Differential regulation of nitric oxide synthases and their allosteric regulators in heart and vessels of hypertensive rats

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Abstract

Objective: Nitric oxide synthase (NOS)-derived nitric oxide (NO) production is regulated posttranslationally through enzyme’s inhibitory interaction with the caveolar coat protein, caveolin and stimulatory interaction with the chaperone heat shock protein, Hsp90. However, changes in the expression of these regulators with the development of hypertrophic cardiomyopathy are unknown.

Methods: Histochemical and immunoblotted signals for the NOS isoforms, caveolin and Hsp90 were compared in left ventricle (LV) and aortic or mesenteric vessels between spontaneously hypertensive rats (SHR; 18 and 63 weeks old) and age-matched normotensive Wistar–Kyoto (WKY) rats. To assess functional impacts on downstream NO signaling, superoxide anions (O\textsubscript{2}\textsuperscript{-}) and cGMP contents were measured in the same tissues by oxidative fluorescent hydroethidine staining and enzyme immunoassay, respectively.

Results: Compared with levels in age-matched WKY rats, endothelial NOS (eNOS) proteins were increased in aorta of SHR at 18 weeks. Conversely, aortic caveolin-1 and -3 were decreased in SHR, whereas Hsp90 remained unchanged. In LV tissue of SHR at 18 weeks, caveolin-1 and -3 were similarly decreased, but Hsp90 upregulated, together with a downregulation of eNOS. However, at 63 weeks, both eNOS and neuronal NOS (nNOS) were markedly upregulated in the LV of SHR, together with an upregulation of Hsp90. No difference in cardiac and aortic cGMP contents was found between the two strains. In LV sections, O\textsubscript{2}\textsuperscript{-} generation was higher in older compared with younger rats from both strains and highest in 63 weeks SHR.

Conclusions: Changes in NOS protein abundance in SHR rats compared with WKY controls are differentially regulated according to the age of hypertension and the tissue examined and are not necessarily correlated with cGMP contents. The coordinate expressional changes in NOS isoforms and their allosteric regulators, such as caveolin and Hsp90, may act as a compensatory mechanism to maintain the production of bioactive NO in the face of increased oxidant stress.

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1. Introduction

Nitric oxide (NO), synthesized from L-arginine by a family of nitric oxide synthases (NOS) is a key regulator of multiple biological processes including the control of blood vessels tone, cardiac and vascular remodeling, platelet aggregation and vascular smooth muscle cell proliferation [1]. At last three distinct NOS isoforms exist in mammalian cells: endothelial (eNOS) expressed both in endothelial cells and cardiomyocytes; neuronal (nNOS) present mainly in neurons, but also in cardiac, skeletal muscle and vascular smooth muscle from some vascular beds, including carotid arteries [2,3]; and inducible (iNOS) expressed in response to bacterial endotoxin and a variety of proinflammatory cytokines.

In addition to transcriptional control, NOS isoforms are importantly regulated at the posttranslational level. The activity of all NOS enzymes is influenced by the availability of their substrate, L-arginine and cofactors such as tetrahydrobiopterin (BH\textsubscript{4}) but also by the dynamic interactions with inhibitory and stimulatory proteins in an iso-
form-specific manner. Upon binding to its consensus site on all three isoforms, calmodulin promotes electron flow from the reductase to the oxygenase domains, thereby enabling catalytic activity. Contrary to iNOS, calmodulin binding to eNOS and nNOS is largely influenced by intracellular concentrations of calcium. NO production by eNOS is negatively regulated by interaction with the caveolar coat membrane proteins, caveolin-1 and -3, the isoforms expressed predominantly in endothelial cells and cardiomyocytes, respectively [4,5]. Direct binding of caveolin-3 to neuronal NOS (nNOS) also attenuates NOS activity [6]. This inhibitory effect has been rationalized as a reciprocal association of caveolin versus calcium/calmodulin on the calcium-sensitive NOS. Conversely, the chaperone protein Hsp90, associates with both eNOS and nNOS and stimulates the enzymes’ catalytic function [7,8], perhaps through a stabilization of calmodulin binding involving changes in phosphorylation states, at least for eNOS [9]. Hsp90 has also been implicated in the balance between the production of NO and $O_2^-$ by eNOS [10].

Previous studies have demonstrated increased myocardial and vascular levels of $O_2^-$ in different animal models of cardiovascular disease, including hypertension [11,12] and pressure overload-induced hypertrophy [13]. $O_2^-$ rapidly reacts with NO and decreases NO bioavailability. The impact of increased $O_2^-$ production and reduced NO bioavailability on the NO/cGMP pathway were suggested to contribute both to abnormalities of endothelium-dependent vascular relaxation [14,15] and impairment of LV relaxation in LV hypertrophy [13]. Thus, an integration of the NOS expression and their posttranslational regulators appears to be an important indication in assessing the enzyme’s activity and in controlling the balance between NO and $O_2^-$. It may also provide an explanation for the paradoxical maintenance of bioactive NO production (as assessed from tissue cGMP levels or sensitivity to NOS inhibitors) despite reduced NOS protein abundance.

In the present study, we therefore examined the concurrent changes in the expression of NOS isoforms and caveolin-1, -3 and Hsp90 proteins in left ventricular, aortic and mesenteric vessel tissues from a rat genetic model of hypertension and hypertension-induced myocardial hypertrophy (SHR), at two stages of development of the disease (i.e., at 18 and 63 weeks of age), compared to WKY controls. In addition, we assessed cardiac production of superoxide anions ($O_2^-$) and cGMP levels to integrate the observed expression modifications in NOS and their regulators with a main determinant of NO bioavailability and the overall functional impact on the NO/cGMP pathway.

2. Methods

2.1. Animals

The study was performed using hearts, aortas and mesenteric arteries from adult (18-week-old) and aging (63-week-old) male SHR (Charles River Laboratories) and age-matched male WKY rats. Both SHR and WKY rats were purchased from Iffa Credo (Belgium). Animals were housed in standard conditions with free access to normal (i.e., at 18 and 63 weeks of age), compared to WKY tissues, care was taken to load between the production of NO and $O_2^-$ by eNOS [10].

Frozen rat LV, mesenteric artery and aortic tissues were homogenized in lysis buffer containing a protease and phosphatase inhibitor cocktail (Sigma) and centrifuged at 12,000 rpm for 10 min. Proteins were quantified using bicinchoninic acid. Equal protein amounts of the boiled supernatant were loaded onto 8 or 12.5% SDS–PAGE gels, and separated proteins were transferred to polyvinylidene difluoride membranes (PVDF; NEN). Naphthol blue black stained with the NOS expression and their posttranslational regulators were carried out in accordance with 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).

2.2. Western blotting analysis

2.3. Determination of NOS activity in left ventricular homogenates

The activity of NOS was determined by conversion of L-$[^{15}$H]arginine to L-$[^{15}$H]citrulline as previously described

 were immediately removed and placed in ice-cold Krebs solution (120 mM NaCl, 5.9 mM KCl, 25 mM NaHCO$_3$, 11.5 mM glucose, 1.2 mM Na$_2$PO$_4$, 1.2 mM MgCl$_2$, 2.5 mM CaCl$_2$). The hearts and vessels were cleaned of all adherent tissues and then frozen in liquid nitrogen and stored at $-80$°C until assay. All experiments were carried out in accordance with 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).

Frozen rat LV, mesenteric artery and aortic tissues were homogenized in lysis buffer containing a protease and phosphatase inhibitor cocktail (Sigma) and centrifuged at 12,000 rpm for 10 min. Proteins were quantified using bicinchoninic acid. Equal protein amounts of the boiled supernatant were loaded onto 8 or 12.5% SDS–PAGE gels, and separated proteins were transferred to polyvinylidene difluoride membranes (PVDF; NEN). Naphthol blue black staining was used to confirm equal protein loading. Immunoblotting analysis was performed as described [16] with primary monoclonal antibodies for eNOS, nNOS, iNOS, caveolin-1, -3 or Hsp90 (1:2500 dilution for each antibody), all purchased from Transduction Laboratories. After incubation with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution), immune complexes were detected using an enhanced chemiluminescence reaction (NEN Life Science Products). The density of the bands was quantified by scanning densitometry. Since the main purpose of the study is to compare protein abundance between WKY and SHR tissues, care was taken to load extracts from both strains on the same gels for equal subsequent processing and accurate comparison. Accordingly, results are presented as comparisons to signals in WKY tissues on the same gels, expressed as 100%. As an additional control, some samples were re-probed for the expression of $\alpha$-skeletal actin to account for cardiac hypertrophy.
2.5. Determination of cyclic GMP content

Frozen tissues (thoracic aorta or left ventricle) were homogenized in ice-cold 6% (w/v) trichloroacetic acid in the presence of the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX; 300 μM). After centrifugation, trichloroacetic acid was extracted by washing supernatants four times with 5 volumes of water-saturated diethyl ether. The remaining aqueous extract was dried under a stream of nitrogen at 60°C. Cyclic GMP contents were assayed using an acetylation protocol of an enzyme immunoassay kit (Amersham–Pharmacia Biotech, Little Chalfont, UK). The mean value was calculated from duplicate measurements of each sample and normalized to total protein content (for thoracic aorta) or to tissue weight (for left ventricle). The amount of protein in the centrifugation pellet from aortic segments was determined by the method using bicinechonic acid (Pierce).

2.6. Detection of superoxide anion generation in rat left ventricles

In situ production of superoxide in the SHR and WKY rats was evaluated using hydroethidine, a reduced precursor of ethidium bromide. Hydroethidine is taken up into cells, and in the presence of O₂⁻ it is transformed into the red fluorescent ethidium bromide, which intercalates with nuclear DNA. The ethidium excited with a wavelength of 480 nm emits light at wavelength of 620 nm. Hydroethidine is particularly sensitive to oxidation by superoxide, as opposed to hydroxyl radical, peroxynitrite, H₂O₂, NO, hypochlorite, or singlet O₂ [17]. Left ventricular segments were embedded in Tissue Tek OCT compound (Miles, Elkhart, IN), snap-frozen in precooled isopentane, and stored at −80°C. Cryosections of 5-μm thick were cut and then placed on Superfrost Plus slides (Menzel-Gläser, Germany). Sections were dried and stored at −80°C. Hydroethidine was first diluted in dimethylsulfoxide (DMSO) as a 2×10⁻² M solution and then diluted to 2×10⁻⁶ M in water before use. Fifty μL of hydroethidine solution was topically applied to each tissue section and the slide coverslipped. The slides were incubated in a light-protected and humidified chamber (37°C, 30 min) before visualizing the fluorescence under a Zeiss Axiosvert S100 (Jena, Germany) inverted microscope coupled to a Pixera 120es digital camera.

2.7. Statistical analysis

Data are presented as mean±S.E.M. Comparison between results from different groups were performed using Student’s test or Bonferroni correction for multiple comparisons where appropriate. Statistical significance was defined as P<0.05.

3. Results

The SHR were significantly hypertensive relative to the WKY rats as early as 18 weeks of age. Body weights increased in both groups during the observation period, but remained significantly lower in the SHR compared with normotensive WKY rats (P<0.0001). Since heart weights also augmented significantly more with age and the development of hypertension in SHR compared to control rats, the heart weight/body weight ratios of SHR were significantly higher compared with age-matched WKY rats (see Table 1), indicating the presence of cardiac hypertrophy in SHR.
Table 1
Systolic blood pressure, body weight and heart weight/body weight ratio in adult (18-week-old) and aging (63-week-old) Wistar–Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR)

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<tr>
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<th>WKY</th>
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<td></td>
<td>18 weeks</td>
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<td></td>
<td>(n=10)</td>
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<tr>
<td>Mean systolic pressure, mmHg</td>
<td>128.0±3.2</td>
<td>–</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>375.0±4.1</td>
<td>623.3±23.3</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>0.91±0.02</td>
<td>1.33±0.05</td>
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<tr>
<td>Heart weight/body weight ratio</td>
<td>2.43±0.05</td>
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* P<0.0001, † P<0.005 and † P<0.01 vs. age-matched WKY rats.

3.1. Expressional changes in NOS isoforms and their allosteric regulators in the left ventricle at 18 and 63 weeks

To examine the expressional modification of NOS isoforms with hypertension we performed Western blot analysis of NOS proteins in extracts from whole left ventricle (Fig. 1). In LV of 18 weeks SHR, eNOS protein expression was significantly lower than that found in age-matched WKY rats (Fig. 1A, left). This decrease in young SHR was even more prominent when eNOS protein abundance was normalized to that of α-skeletal actin on the same blot, as an index of hypertrophy (Fig. 1B). In contrast, eNOS abundance was two times greater in SHR than in WKY at 63 weeks of age (Fig. 1A, right). The expression of neuronal NOS (nNOS) tended to be higher in hearts of 18 weeks SHR (P: NS), and this trend was clearly augmented to reach statistical significance (P<0.05) between WKY and SHR at 63 weeks of age (Fig. 1C).

We next examined changes in expression of caveolin-1 and -3, two allosteric negative regulators of eNOS in endothelial cells and cardiomyocytes, respectively, in the same extracts (Fig. 2). The abundance of caveolin-1 and -3 proteins was markedly lower in whole extracts from LV of 18 weeks SHR compared to control WKY (P<0.0001 and P<0.0005, respectively, Fig. 2A,B, left). A trend towards a similar reduction was observed in LV of 63 weeks SHR compared with age-matched WKY rats (Fig. 2A,B, right), albeit non-significant.

Of interest, the expression of the chaperone protein Hsp90, that positively regulates eNOS (and nNOS) activity, was significantly increased in LV of 63 weeks as well as 63 weeks SHR compared with age-matched control WKY (P<0.01 and P<0.005, respectively; Fig. 2C).

3.2. Caveolin-1 and -3 immunostaining in rat left ventricle

We then examined the pattern of caveolin-1 and -3 immunostaining in LV of 18- and 63-week-old rats from both strains. Immunohistochemical detection with polyclonal anti-caveolin-1 antibody revealed a strong granular signal in the endothelium of large vessels and coronary microvessels in sections of LV from 18 weeks control rats (Fig. 3A). Although the staining pattern was similar in LV vascular endothelial cells from WKY and SHR rats of both ages, it appeared less intense in SHR rats (see Fig. 3B versus A) and in older rats from both strains (Fig. 3A,E versus B,F). However, the limitation of immunohistochemistry precludes from firm quantitative conclusion with this technique.

In WKY rats (both 18 and 63 weeks old), caveolin-3 staining presented as multiple juxtaposed fine granules mainly at the periphery of cardiac muscle cells. No signal was found in microvessels, either in smooth muscle or endothelial cells (Fig. 3C). In SHR rats, the stained granules were more sparsely distributed across the whole cardiomyocyte area (Fig. 3D). Again, no other cell type stained positively. The staining appeared more intense in WKY compared to SHR tissues, regardless of age (see Fig. 3C versus 3D and 3G versus 3H).

3.3. NOS activity in LV extracts at 18 weeks

Ca-dependent NOS activity, as measured by l-[H]3 citrulline formation, was unchanged in LV between the WKY and SHR at 18 weeks of age (Fig. 4A, left). Ca-independent activity remained very low and similar in both strains (Fig. 4A, right, P: NS). Likewise, inducible NOS (iNOS) was undetectable by Western blotting analysis in whole ventricular extracts from either 18 and 63 weeks WKY or age-matched SHR (not shown).

3.4. cGMP contents and superoxide production in hearts extracts

To gain further understanding into the functional correlates to the expressional changes mentioned above, we next assessed the tissular contents of cGMP, a downstream messenger of the NO pathway, in the same LV extracts. There was no difference in LV cGMP content between SHR and WKY rats at any age (Fig. 4B).

Aside from NOS abundance and/or activity, the intracellular production of cGMP depends, among various...
Fig. 1. NOS protein expression in left ventricle (LV) of SHR hearts compared with age-matched WKY control animals. Representative Western blotting (top) and densitometric data (bottom) of immunoblotting of eNOS (A,B) and nNOS (C) in LV extracts from adults (18 weeks; n = 10) and aging (63 weeks; n = 3–5) WKY and SHR. (B) Extracts from young rats were probed on the same filter for eNOS and α-skeletal actin, and densitometric values normalized to actin as an index of hypertrophy. Data in (A,C) are presented as a percentage of values in age-matched control (WKY) rats and are expressed as mean±S.E.M. *P<0.05 and ***P<0.005 versus age-matched WKY rats.

factors, on the bioavailability of the NO produced that is influenced by its oxidative degradation. Therefore, we assessed the tissular production of superoxide anions (\(O_2^-\)) that are known to interact with NO in an equimolar fashion and to decrease its bioactivity. As shown in Fig. 5, \(O_2^-\) generation as measured from ethidium fluorescence in LV sections was higher in 63-week-old compared with 18-week-old rats from both strains. \(O_2^-\) production was also markedly increased in LV tissue sections from 63 weeks SHR compared with age-matched WKY rats.

3.5. Expressional changes in NOS isoforms and their allosteric regulators in aortic and mesenteric vessels at 18 and 63 weeks

A similar expressional analysis was performed in both
Fig. 2. Caveolin and Hsp90 protein expression in LV tissue from adults (18 weeks) and aging (63 weeks) WKY and SHR. Representative Western blot (top) and densitometric data (bottom) of caveolin(cav)-1 (A), caveolin(cav)-3 (B) and Hsp90 (C) protein expression in LV from 18 weeks (n=10; left) and 63 weeks (n=3–5; right) WKY and SHR. Data are presented as a percentage of values in age-matched control (WKY) rats and are expressed as mean±S.E.M. **P<0.01, ***P<0.005, ****P<0.0005 and *****P<0.0001 versus age-matched WKY rats.

Conduit and resistance vessels, where endothelial production of NO modulates peripheral vascular tone. In aortic rings, eNOS protein abundance was markedly increased in 18 weeks SHR compared with age-matched WKY rats (Fig. 6A, left). In 63 weeks SHR that was a trend towards a reduction of eNOS expression, albeit non-significant (Fig. 6A, right). The abundance of nNOS isoform in the same extracts was equally unchanged (100.0±30.1 in control WKY versus 85.6±10.5% in 63 weeks SHR, P: NS), iNOS was undetectable in aortic tissues from 63-week animals of both strains. In mesenteric arteries, the expression of eNOS was similar in 18 or 63 weeks WKY and age-matched SHR (not shown).

Fig. 6B,C illustrates variations in caveolin-1 and -3 in the same aortic extracts. The abundance of caveolin-1 and -3 protein was decreased in 18 weeks SHR (Fig. 6B,C, left) and 63 weeks SHR (Fig. 6B,C, right) compared with age-matched WKY rats.
Fig. 3. Caveolin-1 and-3 immunostaining in LV tissue from adults (18 weeks) and aging (63 weeks) WKY and SHR rats. (A,B,E,F) Positive staining with polyclonal anti-caveolin-1 antibody; (C,D,G,H) positive staining with monoclonal anti-caveolin-3 antibody. (A,C) 18 week-WKY rats; (B,D) 18-week SHR rats; (E,G) 63-week WKY rats; (F,H) 63-week SHR rats. Magnification: ×40; except insets of panels (C,D) (×100).
Likewise, the level of caveolin-1 expression in mesenteric arteries was ~2 times lower in SHR than WKY rats at 18 weeks (52.9±5.2 vs. 100±10.6%; P<0.005, n=9–10 rats), but not at 63 weeks (not shown).

The abundance of Hsp90 was unchanged in aortic extracts across all ages and strains. In mesenteric arteries, a significant increase in Hsp90 expression was observed only in SHR of 63 weeks of age (207.2±15.7 in SHR versus 100±14.6% in WKY group; P<0.005; not shown).

3.6. cGMP contents in aortic tissue

Again, we measured tissular cGMP as an index of the function of NO signaling in the same vessel extracts. No difference in cGMP content was found in aortic rings from adult and aging SHR when compared with age-matched control rats (1.037±0.41 in 18 weeks WKY versus 1.246±0.68 pmol/mg pi in 18 weeks SHR; P: NS, and 0.172±0.03 in 63 weeks WKY versus 0.215±0.04 pmol/mg pi in 63 weeks SHR; P: NS).

The relative changes in eNOS, nNOS, caveolin-1/-3 and Hsp90 expression, in the heart, aorta and mesenteric arteries from adult (18-week-old) SHR and aging (63-week-old) SHR are summarized in Table 2.

4. Discussion

The main purpose of our study was to examine concurrent changes in the abundance of NOS proteins and their allosteric regulators with the development of hypertrophic cardiomyopathy in the SHR model compared with age-matched WKY rats and to assess the tissue specificity of these expression changes. We found that changes in expression of NOS between the two strains with the development of hypertension varies with time (i.e., eNOS abundance is lower in LV from younger, but higher in LV from older SHR), and according to the tissue examined (i.e., LV versus aorta). Contrary to NOS proteins, the abundance of caveolin-1 and -3 is coordinately decreased in LV and vessels. We also observed that changes in NOS abundance are not strictly reflected by concurrent changes in tissue cGMP levels. Notably, the decrease in eNOS proteins in the LV of younger SHR is paralleled with both an increase in Hsp90 protein (positive regulator of eNOS activity) and a decrease in caveolin-1 and -3 proteins (both negative regulators of eNOS activity), which may account for the unchanged cGMP levels. We also show a marked increase of nNOS proteins in the LV of older SHR compared with age-matched WKY, which had not been described before.

In younger SHR (close to the age of 18 weeks chosen in our study), eNOS protein abundance in whole heart was shown to either increase [18] or decrease [19] in previous studies. In contrast, Bayraktutan et al. [20] reported a selective decrease of eNOS proteins in LV cardiac myocytes (but not in coronary microvascular endothelial cells) isolated from 12-week-old SHR. Given the increased mass of cardiomyocytes in hypertrophic hearts, with reduced capillary density, the latter observations would be consistent with our present results, showing a significant reduction in eNOS protein abundance in whole tissue extracts in 18 weeks SHR compared with age-matched control WKY. This reduction in eNOS was even more prominent when the immunoblotting signals were normalized by those for actin (as an index of hypertrophy) in the same extracts (Fig. 1B). Unexpectedly, the reduction in LV eNOS proteins from 18 weeks SHR was not reflected by a reduction in total or calcium-dependent NOS activity (Fig. 4A). Consistent with some [21], but not all [22] previous studies, we found a very low calcium-insensitive NOS activity (reflective of iNOS) in all LV homogenates from 18-week WKY rats similar to age-matched SHR. Accord-
Fig. 5. Comparison of superoxide anion generation in left ventricular sections from adults (18 weeks) and aging (63 weeks) WKY and SHR rats. Superoxide production in unfixed sections of rat hearts was assessed from the transformation of hydroethidine into red fluorescent ethidium bromide for exactly 30 min at 37°C under microscope. (A) 18-week WKY rats; (B) 18-week SHR rats; (C) 63-weeks WKY rats; (D) 63-week SHR rats. Data are representative of three different experiments. Magnification: ×40.

Ingly, no signal for iNOS was detectable in LV extracts of younger or aged animals by Western blot analysis. A potential explanation for the unchanged calcium-dependent NOS activity would be a compensatory increase in the second calcium-sensitive NOS isoform, neuronal NOS (nNOS), known to be expressed in the myocardium. Indeed, the immunoblotted nNOS signal was increased in 5/9 LV extracts of younger SHR and 5/5 LV extracts from aging SHR. The present study is, to our knowledge, the first demonstration of increased nNOS protein expression in the hypertrophic, nonfailing heart of SHR.

The reduction in LV eNOS expression of younger SHR was accompanied with a significant decrease in abundance of the NOS inhibitors, caveolin-1 and -3. Importantly, this contrasted with augmented levels of the heat shock protein, Hsp90, a molecular chaperone known to enhance eNOS and nNOS activity by facilitating calcium–calmodulin binding and promoting Akt phosphorylation (on eNOS [9]). Only a few studies have specifically examined expressional modifications of caveolin-1 and -3 in hypertrophic cardiomyopathy. Recently, we showed a reduction of caveolin-1 and -3 (and eNOS) proteins abundance in dogs with nonfailing, hypertrophic cardiomyopathy induced by perinephritic hypertension [16]. Recently, in the SHR model, Fujita et al. [23] found no change in immunoblotted caveolin-1 and -3 in the LV at 4 weeks, but a reduction in caveolin-3 at 24 weeks of age, i.e., in animals 6 weeks older than those used in the present study.

These changes would be expected to enhance the activity of the residual eNOS (and of nNOS) in the LV. Because our measurements of NOS activity were performed in a buffer containing an excess of exogenous calcium and calmodulin sufficient to dissociate the eNOS–caveolin and nNOS–caveolin complexes, we cannot correlate directly the changes in caveolin expression with calcium-dependent NOS activity in our assay (Fig. 4A), which mostly reflects the $V_{\text{max}}$ proportional to the abundance of Ca-sensitive NOS in the extracts. However, it remains likely that the decreases in caveolin-1 and -3 (and increase in Hsp90) are sufficient to enhance the ability of eNOS and nNOS to produce NO in the intact tissue in vivo. Indeed, we demonstrated that a 25% reduction in caveolin-1 was sufficient to increase basal and agonist-stimulated eNOS activity in intact endothelial cells [24]. Similarly, because caveolin-3 interacts with eNOS and nNOS and negatively regulates their activity [6], it is
Fig. 6. eNOS and caveolin protein expression in aortic tissue from adults (18 weeks) and aging (63 weeks) WKY and SHR rats. Representative Western blot (top) and densitometric data (bottom) of eNOS (A), caveolin(cav)-1 (B) and caveolin(cav)-3 (C) protein expression from 18-week (left; \( n = 7-10 \)) and 63-week (right; \( n = 3-5 \)) WKY and SHR. Data are presented as percentage of values in age-matched control (WKY) rats and are expressed as mean±S.E.M. *\( P<0.05 \), ***\( P<0.005 \) and ****\( P<0.0005 \) versus age-matched WKY rats.

Conceivable that a loss in caveolin-3 abundance potentiates not only eNOS but also nNOS activation, as reported in skeletal muscle from caveolin-3 deficient transgenic mice [25]. Thus, decreases in both caveolin-1 and -3 (and Hsp90 increase) may lead to hyperactivation of both eNOS and nNOS and elevations of NO production in the intact heart.

To address the functional impact of these expressional changes, we measured tissular contents of cGMP, a major downstream messenger of the NOS pathway. Consistent with previous reports [18,26], cGMP contents of the LV were unchanged across both age and strains of the animals (Fig. 4B). Independent factors (e.g., atrial natriuretic peptide or brain natriuretic peptide) may, however, influence cGMP levels in addition to NO production, including in the present study. Previous reports described an age-dependent increase in cardiac soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase type I (cGKI) expression [27] as well as cardiac cGMP content,
but in much older (17- and 22–25-month-old, respectively) WKY rats than those in our experiments. On the contrary, Kojda et al. [28] reported that NO donors-induced activity of the soluble GC–cGMP system in cardiac muscle is not altered in long-term hypertension. These results, together with our present observations indicate that in hypertrophic or failing heart of SHR, the level of bioactive NO can be partially depressed and insufficient to upregulate guanylate cyclase despite potentially increased NOS function.

Several groups reported an exaggerated production of superoxide anion (O$_2^-$) by the vascular wall in SHR and other models of hypertension [11,12] that could contribute to the pathogenesis and/or maintenance of high blood pressure [29]. Because O$_2^-$ equimolarly reacts with NO to form peroxynitrite and other downstream oxygen reactive species, the increase in O$_2^-$ production may account for the reduction of bioactive NO and the endothelial dysfunction observed in SHR [14,15]. To assess the presence of O$_2^-$ in the heart of adult and aging SHR, we adapted an in situ assay using the fluorescent O$_2^-$ indicator hydroethidine [17,30]. The fluorescence signal was markedly greater in the LV from 63 weeks SHR compared with age-matched WKY rats. In addition, we observed greater O$_2^-$ production in older compared with younger animals of both strains. Our findings in the heart also recapitulate increases in O$_2^-$ production with age and chronic hypertension observed by others in rat aortic rings [31]. Therefore, increased O$_2^-$ production plausibly explains the unchanged cGMP levels despite increased NOS abundance and/or activity in the LV of older SHR. The coordinated expression changes in NOS isoforms and their allosteric regulators, such as caveolin and Hsp90, may even be viewed as a compensatory mechanism to maintain the production of bioactive NO in the face of increased oxidant stress. In addition, since the treatment of endothelial cells with H$_2$O$_2$ (a downstream product of O$_2^-$) increases eNOS expression and activity in vitro [32,33], enhanced O$_2^-$ production may directly contribute to the increase in eNOS abundance in the heart of 63 weeks SHR.

Our comparison of expression changes of eNOS in the heart and aortic or mesenteric vessels between the two rat strains confirms the tissue-dependent regulation of its expression; most notably, eNOS abundance increases in the aorta of 18 weeks SHR, whereas it decreases in the LV of the same animals. The upregulation of aortic eNOS in younger SHR is in keeping with some [22,34], but not all [35], previous studies. Possible reasons for the discrepancy include differences in the age of sacrifice and strains used. Nevertheless, ours is the only study evaluating concurrent changes in caveolin-1, -3 and Hsp90 in the same vessels. Contrary to eNOS, caveolin-1 and -3 abundance was decreased in both LV and aortic tissue from younger and older SHR. Caveolin-1 was also decreased in mesenteric arteries of younger SHR. Together with unchanged levels of Hsp90, a downregulation of these eNOS inhibitors would again contribute to maintain NO production in the face of a well-established increased oxidant stress in the vascular wall of hypertensive animals [15,31]. NOS activity may also be reinforced by the simultaneous upregulation of nNOS in the vascular media, as previously observed by others [3,36]. As in LV extracts, the increased oxidant stress likely explains the unchanged aortic cGMP contents.

In conclusion, our results establish the tissue- and isoform-specific regulation of NOS in the SHR, which, in most cases, contrasts with opposite changes in the abundance of allosteric regulators such as caveolins and Hsp90. Such discordance emphasizes the need to assess parallel regulation of NOS regulators in future studies on cardiovascular diseases to gain further understanding in their functional impact on downstream NO signaling.

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