Ventricular Hypertrophy Plus Neurohumoral Activation Is Necessary to Alter the Cardiac β-Adrenoceptor System in Experimental Heart Failure

Kirsten Leineweber, Katja Brandt, Beate Wludyka, Anja Beilfuß, Klaus Pönicke, Ingrid Heinroth-Hoffmann, Otto-Erich Brodde

Abstract—Treatment of rats with monocrotaline (MCT) leads to pulmonary hypertension, right ventricular (RV) hypertrophy, and finally to RV heart failure. This is associated with characteristic changes in right ventricular β-adrenoceptors (β-AR), neuronal noradrenaline transporter (NAT) density and activity (uptake₁), and G protein–coupled receptor kinase (GRK) activity. This study aimed to find out factors that determine β-AR, uptake₁, and GRK changes. Thus, 6-week-old rats were treated with 50 mg/kg MCT subcutaneous or 0.9% saline. Within 13 to 19 days after MCT application (group A), RV weight (222±6 versus 147±5 mg) and RV/left ventricular (LV) weight ratio (0.42±0.01 versus 0.29±0.01) were significantly increased, whereas plasma noradrenaline, RV β-AR density, RV NAT density and activity, and RV GRK activity were not significantly altered. Twenty-one to twenty-eight days after MCT (group B), however, not only RV weight (316±4 versus 148±2 mg) and RV/LV weight ratio (0.61±0.01 versus 0.3±0.01) were markedly increased but also plasma noradrenaline (645±3 versus 278±18 pg/mL); now, RV β-AR density (13.4±1.3 versus 26.5±1.1 fmol/mg protein), RV NAT density (50.9±11.3 versus 79.6±2.9 fmol/mg protein), and RV NAT activity (65.4±7.4 versus 111.8±15.9 pmol [3H]-NA/mg tissue slices/15 min) were significantly decreased and RV-membrane GRK activity (100±15 versus 67±6 [32P]-rhodopsin in cpm) significantly increased. LV parameters of MCT-treated rats were only marginally different from control LV. We conclude that in MCT-treated rats ventricular hypertrophy per se is not sufficient to cause characteristic alterations in the myocardial β-AR system often seen in heart failure; only if ventricular hypertrophy is associated with neurohumoral activation β-ARs are downregulated and GRK activity is increased. (Circ. Res. 2002;91:1056-1062.)

Key Words: monocrotaline • right heart hypertrophy • neurohumoral activation • β-adrenoceptor • G protein–coupled receptor kinase

In human heart failure, there are characteristic alterations in the cardiac β-adrenoceptor system: selective downregulation of β-adrenoceptors with little or no change in β-adrenoceptors, reduced adenylyl cyclase activity, increased G protein–coupled receptor kinases (GRKs) activity, and reduced neuronal noradrenaline transporter.1–6 We and others have recently shown that the monocrotaline rat model of right heart failure exhibits alterations in myocardial β-adrenoceptors that resemble those in human heart failure: a decrease in β-adrenoceptors, a reduction in adenylyl cyclase, an increase in GRK, and a reduction in neuronal noradrenaline transporter.7–10 These changes appear to occur predominately in right ventricles, and are therefore, well comparable with those observed in patients with primary pulmonary hypertension.11

Data from the literature and our own data show that a single injection of the pyrrolizidine alkaloid monocrotaline induces in rats within 14 days after application severe pulmonary hypertension, resulting first in development of right ventricular hypertrophy due to apparent pressure overload and then in right heart insufficiency accompanied by an increase in sympathetic nervous system activity.7–8,10,12–14

Thus, the monocrotaline rat model allows to investigate whether pressure overload evoked hypertrophy per se can induce the characteristic alterations in the cardiac β-adrenoceptor system seen in chronic heart failure, or whether those changes can only occur when hypertrophy is accompanied by neurohumoral activation. In order to study this, we assessed in monocrotaline-treated rats, β-adrenoceptor density and subtype distribution, G protein–coupled receptor kinase activity, neuronal noradrenaline transporter density and activity and plasma noradrenaline levels at two time-points after monocrotaline-treatment: one group (within 13 to 19 days after application) that had
developed right ventricular hypertrophy but did not show signs of neurohumoral activation (no increase in plasma noradrenaline) and one group (within 21 to 28 days after application) that showed right ventricular hypertrophy and increased plasma noradrenaline levels (as index of neurohumoral activation).

Materials and Methods

Animals
All animal experiments were performed according to the German laws for animal welfare and were approved by the local committee for animal studies. Six-week-old male Wistar rats (outbred stock Strain Wistar Hannover) purchased from Schönwalde, Germany) were housed in groups of 3 per cage in a room for animal studies. Six-week-old male Wistar rats (outbred stock strain Wistar Hannover, Germany) were added in a final concentration of 1 μmol/L per 1 mL plasma. Samples were centrifuged with 600 g for 5 minutes at 4°C, the plasma was removed, quickly frozen in liquid nitrogen, and stored at −80°C until further use. Plasma noradrenaline content was assessed by high-pressure liquid chromatography (HPLC) by fluorimetric detection.

[3H]-Noradrenaline Transporter Activity (Uptake)
[3H]-Noradrenaline uptake, was assessed by incubating right and left ventricular tissue slices (0.25×0.25 mm) excluding intraventricular septum taken from saline- and MCT-treated rats at 37°C for 15 minutes with 1.56, 3.125, 6.25, 12.5, and 25 nmol/L [3H]-noradrenaline in the presence of 40 μmol/L corticosterone (specific uptake, antagonist) as recently described. Non-specific accumulation of radioactivity was determined by parallel incubation at 4°C. Specific uptake was defined as total uptake (37°C) minus nonspecific uptake (4°C).

Plasma Noradrenaline Content
For determination of plasma noradrenaline content, blood was drawn from anesthetized rats before excision of the heart by puncture of the ophthalmic venous plexus and collected in a potassium-EDTA S-Monovette (Sarstedt, Germany), glutathione was added in a final concentration of 1 μmol/L per 1 mL plasma. Samples were centrifuged with 600 g for 5 minutes at 4°C, the plasma was removed, quickly frozen in liquid nitrogen, and stored at −80°C until further use. Plasma noradrenaline content was assessed by high-pressure liquid chromatography (HPLC) by fluorimetric detection.

Noradrenaline Transporter Density
Preparation of crude membranes obtained from right and left ventricles excluding intraventricular septum of saline- and MCT-treated rats was performed as recently described. Protein content was determined according to Bradford using γ-globulin as standard.

[3H]-Nisoxetine saturation analysis was performed by incubating 100 μg protein per assay with 6 concentrations of [3H]-nispoxetine ranging from 0.3125 to 10 nmol/L for 3 hours at 4°C as recently described. Non-specific binding of [3H]-nispoxetine was defined as binding in the presence of 1 μmol/L desipramine. Specific binding was defined as total binding minus non-specific binding and amounted to approximately 85% at 2 nmol/L [3H]-nisoxetine.

β-Adrenoceptor Density
Preparation of crude membranes obtained from right and left ventricles (without intraventricular septum) of saline- and MCT-treated rats was performed as recently described. The density of β-adrenoceptors in right and left ventricular crude membranes was determined by (−)-[125I]-iodocyanopindolol (ICYP) binding at 6 concentrations of ICYP ranging from 5 to 200 pmol/L as recently described. Non-specific binding of ICYP was defined as binding to membranes, which was not displaced by a high concentration of the nonselective β-adrenoceptor antagonist (±)-CGP 12177 (125 μmol/L). Specific binding of ICYP was defined as total binding minus nonspecific binding (usually 70% to 80% at 50 pmol/L of ICYP).

Determination of the Relative Amount of β1- and β2-Adrenoceptors in Cardiac Crude Membranes
To determine the relative amount of β1- and β2-adrenoceptors in cardiac crude membrane preparations, the membranes were incubated with 100 pmol/L ICYP in the presence or absence of 6 concentrations of the highly selective β1-adrenoceptor antagonist CGP 20712A ranging from 1 nmol/L to 100 μmol/L and specific binding was determined as described above.

Determination of GRK Activity
Preparation of the cytosolic and membranous GRK was performed as recently described. Protein concentration was determined as described above and enzymatic activity of GRK in the cytosolic and membranous fraction was determined by incubating 20 μg cytosolic and 10 μg membrane protein in duplicates in the dark at 4°C with 200 pmol rhodopsin (prepared from bovine retina), 10 mmol/L MgCl2, 100 μmol/L ATP, 230 pmol βγ-subunits, and γ-[32P]-ATP (20 μCi = 0.08 nmol/L) in a total volume of 60 μL. Light-dependent phosphorylation of rhodopsin was initiated by incubating the sam-

Figure 1. Plasma noradrenaline contents, expressed in pg noradrenaline per mL, in saline- and MCT-treated rats in Group A (13 to 19 days, n = 10/9) and in Group B (21 to 28 days after application, n = 12/12). Values are given as mean ± SEM of [n/n] experiments with ***P < 0.001 vs age-matched saline-treated rats.
iodocyanopindolol (ICYP, specific activity 220 Ci/mmol), and heparin (specific GRK activity inhibitor).

GRK in the cytosolic and membranous fraction prepared from left determined their radioactivity. To verify the phosphorylation of rhodopsin bands (35 kDa) were cut of the gel and Cerenkov counting [3 H]-nisoxetine and ICYP binding were calculated by nonlinear regression analysis using the iterative curve fitting program Prism (Graph-Pad Software). The reaction was terminated with RT and the samples were electrophoresed on 10% SDS-polyacrylamide gels, which were stained with Coomassie brilliant blue according to Reissner et al.


d of 250 µL ice-cold lysis buffer. The samples were immediately centrifuged for 10 minutes at 10 000g at RT and the supernatants were discarded. The pellets were resuspended in 30 µL twice-concentrated Laemmli buffer by vigorous shaking for 30 minutes at 30°C. The samples were electrophoresed on 10% SDS-polyacrylamide gels, which were stained with Coomassie brilliant blue according to Reissner et al.


t by the iterative curve fitting program Prism (Graph-Pad Software) and statistically analyzed using the F-ratio test to measure the goodness of fit of the competition curves for either one or two sites. The significant of differences was estimated by nonpaired two-tailed Student’s t test. All statistical calculations were performed with the Prism program (Graph-Pad Software). A value of P<0.05 was considered to be significant.

Drugs

doI-[-7H(N)]-noradrenaline hydrochloride (13.5 Ci/mmol), [N-methyl-1H]-nisoxetine hydrochloride (80 Ci/mmol), [3H]-iodocyanopindolol (ICYP, specific activity 2200 Ci/mmol), and γ-[32P]-ATP were purchased from Perkin-Elmer Life Sciences (Köln, Germany). Crotaline, (−)-noradrenaline bitartrate, and corticosterone (4-pregene-11β,21-diol-3,20-dione) were purchased from Sigma, Germany. CGP 20712A (1-(1-2-(3-carbamoyl-4-hydroxyphenoxo)-ethyl-amino)-3-(1-methyl-4-trifluoro-methyl-2imidazolylphenoxo)-2-propanol methanesulphonate) and (±)-CGP 12177 hydrochloride (4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-0n) were kindly donated by Ciba-Geigy (Basel, Switzerland). Purified bovine rhodopsin and the βγ-subunit for the GRK assay were kindly provided by Prof Dr M.J. Lohse (University of Würzburg, Germany). All other chemicals were of the highest purity grade commercially available.

Results

Weights of Organs

Effects of MCT treatment on body and organ weights in Group A (13 to 19 days after MCT application) and Group B (21 to 28 days after MCT application) are shown in Table 1. Over the whole treatment period body weight gain was not significantly different between MCT-treated and saline-treated rats. On the other hand, MCT-treated rats exhibited a marked enlargement of the lungs and a significant increase in heart and liver weight compared with saline-treated rats already 13 to 19 days after MCT application (Group A); this remained increased during the following 21 to 28 days (Group B), whereas kidney weight was not all affected.

A more detailed analysis of heart weight revealed that right ventricular weight of MCT-treated rats was about 1.5-fold in Group A and about 2-fold in Group B compared with saline-treated rats. On the other hand, left ventricular weight was not at all affected by the MCT treatment. Accordingly, the RV/body weight ratio, as an index of right ventricular hypertrophy, was increased in Group A, although this just failed to reach statistical significance (P=0.077) and was significantly increased in MCT-treated rats in Group B compared with age-matched controls. The RV/LVS ratio, as an body weight–independent index of right ventricular hypertrophy, was already significantly increased by about 1.4-fold in MCT-treated rats in Group A and further increased by about 2-fold in Group B compared with age-matched saline-treated rats.

Plasma Noradrenaline Content

In Group A, no alteration in plasma noradrenaline content could be detected in MCT-treated rats compared with controls (Figure 1). However, in Group B, noradrenaline content increased by about 2-fold in MCT-treated rats compared with age-matched controls.

Noradrenaline Transporter Density

In Group A, right and left ventricular noradrenaline transporter density was not significantly altered in MCT-treated rats versus controls (Figure 2). However, in MCT-treated rats of Group B, right ventricular noradrenaline transporter den-
cardiac noradrenaline uptake 1 was significantly decreased by about 42% in right ventricular membranes in both groups (Figures 3A and 3B). In Group A, total noradrenaline uptake 1 into right and left ventricular membranes was not significantly altered in comparison to saline-treated rats (Figure 3A). However, in MCT-treated rats, a significant shift in ison to controls (Figure 4). In Group B, total β-adrenoceptor density was significantly reduced by about 50% in right ventricular membranes in both groups (see legend of Figure 4).

**Cardiac β-Adrenoceptors**

In Group A, total β-adrenoceptor density in right and left ventricles of MCT-treated rats was not changed in comparison to controls (Figure 4). However, with the development of right ventricular hypertrophy (Group A) a significant shift in the right ventricular β₁:β₂-adrenoceptor ratio from 73:27% in control to 55:45% in MCT-treated rats with right ventricular hypertrophy was observed (Table 2); under these conditions right ventricular β₁-adrenoceptor density was decreased from 19.6±1.2 to 12.9±0.9 fmol/mg protein (P<0.05), whereas β₂-adrenoceptor density increased from 7.5±0.8 to 10.4±1.0 fmol/mg protein (P<0.05). Left ventricular β₁:β₂-adrenoceptor ratio, however, was not changed.

In Group B, total β-adrenoceptor density was significantly reduced by about 50% in right ventricles of MCT-treated rats compared with age-matched controls (Figure 4), and β₁:β₂-adrenoceptor ratio remained 54:46%, resulting in a significant reduction in right ventricular β₁-adrenoceptor density from 19.8±1.1 to 6.9±0.9 fmol/mg protein (P<0.05), whereas β₂-adrenoceptor density was unchanged (6.6±0.8 versus 6.8±0.9 fmol/mg protein). In addition, in Group B, left ventricular total β-adrenoceptor density was slightly but significantly reduced by about 23% in MCT-treated rats in comparison to saline-treated rats (Figure 4); left ventricular β₁:β₂-adrenoceptor ratio was slightly but not significantly shifted from 72:28% in controls to 66:38% in MCT-treated rats (P=0.07; Table 2), resulting in a significant reduction in β₁-adrenoceptor density from 20.7±1.2 to 14.7±0.7 fmol/mg protein (P<0.05), whereas β₂-adrenoceptor density (8.1±0.6 versus 7.6±0.8 fmol/mg protein) was unchanged. K<sub>D</sub>-values for ICYP, however, were not altered in right and left ventricular membranes in both groups (see legend of Figure 4).

**Cytosolic and Membrane GRK Activity**

As demonstrated in Figure 5A, incubation of rhodopsin with the cytosolic and membranous fraction obtained from left ventricles of saline-treated rats in the absence of light resulted in a marked reduction in phosphorylated rhodopsin. Moreover, 1 mmol/L heparin caused an almost identical reduction in light-dependent phosphorylation of rhodopsin (Figure 5A). The results of assessment of GRK activity in right and left ventricles obtained from saline- and MCT-treated rats of both groups are shown in Figure 5B. Right and left ventricular...
cytosolic as well as membrane-associated GRK activity was not altered in MCT-treated rats in Group A in comparison to age-matched controls (Figure 5B). However, in MCT-treated rats in Group B right ventricular GRK activity was significantly increased in the cytosolic fraction by about 28% (Figure 5B) and in the membrane fraction by about 34% (Figure 5B) compared with saline-treated rats. On the other hand, left ventricular GRK activity was not at all affected (Figure 5B).

Discussion

The main findings of this study was that within 13 to 19 days after MCT application (Group A), right ventricular hypertrophy had developed without significant changes in right ventricular β-adrenoceptor density, noradrenaline uptake, (sites and activity), and GRK activity, whereas at later time points (21 to 28 days after MCT application, Group B) where right ventricular hypertrophy was accompanied by neurohumoral activation (indicated by increased plasma noradrenaline levels), the β-adrenoceptor density and noradrenaline uptake, (sites and activity) were decreased while GRK activity was increased.

Within 13 to 19 days after MCT application, right ventricular hypertrophy had developed as indicated by increased right ventricular weight and RV/body weight and RV/LVS ratio obviously due to local pressure overload induced by the MCT treatment (Group A). Correspondingly, Yoshi et al observed at this time-point a significant increase in right ventricular systolic pressure in MCT-treated rats. On the other hand, within this time range neither marked changes in total β-adrenoceptor density or noradrenaline uptake, (sites and activity) nor GRK activity could be observed. However, we found a significant shift in the right ventricular β1:β2-adrenoceptor ratio toward β2-adrenoceptors within this time range, but no alterations in left ventricular β1:β2-adrenoceptor ratio. These results indicate that there might be a chamber-specific decrease in β2-adrenoceptor density accompanied by an increase in β1-adrenoceptor density in right ventricles within this time range. The reason for this right ventricular β-adrenoceptor subtype redistribution is not known; however, it should be considered that we have assessed β-adrenoceptor density and subtype distribution in crude membranes prepared from whole right ventricles. These membranes are composed of not only cardiomyocytes (that contain 75% β-adrenoceptors and about 25% β2-adrenoceptors) but also of other cell-types like endothelial cells and fibroblasts that contain predominately β1-adrenoceptors.22–24 Thus, it might be possible that in right ventricular hypertrophy there is an increased number of fibroblasts that results in an increased amount of β2-adrenoceptors and in a relative decrease in cardiomyocytes with the consequence of a reduced amount of β1-adrenoceptors.

Taken together, these data clearly indicate that hypertrophy per se is not sufficient to induce the characteristic alterations in the myocardial β-adrenoceptor system often observed in human heart failure, ie, selective downregulation of β1-adrenoceptors with little or no change in β2-adrenoceptors, reduced adenylyl cyclase activity, reduced noradrenaline uptake, sites, and increased G protein–coupled receptor kinases (GRKs) activity.1–6 However, these data are in agreement with Choi et al,23 who demonstrated, in mice, that even overexpression of the cardiac-targeted oncogenic ras (known to develop severe cardiac hypertrophy in the absence of hemodynamic overload) did not alter the β-adrenoceptor system and GRK2 activity although the left ventricle to body weight ratio in these transgenic mice was increased by about 62%.

However, 21 to 28 days after MCT application, right ventricular hypertrophy had further increased, and this was now accompanied by increased plasma noradrenaline levels.

**TABLE 2.** Alterations in Cardiac β1:β2-Adrenoceptor Ratio Due to the Development of Right Ventricular Hypertrophy Within 13 to 28 Days After MCT Treatment

<table>
<thead>
<tr>
<th></th>
<th>Saline vs MCT</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV β1:β2-AR ratio, %</td>
<td>73±1.27±1.6 vs 55±3.45±3.7***</td>
<td>75±1.42±1.45 vs 54±3.46±3.6***</td>
<td>75±1.42±1.45 vs 54±3.46±3.6***</td>
</tr>
<tr>
<td>LV β1:β2-AR ratio, %</td>
<td>73±1.42±1.4 vs 74±1.42±1.47</td>
<td>72±2.22±2.2 vs 66±3.34±3.86</td>
<td>72±2.22±2.2 vs 66±3.34±3.86</td>
</tr>
</tbody>
</table>

Group A (13 to 19 days) and Group B (21 to 28 days) after saline or MCT application. RV indicates right ventricle; LV, left ventricle; AR, adrenoceptor.

Values are given as mean±SEM of [n] experiments with ***P<0.001 vs age-matched saline-treated rats.
An increase in plasma noradrenaline levels is generally taken as an index of increased sympathetic activity. Moreover, Ceconi et al. had previously shown that in rats 4 weeks after MCT application, severe right ventricular hypertrophy was accompanied by a markedly decreased amount of right ventricular noradrenaline content. However, an increase in plasma noradrenaline levels and a decrease in ventricular noradrenaline content is a classical pattern in chronic heart failure and indicates increased sympathetic drive to the heart. Under these conditions of right ventricular hypertrophy plus neurohumoral activation, right ventricular β-adrenoceptor density was decreased, whereby this decrease was predominately due to a reduction in β1-adrenoceptors. Moreover, noradrenaline uptake, sites and activity was decreased, and GRK activity was increased. There was in addition a small decrease in left ventricular β-adrenoceptor density (again predominately due to a decrease in β1-adrenoceptors); however, left ventricular noradrenaline uptake, and GRK activity were not at all altered. It is interesting to note, however, that β-adrenoceptor alterations were markedly more pronounced in right versus left ventricles. These results can be taken as an indication that only the combination of increased sympathetic drive and decreased noradrenaline reuptake activity is capable of increasing noradrenaline in the synaptic cleft to concentrations that downregulate the β-adrenoceptor and simultaneously lead to increases in GRK activity. Moreover, studies have demonstrated a statistically significant negative correlation between noradrenaline reuptake activity and/or interstitial noradrenaline concentration and β-adrenoceptor density. However, it should be noted that in Group B (rats 21 to 28 days after MCT application) right ventricular hypertrophy was significantly more pronounced as in Group A rats (cf, Table 1). Thus, we cannot completely exclude the possibility that the progression in hypertrophy might have (at least partly) contributed to the alterations in right ventricular GRK activity.

In conclusion, the data of the present study in MCT-treated rats with severe right ventricular hypertrophy is phosphorylated with the relative order of potency: GRK2>GRK3=GRK5. Furthermore, it should be noted that the marginal alterations in the left ventricular β-adrenoceptor system described in the present study are at variance with our previous data with a markedly reduced β-adrenoceptor density and significantly reduced adenylyl cyclase activity in left ventricles of MCT-treated rats with severe right heart hypertrophy. Moreover, in our previous study, no alterations in the β2:β1-adrenoceptor ratio could be detected in MCT-treated rats. However, Yoshi et al. had previously shown that in MCT-treated rats with severe right ventricular hypertrophy β-adrenoceptor density and adenylyl cyclase activity were decreased not only in right ventricles, but also in the intraventricular septum. Thus, the discrepancy between the present and our previous data can be explained by the fact that, in the present study, we assessed β-adrenoceptor density and subtype distribution in left ventricles without intraventricular septum (see Material and Methods), whereas in our previous experiments, left ventricular preparations, including intraventricular septum were used. We have recently observed similar discrepancies in β-adrenoceptor alterations in preperations of left ventricles with and without intraventricular septum in chronic uremic rats.

In conclusion, the data of the present study in MCT-treated rats show that ventricular hypertrophy per se is not sufficient to cause the characteristic alterations in the myocardial β-adrenoceptor system often seen in heart failure; only if ventricular hypertrophy is associated with neurohumoral activation, β-adrenoceptors are downregulated and GRK activity is increased. This appears more likely to be a local than a systemic effect in the MCT model of pulmonary hypertension: only in right ventricles, β1-adrenoceptors and neuronal noradrenaline transporter activity were decreased.
and GRK was increased, whereas only marginal changes of these parameter could be observed in left ventricles. Thus, the MCT rat model of pulmonary hypertension resemble that of human primary pulmonary hypertension where also chamber-specific changes of the β-adrenoceptor system have been described only in right ventricles.11

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References


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