Sympathectomy or Doxazosin, But Not Propranolol, Blunt Myocardial Interstitial Fibrosis in Pressure-Overload Hypertrophy

Stefano Perlini, Giuseppina Palladini, Ivana Ferrero, Rossana Tozzi, Silvia Fallarini, Angelica Facoetti, Rosanna Nano, Francesca Clari, Giuseppe Busca, Roberto Fogari, Alberto U. Ferrari

Abstract—The adaptive changes that develop in the pressure-overloaded left ventricular (LV) myocardium include cardiomyocyte hypertrophy and interstitial fibrosis. Although the former is known to depend to a sizeable extent on sympathetic (over)activity, little information exists whether the same applies to the latter, ie, whether excess catecholamine exposure contributes to the imbalance between collagen deposition by fibroblasts and degradation by matrix metalloproteases (MMPs), eventually leading to LV collagen accumulation. Sprague-Dawley rats were subjected to abdominal aortic banding (B) or sham operation (S) and treated with β-blockade (Bb, oral propranolol, 40 mg/kg per day), chemical sympathectomy (Sx, 6-hydroxydopamine, 150 mg/kg intraperitoneal twice per week) or vehicle (Vh). Ten weeks later, systolic blood pressure, LV weight, collagen abundance (computer-aided histology), zymographic matrix metalloproteasine (MMP)-2 activity and its specific tissue inhibitor concentration (TIMP-2) were measured. Both sympathectomy and β-blockade failed to attenuate the banding-induced blood pressure elevation but significantly attenuated the attendant LV hypertrophy. As expected, pressure-overload hypertrophy was associated with interstitial fibrosis (collagen: $4.37 \pm 1.23\% \text{ BVh versus } 1.23 \pm 0.44\% \text{ SVh, } P<0.05$), which was abolished by sympathectomy ($2.55 \pm 1.31\%, P=\text{not significant versus SSx}$) but left unchanged by β-blockade ($4.11 \pm 1.23\%, P<0.05$ versus both SBb and BSx). β-blockade, but not sympathectomy, was also associated with an increased TIMP-2/MMP-2 ratio ($P<0.05$), indicating reduced interstitial collagenolytic activity. In separate groups of banded and sham-operated rats, treatment with the α-receptor blocker doxazosin (10 mg/kg per day) displayed similar antifibrotic and biochemical effects as sympathectomy. Thus in the course of experimental pressure overload, the sympathetic nervous system plays a major pro-fibrotic role, which is mediated via α-adrenergic but not β-adrenergic receptors. (Hypertension. 2005;46:1213-1218.)

Key Words: collagen ■ extracellular matrix ■ fibrosis ■ heart failure ■ hypertrophy ■ sympathectomy

There is extensive evidence that sympathetic nerve overactivity consistently accompanies hypertension in its uncomplicated stage and even more so after the progressive development of left ventricular (LV) hypertrophy, dysfunction, and failure. This has deleterious consequences on the severity of hypertension itself, because it produces peripheral vasoconstriction, as well as on the heart, because: (1) it favors activation of other potentially cardiotoxic neurohumoral systems (renin-angiotensin system, endothelin, etc)3,4; (2) it increases myocardial oxygen consumption; and (3) it directly exerts toxic (pro-apoptotic, pro-necrotic) effects on the myocardium.5–7 Accordingly, it is now firmly established that administration of β-adrenergic receptor blockers favorably affects hypertension and heart failure,8 most likely via correction of the aforementioned pathophysiological alterations.

However, although it is well documented that β-blockers attenuate the inappopriate increase in neurohumoral activity and in myocardial oxygen consumption, thus protecting the cardiomyocyte, much more limited and controversial information exists about the effects of β-blockers on the cardiac interstitium; however, some reports suggest that these drugs inhibit interstitial fibrosis,9,10 this is not confirmed by other studies. In recent experiments designed to address the mechanisms underlying the antihypertrophic effect of propranolol in pressure-overloaded rats, it has been incidentally observed that while effectively inhibiting cardiomyocyte enlargement, this agent fails to oppose excess collagen deposition.11 Similar findings were obtained in pressure-overloaded mice, in which propranolol prevented development of LV hypertrophy and progression to LV dysfunction but failed to inhibit the attendant myocardial fibrosis.12 A first goal of the present study was therefore to reassess and more in-depth characterize the effects of β-blockers on the cardiac structural alterations associated with chronic hypertension.
pressure overload. A second goal was to test the hypothesis that an anti-sympathetic intervention able to more extensively interfere with sympathetic influences than β-receptor blockade alone, namely chronic chemical sympathectomy, may effectively inhibit the development of LV hypertrophy and dysfunction, as well as of LV interstitial fibrosis. These issues were addressed in rats with aortic banding-induced pressure overload and chronic treatment with either propranolol or 6-hydroxydopamine, in which both computer-aided histological quantification of fibrosis and biochemical assessment of myocardial collagenolytic processes were performed. The results disclosed that the latter but not the former anti-sympathetic intervention very effectively blunts cardiac fibrosis. Considering these results, a third goal of the study arose, namely to gain mechanistic insight into the differential antifibrotic effects of sympathectomy versus β-blockade: because the more likely hypothesis was that in the banding rat model a profibrotic action is exerted via α-receptors, we additionally studied separate groups of banded rats treated with the α-receptor blocker doxazosin.

Methods

Animal Preparation and Surgery
The experiments were performed on 8-week-old Sprague-Dawley male outbred rats, weighing 200 grams (Charles River, Calco, Italy). All procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with the international policies according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85 to 23, 1996).

Left ventricular pressure-overload hypertrophy was induced by subjecting the animals to abdominal aortic banding. Surgery was performed 24 to 72 hours after arrival. Rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). The suprarenal portion of the aorta was exposed and a ligature (5-0 silk) was snugly tied around both the aorta and the needle. The needle was then removed, leaving the internal diameter of the aorta approximately equal to that of the needle. Sham-operated animals had an untied ligature placed in the same location. The animals were housed under controlled environmental conditions, with food (Purina

Collagen Evaluation
Collagen evaluation was performed on frozen formalin fixed sections of the left ventricle. Sections were stained with Masson’s trichrome stain and with Sirius red.16 For each animal, the stained area was quantified by averaging 9 random high-power fields from the analyzed tissue. Interstitial collagen quantification was performed with a computer-based quantitative color image analysis system (Image-Pro-Plus).37

MMP Zymography
The myocardial extracts were normalized by a final protein concentration of 400 µg/mL using sample loading buffer (0.25 mol/L Tris-HCl, 4% sucrose w/vol, 10% SDS w/vol and 0.1% bromphenol blue w/vol, pH 6.8). The samples after dilution were loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin, under nonreducing conditions.14–15 The gels were run at 15 mA/gel trough the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), at the temperature of 4°C in a running buffer. After the SDS-PAGE the gels were washed twice in 2.5% Triton X-100 for 30 minutes each to allow proteins to renature, rinsed in water, and incubated for 18 hours in an incubation buffer at 37°C (50 mmol/L Tris-HCl, CaCl2 5 mmol/L, NaN3 0.02% w/vol, pH 8). After incubation, the gels were stained using Coomassie blue R-250 for 30 minutes, destained for 1 hour with the change of the solution after 30 minutes, to reveal zones of lysis; the gels were then dried and analyzed. Positive control for SDS-PAGE zymography was included in all zymograms. The zymograms were analyzed by densitometer (GS 710 Densitometer BioRad) and data were expressed as optical density normalized to total protein content.

MMP Imunoblotting
The same samples as used for zymography were also used for immunoblotting analyses to validate the zymography. The samples were normalized by a final protein concentration of 400 µg/mL using sample buffer (pH 6.8). The samples after dilution were loaded onto an 8% SDS-polyacrylamide gel and separated at 40 mA under reducing conditions. The separated proteins were electroblotted at 360mA onto a nitrocellulose membrane in a transfer medium (pH 8.2). Membranes were blocked with a 50 mmol/L Tris HCl buffer pH 7.4 containing 5% powdered goat milk for 1 hour at room temperature to block nonspecific binding, and then membranes were incubated overnight at 4°C with specific monoclonal antibodies to MMP-2 (1 µg/mL) diluted in 50 mmol/L Tris HCl buffer, pH 7.4 containing 1% powdered goat milk. After washing, the membranes were incubated for 1 hour in horseradish peroxidase conjugated secondary goat anti-mouse antibody (1:5000 dilution). The reactions were developed with Amplified Opti-4 CN Detection Kit (BioRad). Positive controls for MMP-2 were included in all immunoblotting.

TIMPs
The supernatants of the myocardial extracts were assayed for TIMP-2 content with a commercially available ELISA kit (Biotrak; Amersham Pharmacia Biotech), able to detect total TIMP-2, ie, both free and complexed with metalloproteinases. The concentration of total MMPs in samples was determined by interpolation from a standard curve and expressed in mg/mL reported to mg/mL of proteins of each sample. TIMP-2/MMP-2 ratio was computed as a crude index of the tissue inhibition of MMP activity, ie, of extracellular matrix lytic activity.
Effects of Sympathectomy, \(\beta\)-Blockade, and \(\alpha\)-Blockade on Hemodynamic and Biochemical Variables in Sham-Operated and Aortic-Banded Rats

<table>
<thead>
<tr>
<th>Hemodynamic and Biochemical Parameters</th>
<th>SVh (n=9)</th>
<th>BVh (n=10)</th>
<th>SSx (n=10)</th>
<th>BSx (n=15)</th>
<th>SbB (n=10)</th>
<th>BBb (n=11)</th>
<th>SAb (n=8)</th>
<th>BAB (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>102±12</td>
<td>154±19*</td>
<td>96±15</td>
<td>159±18*</td>
<td>100±15</td>
<td>150±12*</td>
<td>103±12</td>
<td>152±13*</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>258±16</td>
<td>252±14</td>
<td>231±20</td>
<td>238±21</td>
<td>238±14</td>
<td>235±16</td>
<td>257±13</td>
<td>250±12</td>
</tr>
<tr>
<td>MMP-2 activity (ng/mL per mg protein)</td>
<td>0.35±0.05</td>
<td>0.42±0.04</td>
<td>0.38±0.03</td>
<td>0.48±0.04</td>
<td>0.19±0.02</td>
<td>0.30±0.04</td>
<td>0.51±0.07</td>
<td>0.78±0.07†</td>
</tr>
<tr>
<td>TIMP concentration (ng/mL per mg protein)</td>
<td>1.67±0.15</td>
<td>1.79±0.11</td>
<td>1.54±0.09</td>
<td>1.81±0.09*</td>
<td>1.53±0.06</td>
<td>1.89±0.09*</td>
<td>1.56±0.13</td>
<td>2.04±0.13*</td>
</tr>
</tbody>
</table>

\(P<0.05\) vs. respective sham-operated control. 
†\(P<0.05\) vs. respective vehicle-treated control.

Statistical Analysis
ANOVA for repeated measurements followed by either the Dunnnett or Student-Newman-Keuls test was used to determine statistical significance within each experimental group, and factorial analysis of variance was used for comparisons between the different interventions. Student t test was applied to paired comparisons. All data are expressed as means±SEM. \(P<0.05\) was used to indicate statistical significance. All statistical procedures were performed using the STATVIEW statistical software package.

Results
Blood Pressure, LV Hypertrophy, and Pulmonary Congestion
As expected, abdominal aortic banding was associated with a significant increase in systolic blood pressure, left ventricular weight index and lung weight index, indicating the development of pressure-overload hypertrophy and of its progression to left ventricular failure. These data are shown in the Table and Figure 1. As also shown in the Table, neither sympathectomy nor \(\alpha\)- or \(\beta\)-blockade attenuated the degree of hypertension. However, the adaptive left ventricular hypertrophic response was blunted in sympathectomized as well as in propranolol- or doxazosin-treated animals (Figure 1). Moreover, none of the active treatments was associated with an increase in lung weight index, indicating that each one was able to prevent the development of heart failure (Figure 1).

Collagen Content, MMP Activity, and TIMP Concentration
Pressure-overload hypertrophy was associated with left ventricular fibrosis, as shown by a marked increase in cardiac collagen fraction (examples in Figure 2A). Chronic \(\beta\)-blockade did not affect the extent of interstitial fibrosis (Figure 2, panel C). In contrast, no collagen accumulation was observed in sympathectomized and doxazosin-treated pressure overloaded animals whose cardiac collagen fraction was consistent in sham-operated controls (Figure 2B and 2D). The results of computer-aided interstitial collagen quantification are shown in the bar graphs of Figure 3. Perivascular collagen fraction and the ratio of epicardial versus endocardial collagen accumulation showed similar trends in the different experimental groups (data not shown).

Regarding extracellular matrix biochemistry (data shown in Table), aortic banding was associated with no significant change in MMP-2 activity as well as in TIMP-2 concentration in vehicle-treated animals; \(\beta\)-blockade did per se significantly reduce MMP-2 activity in sham-operated animals, with no further change associated with aortic banding; it was also associated with a significant increase in TIMP-2 concentra-

in aortic-banded rats. In contrast, sympathectomized banded rats displayed no change in MMP-2 activity with increased TIMP-2 tissue concentration. At variance with \(\beta\)-blockade, doxazosin did per se increase MMP-2 activity in sham-operated animals with a further increase in banded rats.

The differential effects of sympathectomy and \(\alpha\)-blockade versus \(\beta\)-blockade on extracellular matrix biochemistry is even better documented by the opposite trends of TIMP2/ MMP2 ratio associated with these interventions: as shown in the bottom panels of Figure 3, this ratio was unchanged with...
sympathectomy, whereas it was increased by \textit{\beta}-blockade. With \textit{\alpha}-blockade, the increased MMP activity was paralleled by an increased TIMP concentration so that their ratio was not significantly reduced in the banded compared with their sham-operated counterparts, quite similarly to the pattern observed in sympathectomized animals.

**Discussion**

Our study strengthens and extends the notion that early interference with sympathetic overactivity has beneficial effects on the development of LV hypertrophy/dysfunction/failure in aortic banded rats, and shows for the first time that different antiadrenergic interventions have differential effects on the pressure-overload associated interstitial fibrosis, which was almost completely abrogated in sympathectomized rats but totally unaffected in rats with \textit{\beta}-blockade. This was unlikely to reflect differences in the prevailing hemodynamic load or functional conditions, because arterial blood pressure, left ventricular weight and the severity of lung congestion (as reflected by lung weight index) were virtually identical in the two groups, and is thus to be ascribed to a different influence of the two interventions on the mechanisms controlling extracellular matrix turnover.\(^\text{18}\)

To our best knowledge, there has been no previous demonstration that an antiadrenergic intervention can shift the balance of extracellular matrix turnover toward a markedly enhanced collagenolytic activity, and to do so to such a large extent to completely offset the powerful stimulus to collagen deposition represented by pressure overload. Cardiac collagen synthesis may be influenced, among many other factors, by the activity of the adrenergic nervous system, and fibrosis is a well recognized feature of experimental isoproterenol-induced cardiac injury.\(^\text{10}\) Intriguingly, however, it has been reported that the \textit{\beta}-blocker atenolol promotes spontaneous myocardial fibrosis in Sprague-Dawley rats.\(^\text{19}\) In contrast, \textit{\alpha}-blockade with doxazosin depressed aortic collagen synthesis in SHR rats.\(^\text{20}\)

Although our study was not designed to exhaustively address the mechanism underlying the differential effect of sympathectomy and \textit{\beta}-blockade on interstitial fibrosis, the results of the doxazosin experiments demonstrate that \textit{\alpha}-adrenoceptors are involved in extracellular matrix degradation, namely they oppose this process. Not necessarily alternative to this, it is also to be considered that having...
shown the prominent profibrotic role of α-adrenoceptors makes it less likely, but cannot rule out, the possibility of a contribution to fibrosis by catecholamine-mediated activation of those β-adrenergic pathways known to be resistant to propranolol (a drug we selected for the present study as the prototype of the β-blockers used in the clinical setting rather than as a sophisticated pharmacological tool), namely those operated through the β1-adrenoceptor or the so-called β2-adrenoceptor (later and more properly referred to as the propranolol-insensitive state of the β1-adrenoceptor).21,22 It may also be that other synaptic mechanisms (which would be disrupted by sympathectomy and not by β-blockade) play an inhibitory role on collagenolytic processes. These mechanisms may relate to adrenergic cotransmitters such as neuropeptide Y, ATP, or others. A further possible explanation would simply be that sympathectomy more strongly interferes with β-adrenoceptor-mediated effects than β-blockade, although we believe this not to be very likely because in preliminary, model setup experiments we showed 100% blockade of isoproterenol-induced tachycardia with propranolol at the doses used in the present study and not >90% suppression of tyramine-induced tachycardia in sympathectomized rats23,24; moreover, other inhibitory effects (eg, the antihypertrophic effect) of the two interventions were quantitatively similar; finally, there was not even a tendency for propranolol to attenuate cardiac fibrosis, a feature that supports a qualitative rather than quantitative difference between the effects of β-blockade versus sympathectomy with regard to extracellular matrix turnover.

In perspective, these findings may have a twofold clinical implication. First, they may raise concern on the long-term cardioprotective effects of β-blockade for the treatment of hypertension and heart failure, although (needless to say) these experimental animal observations do not necessarily apply to humans nor to the effects of β-blockers other than propranolol. It is nonetheless interesting to mention very recent evidence in hypertrophic hypertensive patients in whom ultrasound indices of cardiac fibrosis were ameliorated by long-term losartan but not atenolol-based treatment.25 Second, one may speculate about a possible antifibrotic effect of α-blockers in cardiovascular therapy, although, again, ad hoc human studies will be needed to confirm or disprove this possibility.

Our study also has limitations. First, the observed pharmacological effects deal with inhibition of development and should not be arbitrarily extrapolated to possible reversal effects on left ventricular hypertrophy, dysfunction, and interstitial fibrosis. They, however, have the advantage of showing the beneficial effects of an antiadrenergic intervention since the very onset of hypertensive heart disease. Second, the observation period was only 10 weeks so that we are at present unable to establish whether the protective effects of sympathectomy and α-blockade will be maintained in the long term. Third, the analysis of the observed interstitial effects have so far not included measurements of other (neuro-)humoral factors such as angiotensin, endothelin, and vasopressin. This information will clarify whether the interstitial phenomena observed in the present experiments directly depend on modulation of sympathetic activity rather than being mediated via the recruitment of other systems known to influence extracellular matrix biology.

In conclusion, the present study shows that: (1) the adaptive sympathetic activation associated with pressure overload by no means exerts cardioprotective effects and indeed promotes unfavorable changes such as development of cardiomyocyte hypertrophy, interstitial fibrosis and evolution toward heart failure; and (2) conversely, interfering with sympathetic activity during the course of experimental hypertensive heart disease is markedly beneficial to LV function, structure, and interstitial biochemistry; however, although β-blockade by propranolol can only oppose cardiomyocyte hypertrophy, the favorable interstitial effects are selectively exerted by sympathectomy and doxazosin, ie, by interventions that inhibit the function of α-receptors; thus, the latter are shown to significantly contribute to the pathophysiology of cardiovascular damage in this model.

References
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