Endothelin receptor blockade combined with phosphodiesterase-5 inhibition increases right ventricular mitochondrial capacity in pulmonary arterial hypertension

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Mouchaers KT, Schalij I, Versteilen AM, Hadi AM, van Nieuw Amerongen GP, van Hinsbergh VW, Postmus PE, van der Laarse WJ, and Vonk-Noordegraaf A. Endothelin receptor blockade combined with phosphodiesterase-5 inhibition increases right ventricular mitochondrial capacity in pulmonary arterial hypertension. Am J Physiol Heart Circ Physiol 297: H200–H207, 2009. First published April 24, 2009; doi:10.1152/ajpheart.00893.2008.—Pulmonary arterial hypertension (PAH) is often treated with endothelin (ET) receptor blockade or phosphodiesterase-5 (PDE5) inhibition. Little is known about the specific effects on right ventricular (RV) function and metabolism. We determined the effects of single and combination treatment with Bosentan [an ET type A (ETα) and type B (ETβ) receptor blocker] and Sildenafil (a PDE5 inhibitor) on RV function and oxidative metabolism in monocrotaline (MCT)-induced PAH. Fourteen days after MCT injection, male Wistar rats were orally treated for 10 days with Bosentan, Sildenafil, or both. RV catheterization and echocardiography showed that MCT clearly induced PAH. This was evidenced by increased RV systolic pressure, reduced cardiac output, increased pulmonary vascular resistance (PVR), and reduced RV fractional shortening. Quantitative histochemistry showed marked RV hypertrophy and fibrosis. Monotreatment with Bosentan or Sildenafil had no effect on RV systolic pressure or cardiac function, but RV fibrosis was reduced and RV capillarization increased. Combination treatment did not reduce RV systolic pressure, but significantly lowered PVR, and normalized cardiac output, RV fractional shortening, and fibrosis. Only combination treatment increased the mitochondrial capacity of the RV, as reflected by increased succinate dehydrogenase and cytochrome c oxidase activities, associated with an activation of PKG, as indicated by increased VASP phosphorylation. Moreover, significant interactions were found between Bosentan and Sildenafil on PVR, cardiac output, RV contractility, PKG activity, and mitochondrial capacity. These data indicate that the combination of Bosentan and Sildenafil may beneficially contribute to RV adaptation in PAH, not only by reducing PVR but also by acting on the mitochondria in the heart.

Bosentan; Sildenafil; mitochondria; oxidative capacity; chronic heart failure

PULMONARY ARTERIAL HYPERTENSION (PAH) is a severe progressive disease of the small lung vasculature leading to increased pulmonary vascular resistance (PVR). The right ventricle (RV) hypertrophies to withstand the rise in PVR and maintain cardiac output (CO) (16, 48). Subsequently, RV contractile dysfunction occurs, and failure may develop (8, 51). Many factors influence the capacity of the RV to adapt to PAH, such as the degree of RV wall stress, myocardial ischemia, or microvascular endothelial dysfunction. However, the exact sequence of events leading the RV toward failure, and the effects of treatments to counteract this process remain uncertain (51).

Various treatments for PAH have been developed over time, which primarily act as vasodilators of the pulmonary vasculature. These treatments include anticoagulant therapy, prostacyclin infusion, endothelin (ET) receptor blockade, and phosphodiesterase-5 (PDE5) inhibition (9, 25). ET receptor blockade by Bosentan and PDE5 inhibition by Sildenafil are both common strategies in PAH treatment (9, 25, 43). The ET system is highly active in PAH and causes sustained vasostriction of pulmonary arteries. It increases the mitogenic activity of smooth muscle cells and fibroblasts in the pulmonary vessel wall, thereby decreasing the lumen of pulmonary vessels, also contributing to increased pulmonary vascular resistance (PVR) (24, 40). In addition, it is known that ET receptor expression in the myocardium of the RV increases upon PAH (47). Similarly, PDE5 is overactive in the pulmonary vasculature of PAH patients, where it prevents normal relaxation of smooth muscle cells in the vessel wall by excessive degradation of cGMP. PDE5 inhibition causes relaxation of pulmonary vessels and also exerts antiproliferative effects on pulmonary artery smooth muscle cells (24, 53). In addition, it has been shown that PDE5 is highly expressed in the RV of PAH patients and rats, and that acute inhibition increases RV contractility (35).

Although these reports have shed light on the etiology and treatment of the arterial pathology and pulmonary hypertension, limited information is available regarding counteracting the (mal)adaptation of the heart in PAH (34, 46). Central in this (mal)adaptation is the hypertrophy of RV cardiomyocytes that is needed to generate sufficient power to perfuse the lung vascular bed. However, the increased demand of power output of the RV in PAH requires a rise in O2 delivery to RV cardiomyocytes to maintain sufficient ATP synthesis. The increased O2 consumption and diffusion distances for O2 in hypertrophied cardiomyocytes, in combination with the reduced capillary density, can lead to a mismatch between O2 supply and demand (15, 54). In turn, this can cause hypoxia in individual cardiomyocytes and may lead to cellular damage, contributing to RV failure (45, 51).

Bosentan and Sildenafil have been extensively studied in regard to their effects on pulmonary vessels. However, knowledge about their specific effects on the RV is still limited. We
hypothesized that treatment with Bosentan, Sildenafil, or their combination affects cardiac function and determinants for RV contractility and oxidative metabolism in a rat reversal model of monocrotaline (MCT)-induced PAH. To this end, we studied RV contractility using echocardiography and measured RV myocardial activities were measured in 40 cardiomyocytes of the RV and LV. SDH activity (15), and adjacent sections were incubated for cytochrome c oxidase activity (39). SDH and cytochrome c oxidase activities were measured in 40 cardiomyocytes of the RV and LV. SDH activity determined in this way is proportional to the maximum activities were measured in 40 cardiomyocytes of the RV and LV. (SDH) activity (15), and adjacent sections were incubated for cytochrome activity (15), and adjacent sections were stained with hematoxylin and eosin. Mean RV and LV cardiomyocyte cross-sectional area (CSA) of 40 cardiomyocytes were measured randomly. The myoglobin concentration was determined in both ventricles by calibrated histochemistry (47a). The number of capillaries per cardiomyocyte was determined using collagen type IV staining (15). Picrosirius red-stained sections were used to determine the amount of fibrosis using polarization filters. Cytochrome c release was determined as an indicator of mitochondrial membrane integrity (47b).

RV PKG activity. The RV activity of PKG was studied by measuring the Ser239 phosphorylation of VASP, a myocardial PKG-1 target (29), using immunological Western blot analysis. Densitometry (over total VASP expression) was performed and is presented as previously described (33, 35, 49). Myocardial tissue of the RV was homogenized. Detection of VASP (C17) and phosphorylated VASP (Ser239) (both 1:200, Santa Cruz Biotechnology) was performed. VASP phosphorylation is known to cause a migrational shift on SDS-PAGE, which causes a separation of VASP over two bands (6). Both bands together served as total VASP. Before the experiments, we performed a signal linearity control for protein loading (Ponceau). We used horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1,000, DAKO Cytomation). Horseradish peroxidase was detected using the ECL plus Western blot detection system (Amersham Biosciences). Relative density was determined as previously described (49). Sample densities were expressed as a ratio between phospho-VASP and total VASP, where MCT and MCT + treatment samples are expressed relative to the ratio of the control samples, which was set to 1.

Statistical analysis. Mean values ± SE are given. Since comparisons in this study were planned, Student’s t-test was used to determine differences between means. Two-way ANOVA was used to detect interactions between Bosentan and Sildenafil treatments. P values of <0.05 were considered to be statistically significant.

RESULTS

Effects of treatment on hemodynamics, body mass, and lung mass. Table 1 shows hemodynamic parameters and body and lung masses of the various groups of animals studied. MCT clearly induced PAH, which was clearly demonstrated by the higher RVSP on day 25 than in control animals. Neither single treatment with either Bosentan or Sildenafil nor simultaneous treatment with both drugs significantly reduced MCT-induced RVSP significantly. Nontreated MCT rats and both groups of single drug-treated MCT rats had lower systemic blood pressures than control animals.

Body masses of all PAH rats, including treated rats, were significantly lower on day 25 compared with their control counterparts (Table 1). In addition, lung wet masses were significantly increased in MCT-, Bosentan + MCT-, and Sildenafil + MCT-treated rats, whereas the combination treatment normalized lung mass.

Effects of treatment on RV and LV dimensions and contractility, CO, SV, and PVR. To evaluate the effects of MCT-induced PAH and treatment by Bosentan and Sildenafil on the heart, functional echocardiography was performed in anesthetized rats. Representative pictures of M-mode echocardiographic recordings of the RV are shown in Fig. 1. Echocardiographic data are shown in Table 1. After 25 days, MCT rats showed marked dilation of the RV, as demonstrated by increased RVEDDs and RVESDs. RV FS significantly decreased. RVEDD and RVESD values of Bosentan + MCT- and Sildenafil + MCT-treated rats were not different from those of untreated MCT rats, but the calculation of RV FS revealed a further decrease. Combined Bosentan and Sildenafil treatment of MCT rats resulted in less RV dilation, as evidenced by smaller RVEDD and RVESD values, resulting in normalized RV FS. LVEDD and LVESD significantly decreased upon MCT exposure and further decreased upon Bosentan monotherapy, which was not observed in Silde-

1 Supplemental Material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website.
untreated MCT, MCT

Table 1. Hemodynamic and echocardiographic data for the RV and LV, body mass, and lung mass of rats from the control, untreated MCT, MCT + Bosentan, MCT + Sildenafil, and combination treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Untreated (Placebo)</th>
<th>MCT + Bosentan</th>
<th>MCT + Sildenafil</th>
<th>MCT + Bosentan + Sildenafil</th>
<th>P Value of the Interaction of Bosentan and Sildenafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV systolic pressure, mmHg</td>
<td>28±2†</td>
<td>62±7*</td>
<td>64±5*</td>
<td>55±5*</td>
<td>55±10*</td>
<td>NS</td>
</tr>
<tr>
<td>Systemic pressure, mmHg</td>
<td>89±3</td>
<td>70±4*</td>
<td>67±7*</td>
<td>74±5*</td>
<td>76±7</td>
<td>NS</td>
</tr>
<tr>
<td>RVEDD, mm</td>
<td>2.48±0.05†</td>
<td>4.18±0.52*</td>
<td>5.24±0.50*</td>
<td>4.91±0.44*</td>
<td>3.37±0.55</td>
<td>0.0003</td>
</tr>
<tr>
<td>RVESD, mm</td>
<td>1.07±0.06*</td>
<td>2.39±0.38*</td>
<td>3.52±0.38*</td>
<td>3.16±0.27*</td>
<td>1.60±0.21*</td>
<td>0.0003</td>
</tr>
<tr>
<td>RV FS, %</td>
<td>57±3‡</td>
<td>44±3*</td>
<td>33±3*†</td>
<td>35±2†</td>
<td>52±2‡</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>6.75±0.17†</td>
<td>5.86±0.22*</td>
<td>5.43±0.21*</td>
<td>5.99±0.45</td>
<td>6.65±0.18†</td>
<td>0.043</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.97±0.17†</td>
<td>2.32±0.20*</td>
<td>1.86±0.15†</td>
<td>2.39±0.26</td>
<td>2.63±0.26</td>
<td>NS</td>
</tr>
<tr>
<td>LV FS, %</td>
<td>56±2</td>
<td>61±2</td>
<td>66±2†</td>
<td>60±4</td>
<td>61±3</td>
<td>NS</td>
</tr>
<tr>
<td>Stroke volume, µL</td>
<td>296±21</td>
<td>201±21</td>
<td>164±18</td>
<td>228±44</td>
<td>234±19</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>116±10‡</td>
<td>71±10*</td>
<td>47±1*†</td>
<td>74±14*</td>
<td>112±8*‡</td>
<td>0.0048</td>
</tr>
<tr>
<td>Pulmonary vascular resistance, dyn·s·cm⁻⁵</td>
<td>15,005±780†</td>
<td>46,224±8,451*</td>
<td>69,276±8,913†</td>
<td>60,350±5,983†</td>
<td>22,732±2,687†‡</td>
<td>0.0005</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>392±13‡</td>
<td>345±15*</td>
<td>291±11‡</td>
<td>334±16*</td>
<td>387±9‡</td>
<td>0.015</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>363±98</td>
<td>311±12†</td>
<td>297±11†</td>
<td>305±12†</td>
<td>330±9*</td>
<td>NS</td>
</tr>
<tr>
<td>Lung mass, g</td>
<td>1.69±0.11†</td>
<td>2.06±0.06*</td>
<td>2.21±0.11†</td>
<td>2.25±0.13†</td>
<td>1.96±0.13</td>
<td>0.048</td>
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Values are means ± SE; n = 5–7 rats/group (also see the online Supplemental Material). RV, right ventricle; LV, left ventricle; MCT, monocrotaline; RVEDD and LVEDD, RV and LV end-diastolic diameter, respectively; RVESD and LVESD, RV and LV end-systolic diameter, respectively; FS, fractional shortening; NS, not significant. *P < 0.05 and †P < 0.005 vs. control; ‡P < 0.05 and §P < 0.005 vs. untreated MCT rats.

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mean RV cardiomyocyte CSA, Bosentan, Sildenafil, and also the combination treatment further increased cardiomyocyte hypertrophy (Figs. 2A and 3A). The myoglobin concentration did not differ between cardiomyocytes of control, nontreated, and drug-treated MCT rats (Figs. 2B and 3B). Nontreated MCT rats showed a trend toward an increase in capillary density. The number of capillaries per cardiomyocyte increased significantly in MCT rats exposed to both single drug and combination treatments compared with control rats (Figs. 2C and 3C).

In addition, MCT increased the formation of fibrosis in the RV, which reduced significantly upon single treatments with either Bosentan or Sildenafil and normalized upon combination treatment (Figs. 2D and 3D). The mean RV cardiomyocyte SDH activity of control, nontreated MCT, or single drug-treated MCT rats was similar, whereas the combination treatment significantly increased SDH activity (Figs. 2E and 3E). Cytochrome c oxidase activity revealed a similar pattern (Figs. 2F and 3F). SDH and cytochrome c oxidase activities were proportional ($P < 0.0001$, $r^2 = 0.66$).

For comparison, mean LV cardiomyocyte CSA, myoglobin concentrations, and capillary densities were similar in all groups (382 ± 13 μm$^2$, 0.74 ± 0.03 mM, and 1.60 ± 0.03 capillaries/cell, respectively, $n = 31$). However, LV fibrosis significantly increased in nontreated MCT rats compared with control rats (2.9 ± 0.8% vs. 1.1 ± 0.4%, respectively, $P = 0.0002$) and was only lowered by the combined treatment of Bosentan and Sildenafil (to 1.9 ± 0.3%, $P = 0.012$ vs. MCT rats). In addition, SDH activity increased in Sildenafil- and combination-treated rats [absorbance (A) at 660 nm = 0.172 ± 0.006, $n = 14$; pooled value for the two groups compared with
pooled control, nontreated, and Bosentan treated MCT rats, which were similar: \( A_{660nm} = 0.133 \pm 0.005, P < 0.0001, n = 17 \), which was also observed for cytochrome \( c \) oxidase activity \( A_{436nm} = 0.223 \pm 0.004, n = 14 \); pooled value for the two groups compared with pooled control, nontreated, and Bosentan treated MCT rats, which were similar: \( A_{436nm} = 0.193 \pm 0.005, P < 0.0001, n = 17 \).

Effect of treatment on RV PKG activity. Subsequently, we studied whether the effects of treatment on the RV could be explained by a change in cGMP. To this end, the activity of PKG in the RV was measured by determining the degree of Ser239 phosphorylation of VASP. Western blot analysis showed that the RV of MCT rats revealed no difference in RV PKG activity compared with control rats. This shows that PAH induction by MCT itself does not alter PKG activity in RV cardiomyocytes. However, while single treatment with Bosentan or Sildenafil had no significant effect on VASP phosphorylation, the combination of both drugs significantly increased PKG activity by 4.6-fold (Fig. 4).

Interactions between Bosentan and Sildenafil. Two-way ANOVA detected significant interactions between Bosentan and Sildenafil treatment. In addition to those shown Table 1, interactions were found for mean RV CSA \( (P = 0.034) \), Ser239 phosphorylation of VASP \( (P = 0.023) \), and cytochrome \( c \) oxidase activity \( (P = 0.0035) \).

**DISCUSSION**

The main finding of our study is that, in this model of MCT-induced PAH, the combination of orally administered Bosentan and Sildenafil, but not single treatments, improved the RV adaptation to severe PAH with respect to the number of...
capillaries per cardiomyocyte and mitochondrial capacity, as reflected by SDH and cytochrome c oxidase activities. This was associated with a lowering of PVR and normalization of CO, RV FS, and fibrosis.

In this PAH model, RVSP is already elevated by day 14 (22). We specifically chose to study the treatment of already developed PAH and examined the effects of 10 days of treatment with Bosentan and Sildenafil. Compared with previous studies (11, 23, 46), we used a relatively short period of treatment and low dosages of drugs, which may have precluded effects on the reduction of RVSP. In addition, compared with these studies, we used relatively young rats, which may have resulted in more progressive PAH. Furthermore, the biological effects for Sildenafil may differ depending on the experimental setup. A recent report by Andersen et al. (2) has shown that high-dose Sildenafil treatment (100 mg/kg/day) in a model for pulmonary trunk banding was not able to attenuate PAH development; in fact, it increased RV hypertrophy. Our determination of SV, CO, and PVR is in agreement with previous studies (21, 27) using different methodologies and showed similar changes upon RV pressure overload. Despite unaltered PAH, we demonstrated beneficial effects of mainly combination treatment on PVR, SV, CO, and RV dimensions and contractility. Rats who received the combination of Bosentan and Sildenafil were able to maintain CO and showed a lower PVR. In addition, RV dilatation was significantly less, as indicated by lower RVEDD and RVESD values, resulting in restored RV FS. Because RVSP was unaltered after combination treatment, these results indicate, on the basis of the law of Laplace, that RV wall stress was lower after combination treatment. Interestingly, only combination treatment significantly lowered PVR. Taken together, these data indicate that combined use of ET receptor blockade and PDE5 inhibition did reduce RV afterload (38) and can be effective even at suboptimal concentrations for single treatments.

We hypothesized that treatment of PAH rats with Bosentan or Sildenafil would affect determinants for RV O2 supply and demand. We therefore determined the myoglobin concentration and the capillary density of the RV as a measure of the O2 supply and measured CSA, SDH activity, and cytochrome c oxidase activity as determinants of the maximal O2 demand of cardiomyocytes.

One mechanism to enhance O2 supply is increased blood supply by angiogenesis. We found that both single and combination treatments with Bosentan and Sildenafil significantly increased the number of capillaries per cardiomyocyte. Indeed, PDE5 inhibition with Sildenafil can induce an angiogenic response through a PKG-mediated pathway (44, 50). While ET-1 can enhance angiogenesis (12), blockade of both ETA and ETB receptors by Bosentan also improved angiogenesis in ischemic hindlimbs of Wistar rats via the activation of the VEGF-nitric oxide (NO) pathway (26). This paradoxical response occurs probably via the inhibition of ETB receptors, which is known to enhance angiogenesis (28), whereas ETA receptor blockade inhibits it (5). Because RV VEGF expression was found to be decreased in MCT-injected rats (41), it is plausible that Bosentan had similar effects on the activation of the VEGF-NO pathway in our study. Despite the increase in capillary density in Bosentan- and Sildenafil-treated animals, an improvement of RV FS was only observed in the combination treatment, indicating that other determinants had a more dominant effect on the improvement of RV contractility.

A second possibility to counteract hypoxia in hypertrophied cardiomyocytes is to increase the myoglobin concentration (18, 19, 34). In the RV of MCT rats, the amount of myoglobin increased in proportion to the increase in cardiomyocyte CSA. However, the myoglobin concentration remained unaltered, a finding in agreement with previous results (15). This pattern was also observed in all the interventions studied.

The maximal power output of cardiomyocytes is determined by the cardiomyocyte size and oxidative capacity to synthesize ATP. These parameters of maximal O2 demand are reflected by CSA, SDH activity, or cytochrome c oxidase activity. The activities of SDH and cytochrome c oxidase are proportional, and we (15) have previously shown that SDH activity is proportional to the maximum rate of O2 consumption. We found that all treatments increased CSA further compared with the hypertrophy induced by MCT. In contrast, RV SDH and cytochrome c oxidase activities increased only in rats treated simultaneously with both Bosentan and Sildenafil. As only the combination of Bosentan and Sildenafil resulted in a major improvement of RV function, we conclude that increased cardiomyocyte power output is an additional mechanism contributing to restored RV function in PAH rats. It is of interest to note that the mitochondrial capacity in the RV was not altered by the administration of MCT itself. This indicates that the restoration of RV function reflects a compensatory reaction rather than inhibiting the cause of PAH.

Our findings also indicate synergistic actions of Bosentan and Sildenafil on several parameters, such as PVR, RV dimensions (EDD and ESD), RV contractility (FS), RV hypertrophy, cytochrome c oxidase activity, and CO. Because Bosentan and Sildenafil affect different pathways involved in PAH pathophysiology, this suggests that the synergy of their intracellular signals is particularly important in counteracting the adverse effects of PAH on RV function. Previous clinical observations have shown normalization of NO bioavailability by Bosentan in PAH (20). It is likely that PDE5 inhibition by Sildenafil augments the NO-mediated effects. The elevated PDE5 in the
RV of PAH patients and MCT rats (35) would locally reduce cGMP generated by NO-stimulated guanylate cyclase. Such synergy of Bosentan and Sildenafil on NO/cGMP-mediated processes may also underlie the observed improvement of mitochondrial density reflected by SDH and cytochrome c oxidase activities. Indeed, it has been shown that NO stimulates mitochondrial biogenesis through the activation of the mitochondrial transcription factors peroxisome proliferator-activated receptor-γ coactivator-1α, nuclear respiratory factor-1, and mitochondrial transcription factor A in a cGMP-dependent manner (10, 36, 37). We therefore investigated whether the combination of Bosentan and Sildenafil would be able to augment the NO-cGMP pathway by investigating the RV Ser239 phosphorylation of VASP. Indeed, we found an increase in RV VASP phosphorylation only with the combination of Bosentan and Sildenafil and not with the single treatments. These data confirm the synergism and indicate that an increase in mitochondrial capacity is likely an effect of the activation of PKG through the rescue of the NO-cGMP pathway.

The observed effects in our study were predominant for the RV, as treatments did not affect LV histological characteristics except for SDH and cytochrome c oxidase activities in both groups that received Sildenafil. Recent studies have shown that Sildenafil is able to reduce the production of ROS by mitochondria (17) and that it exerts cardioprotective effects through the activation of the NO-cGMP pathway. In addition, we observed altered LV dimensions as indicated by reduced LVEDD and LVESD, which was also observed in Bosentan-treated rats. This suggests that the LV of PAH rats was also subjected to mechanical changes secondary to the pressure-overloaded RV, such as impaired LV filling (30, 32).

In conclusion, we demonstrated that the combination of ET receptor blockade by Bosentan and PDE5 inhibition by Sildenafil, at dosages that are ineffective in single treatments, is able to increase RV mitochondrial enzyme activity in a progressive model of MCT-induced PAH, likely through the activation of PKG by enforced expression of the NO-cGMP pathway. This was associated with a lowering of PVR, a restoration of RV contractility, a normalization of RV fibrosis, and a significant increase in RV capillarization, resulting in the maintenance of normal CO. Our data confirm the synergism between Bosentan and Sildenafil and indicate that combination treatment with both drugs may act at the mitochondrial level in the heart.

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