compared with NWR (Fig. 4). These results suggest the decrease in eNO at 2 weeks and attenuation of myocytic

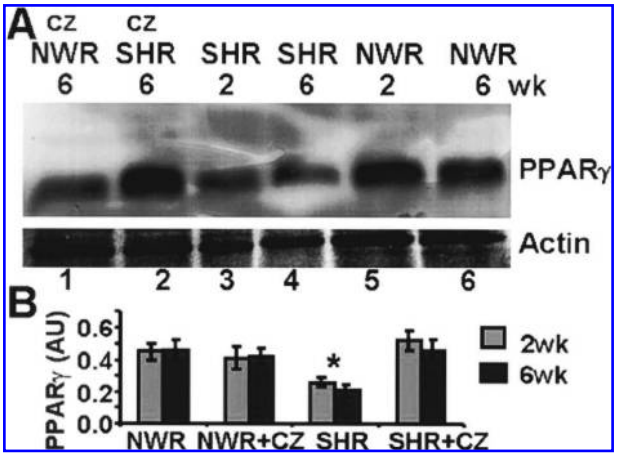

FIG. 1. The levels of PPARγ in SHR and NWR. (A) Western blot analysis. Lane 1, NWR6wk treated with CZ; lane 2, SHR6wk treated with CZ; lane 3, SHR2wk; lane 4, SHR6wk; lane 5, NWR2wk; lane 6, NWR6wk. The bands scanned are normalized by actin. **(B)** The scanned band intensity in arbitrary units (AU) is shown in histograms. Each bar represents the mean ± SD from $n = 6$. *$p < 0.001$.

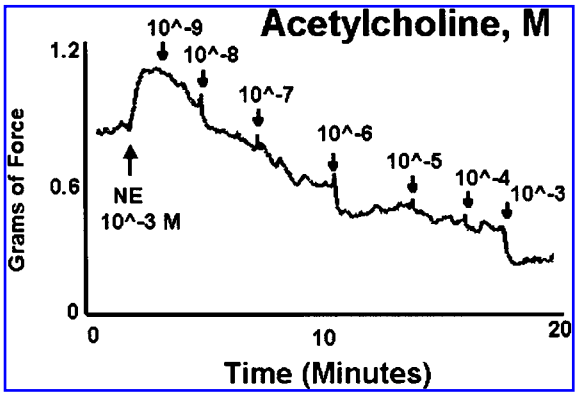

FIG. 2. A representation and time course of LV ring contraction and relaxation. The LV ring from SHR6wk was stretched and brought to resting tension, and different doses of NE were added. The up arrow indicates the addition of 1 m*M* NE. The down arrows indicate different doses of ACH, added to the myobath containing ring. The contraction was measured in grams.

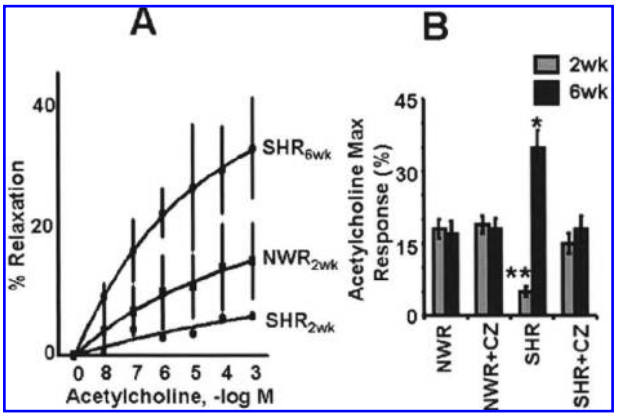

FIG. 4. **(A)** ACH-dependent cardiac relaxation. NE-precontracted rings were relaxed with different doses of ACH. The % relaxation was measured based on the maximum contraction after 1 m*M* NE addition. The lines are the best to data to a nonlinear least squares. Each data point is an average of six independent experiments. **(B)** Histographic presentation of ACH maximum response (%) in LV rings prepared from NWR, NWR+CZ, SHR, and SHR+CZ at 2 weeks and 6 weeks. The means ± SD are reported; $n = 6$ was used in each group. $^{**}p = 0.005$; $^{*}p = 0.001$.

cholinergic response at 6 weeks in SHR, causing a paradoxical increase in cardiac relaxation in SHR. Treatment with CZ ameliorated the attenuation in ACH response in SHR.

Response to NP

There were no differences in the EC_{50} or the maximum response to NP in cardiac rings prepared from either group of rats at 2 weeks. However, at 6 weeks in SHR there was significant attenuation of cardiac relaxation to NP (Fig. 5). These results suggest that at 2 weeks no significant damage has occurred to cells responsible for contractile function; however, at 6 weeks there was a significant decrease in the number of contractile cells. Treatment with CZ reversed the cell damage and improved contractile function in SHR.

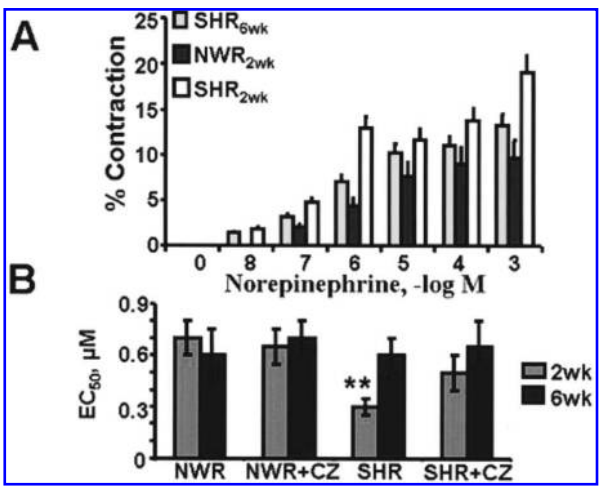

FIG. 3. **(A)** NE dose responses of cardiac rings prepared from NWR2wk, SHR2wk, and SHR6wk. The tension (*y*-axis) generated by the addition of NE was normalized to the weight of the tissue; the % contraction was calculated based on the maximum contraction to 10 m*M* NE. The data are presented as histograms. The means ± SD are reported ($n = 6$). **(B)** Histographic presentation of EC_{50} (µ*M*) data for NE to NWR, NWR+CZ, SHR, and SHR+CZ at 2 weeks and 6 weeks. The means ± SD are reported; $n = 6$ was used in each group. $^{**}p = 0.005$.

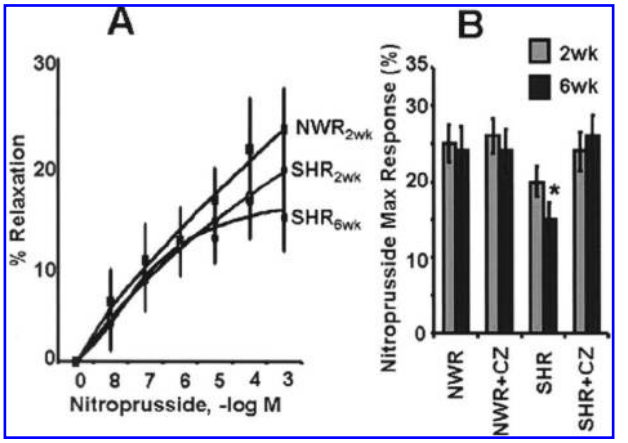

FIG. 5. **(A)** Dose response to NP. Endothelial-independentresponse in cardiac relaxation was estimated by the dose-response titration of NP in the tissue myobath containing LV rings prepared from NWR2wk, SHR2wk, and SHR6wk. The ring was precontracted with 1 m*M* NE, and different doses of NP were added. The % relaxation was estimated with respect to maximum contraction to NE. The lines are the best to data to a nonlinear least squares. Each data point is an average of six independent experiments. **(B)** Histographic presentation of NP maximum response (%) in LV rings prepared from NWR, NWR+CZ, SHR, and SHR+CZ at 2 weeks and 6 weeks. The means ± SD are reported; $n = 6$ was used in each group. $^{*}p = 0.01$.

Cardiac fibrosis

The collagen expression was increased twofold, significantly, in SHR6wk as compared with any other groups. Treatment with CZ tended to regress the collagen content, but insignificantly (Fig. 6).

LVH

At 6 weeks of age, SHR endothelium was disrupted, myocytes began to enlarge, and fibrosis began to appear. Also, the LV of the SHR showed a marked hypertrophy compared with NWR. The average myocyte lengths were 14.53 ± 2.28 μm, 11.47 ± 1.85 μm, and 26.19 ± 7.88 μm in NWR2wk, SHR2wk, and SHR6wk, respectively (Fig. 7). There was no statistically significant increase in myocyte cross-sectional area between NWR and SHR at 2 weeks; however, there was significant cardiomyocyte hypertrophy in SHR at 6 weeks as compared with any other groups at 2 or 6 weeks. Treatment with CZ reversed the myocyte hypertrophy (Fig. 7).

LV pressure

The LV pressure in SHR6wk was increased to an average of 173 ± 10 mm Hg as compared with SHR2wk or NWR, which had an average of 120 mm Hg. There was a 60% increase in LV pressure in the SHR6wk test group over SHR2wk and NWR, and no change in the LV pressure in NWR of either age. Treatment with CZ decreased the LV pressure to normal and prevented the increase in LV pressure in SHR (Fig. 8).

DISCUSSION

High homocysteine is associated with neurogenic disorder (14, 28, 35). Homocysteine decreases eNO (41). The levels of homocysteine are twofold higher in the heart of SHR as compared with NWR (23, 24). A negative correlation between homocysteine and PPAR has been suggested (4, 16). Although the treatment of PPAR agonists ameliorated the homocysteine-mediated cardiovascular dysfunction, the levels of homocysteine were not decreased (9). In fact, the levels of homocysteine were increased (9). Results from our laboratory have suggested competition between PPAR agonist and homocysteine for binding to PPAR (15). Here we suggest that decreased PPARγ expression is one of the causes of LV dysfunction in

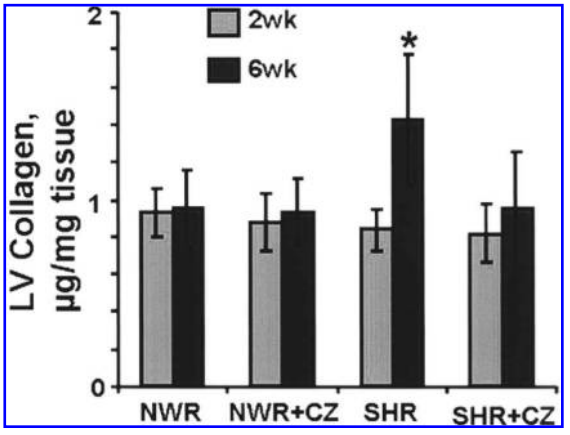

FIG. 6. Collagen content. Total hydroxyproline was measured in identical amounts of LV tissue from NWR, NWR+CZ, SHR, and SHR+CZ at 2 and 6 weeks. The means ± SD are reported; $n = 6$ was used in each group. *$p = 0.05$.

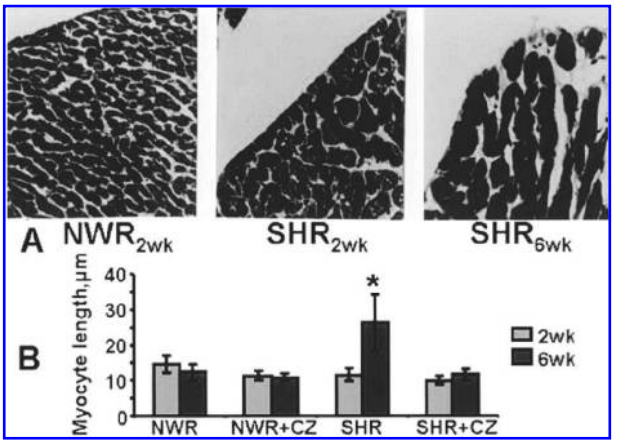

FIG. 7. **(A)** Histological analysis of endocardium and LVH. Identical tissue sites were prepared for histological analysis from NWR2wk, SHR2wk and SHR6wk. All light micrographs were taken at a magnification of 40×. **(B)** LVH was estimated by measuring myocyte length (μm). Histograms show myocyte length in endocardial tissue section prepared from NWR, NWR+CZ, SHR, and SHR+CZ at 2 weeks and 6 weeks. The means ± SD are reported; $n = 6$ was used in each group. *$p = 0.001$.

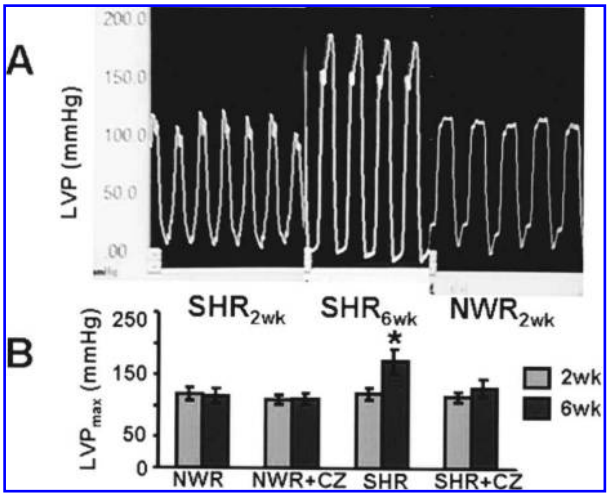

FIG. 8. **(A)** LV pressure (LVP). Representative LV pressure waves from SHR2wk, SHR6wk, and NWR2wk. **(B)** The LVP maximum, in mm Hg, was measured by an intraventricular PE-50 catheter in Inactin-anesthetized rats. Histograms show LVP-max in NWR, NWR+CZ, SHR, and SHR+CZ at 2 weeks and 6 weeks. The means ± SD are reported; $n = 6$ was used in each group. *$p = 0.005$.

SHR. The development of LVH associated with the decrease in eNO availability. This leads the muscarinic response to dwindle, causing cardiomyocyte hypertrophy and increase in LV pressure in SHR. NE receptors are up-regulated at 2 weeks, suggesting activation of the sympathetic pathway in LVH. NE regulates vasoconstriction through its binding to α-adrenoreceptors on vascular smooth muscle cells and chronotropic and ionotropic contractile responses through its binding to β-adrenoreceptors on cardiac myocytes. Chronic stimulation of adrenoreceptors leads to cardiac dysfunction by causing ischemia and by causing elevated levels of cyclic AMP in cardiac myocytes, resulting in hypertrophy or apoptosis (6). Although treatment with PPAR agonist ameliorated LVH (11, 31), it was unclear whether PPAR agonist augments the NE effects. Our results demonstrate that PPAR agonist decreases the NE effect, in part, by decreasing NE affinity to its receptor (Fig. 3).

Others have shown generalized endothelial dysfunction in SHR (19, 20). Our results with ACH response demonstrated a significant abrogation of NO generation in the endocardium of SHR at 2 weeks. However, at 6 weeks a robust response to ACH suggests degeneration of eNO, leading to the attenuation of muscarinic cholinergic receptor in cardiac muscle cells (13, 43) and, therefore, increased ACH response (Fig. 4). These data suggest a relationship between eNO and adrenergic responsiveness, and demonstrate a role of PPAR agonist in protection against eNO degeneration and amelioration of sympathetic activation in the development of HT.

The response to NP elicits normal cell contractile function to exogenous NO at 2 weeks in all groups. However, the response was abrogated at 6 weeks in SHR (Fig. 5). These results suggest that at 2 weeks, the number of contractile cells is normal. However, at 6 weeks in SHR, the number of contractile cells is decreased and, therefore, the response to NP is attenuated. Although at 2 weeks eNO is decreased (Fig. 4), it does not play a role in NP-mediated (*i.e.,* endothelial-independent) cardiac relaxation. The reversal of the NP response by CZ suggests a role of myocytic cell damage in the development of HT.

Decreased NO availability leads to activation of matrix metalloproteinases (MMPs) (29). The levels of MMP activity were increased in SHR as compared with NWR (24, 26, 27). It is a paradox that increased MMP activity and fibrosis track together in SHR. However, it can be the scenario that during increases in load, in the absence of NO, latent MMP is activated to dilate the heart in an attempt to reduce the wall stress. The myocardium compensates by developing hypertrophy and rearranging the extracellular matrix. However, perivascular, microvascular, and interstitial fibrosis are the primary entities manifested in SHR. The medium of vessels, the basement membrane of capillary endothelium containing a substantial amount of elastin, and the ultrastructural collagen are responsible for interstitial connections (32). The constitutively expressed MMP-2 and inducible MMP-9 degrade elastin (34), as well as ultrastructural (*i.e.,* newly synthesized) collagens efficiently (1). Because the turnover of ultrastructural collagen and elastin is remarkably lower than that of oxidized collagen (33), the degraded ultrastructural collagen and elastin are replaced by oxidatively modified, stiffer collagen. In addition, PPAR agonist decreases MMP activity (21, 30), and amelioration of adrenergic dysfunction is associated with decrease in MMP activity (3, 36). Cardiac fibrosis and myocyte hypertrophy are apparent at 6 weeks in SHR (Figs. 6 and 7). Others have demonstrated decreased cell adhesive molecules (12) and fibrinogen expression by PPAR agonist (17). Also, studies have suggested that a decrease in nitric oxide production leads to an increase in MMP activity (29) and agonist of PPAR decreases MMP activity (21, 30). In SHR, however, CZ tends to reduce collagen expression, but insignificantly. *In vitro* PPAR reduces myocyte hypertrophy (44), and here we demonstrate inhibition of LVH by CZ *in vivo*. Inactivation of γ-aminobutyric acid (GABA) receptor in hypertrophy has been suggested (10). GABA receptor may be desensitized in SHR, and CZ activates the GABA receptor. The LVH and LV pressure were regressed, significantly, by CZ treatment in SHR (Figs. 7 and 8). Studies in human and rodent have demonstrated reduction in blood pressure by the treatment with PPAR agonist (11, 31). The L-arginine administration in SHR reduces cardiac hypertrophy, but has no effect on blood pressure (22). Our study suggests a connection between eNO degeneration and muscarinic inactivation in the development of fibrosis and hypertrophy. The treatment of PPAR agonist disrupts this connection.

Limitation

Although the role of GABA receptor is suggested in the attenuation of ACH response in LVH in SHR6 wk, the levels of GABA are not measured.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ACH, acetylcholine; CZ, ciglitazone; EC_{50}, half effective concentration; EE, endocardial endothelial; eNO, endothelial nitric oxide; GABA, γ-aminobutyric acid; HT, hypertension; LV, left ventricle; LVH, left ventricle hypertrophy; MMP, matrix metalloproteinase; NE, norepinephrine; NP, nitroprusside; NWR, normotensive Wistar rats; NWR2wk and NWR6wk, NWR at 2 and 6 weeks, respectively; PPAR, peroxisome proliferator-activated receptor; RV, right ventricle; SHR, spontaneously hypertensive rats; SHR2wk and SHR6wk, SHR at 2 and 6 weeks, respectively.

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