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Bosentan Attenuates Right Ventricular Hypertrophy and Fibrosis in Normobaric Hypoxia Model of Pulmonary Hypertension

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Abstract

Maladaptive right ventricular (RV) hypertrophic responses lead to RV dysfunction and failure in patients with pulmonary arterial hypertension, but the mechanisms responsible for these changes are not well understood. The objective of this study was to evaluate the effect of treatment with bosentan on RV hypertrophy (RVH), fibrosis and expression of protein kinase C (PKC) isoforms in the RV of rats exposed to chronic hypoxia.

Methods—Adult Sprague Dawley rats were housed in normoxia or hypoxia (FiO₂: 10%) and administered vehicle or 100mg/kg/day of bosentan. After 3 weeks, echocardiographic, hemodynamic assessment was performed. PKC, procollagen-1, and collagen expression was assessed using immunoblot or colorimetric assay.

Results—RV systolic pressure (RVSP) and RVH were higher in hypoxic compared to normoxic animals (RVSP: 72 ± 4 vs. 25 ± 2 mmHg, p<0.05; RVH: 1.2 ± 0.06 vs. 0.5 ± 0.03 mg/g body wt., p<0.05). Bosentan had no effect on RVSP or mass in normoxic animals, but did attenuate RVH in hypoxic animals (Hypoxic/vehicle: 1.2 ± 0.06; Hypoxic/bosentan: 1.0 ± 0.05 mg/g body wt., p<0.05). Hypoxia increased RV procollagen-I, and total collagen expression, effects that were attenuated by bosentan treatment. Hypoxia increased RV total and cytosolic PKC-δ protein expression, but had no effect on PKC-α or -ε isoforms. Administration of bosentan did not affect total PKC-δ protein expression. However, animals treated with bosentan had an increase in membranous PKC-δ when exposed to hypoxia.

Conclusion—Bosentan inhibits RVH and RV collagen expression in rats exposed to chronic hypoxia possibly via alteration of PKCδ activity.

Keywords

Endothelin; Endothelin Receptor blocker; Chronic Hypoxia; Pulmonary Hypertension; Right Ventricle; Protein Kinase C

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Introduction

Right ventricular failure is the primary cause of morbidity and mortality in patients with pulmonary arterial hypertension (PAH). As an adaptive response to increased afterload, the right ventricle (RV) initially hypertrophies. However, persistently increased afterload leads to maladaptive hypertrophic responses that include myocardial apoptosis and increased expression of non-contractile proteins in the myocardial interstitium that may contribute to decreased RV systolic function. The mechanisms responsible for modulating RV maladaptive hypertrophic responses to increased pulmonary arterial pressure are not well known. Endothelin-1 (ET-1) is a potent vasoconstrictor and mitogen that has been shown to have hypertrophic effects on the heart. Pulmonary expression of ET-1 is increased in patients with PAH and circulating levels of ET-1 correlate inversely with survival (1, 2). Investigations have confirmed the role of ET-1, in the hypertrophy of cultured cardiomyocytes (3, 4). Moreover, the hypertrophic effects of epinephrine and angiotensin II have been shown to be mediated by an increased production of endogenous ET (5).

The role of ET-1 in mediating RVH associated with pulmonary hypertension is not well understood. Endothelin receptor antagonists (ERA) have been shown to blunt the increase in RV mass associated with several animal models of pulmonary hypertension; including hypoxia- and monocrotaline-induced pulmonary hypertension (6–8). However, it is unclear if this occurs as a result of blocking the hypertrophic effects of ET-1 on the heart or as the result of decreased RV afterload caused by blocking the vascular remodeling effects of ET-1 on the pulmonary circulation.

ET receptor activation causes increased diacylglycerol signaling, mediated via $G\alpha_q$ protein coupled receptors that can subsequently activate protein kinase C (PKC), a family of serine/threonine kinases that has been linked to cardiac hypertrophy both *in vivo* and *in vitro* (9–11). ET-mediated activation of PKC, particularly the α and δ isoforms, has been demonstrated in the vascular smooth muscle of systemic vessels (12) and in neonatal cardiomyocytes (13, 14). An increase in RV PKC- δ expression has been reported after pulmonary artery banding and exposure to hypobaric hypoxia in rats, suggesting that this PKC isoform may be particularly important in modulating RV hypertrophic responses (6, 15). However, the effect of endothelin receptor antagonism, a known therapy for PAH, on the expression of PKC isoforms in RVH is not known.

In this study, we hypothesized that increased ET-1 signaling during the development of pulmonary hypertension causes increased activation of PKC and increased expression of non-contractile proteins in the RV myocardium. To test this hypothesis, we examined the expression of PKC isoforms and collagen in the right and left ventricles of rats exposed to chronic hypoxia in the presence or absence of the non-selective ERA, bosentan.

Methods

The animal protocol was approved by the Institutional Animal Use and Care Committee of the Providence VA Medical Center.

Experimental Protocol

Adult male CD IGS rats (200–250g) (Charles River Laboratories International Inc., Wilmington, MA) were placed in either normoxic or normobaric hypoxic (10% FiO₂) environment for 3 wks. Normobaric hypoxia was achieved by placing the rats in Biospherix A-chamber with Pro-Ox oxygen controller (Biospherix Ltd., Lacona, NY). Half of the animals exposed to either normoxia or hypoxia were administered either vehicle (5% gum arabic) or 100 mg/kg bosentan (Actelion Pharmaceuticals, Switzerland) by gavage, daily

during the course of the experiment. After 3 weeks of exposure to normoxia or hypoxia, transthoracic echocardiogram was performed on the rats using a Visualsonics Vevo 2100 machine after general anesthesia with isoflurane to perform 2D, Doppler and M-mode recordings to measure LV fractional shortening, LV and RV dimensions, right ventricular outflow tract (RVOT) diameter, and heart rate. The pulse-wave Doppler recording at the RVOT was used to measure pulmonary acceleration time (PAT) and the velocity time integral (VTI). Stroke volume was calculated by multiplying the VTI with the area of RVOT. Cardiac output (CO) was calculated by multiplying the stroke volume with the heart rate. Tricuspid annular plane systolic excursion (TAPSE) was measured using M-mode across the tricuspid valve annulus at the RV free wall. TAPSE was determined by measuring the excursion of the tricuspid annulus from its highest position to the peak descent during ventricular systole.

Rats were then weighed and anesthetized with ketamine (50–75 mg/kg ip) and medetomidine (0.3–0.5 mg/kg ip). Subsequently, a fluid filled catheter or Millar catheter (Millar Instruments, Inc., Houston, TX) was inserted into the right jugular vein to measure right atrial pressure (RAP) and subsequently advanced into the RV to measure right ventricular systolic pressure (RVSP). A second catheter was placed in the right carotid artery to measure mean systemic arterial pressure. The pressure was transduced using Grass pressure transducers (Grass Instruments, Warwick RI) and data was acquired on a PC and analyzed using Polyview software (Grass Instruments). After hemodynamic measurements, the animals were euthanized. The heart and lungs were removed en bloc. The RV was separated from the left ventricle (LV) and interventricular septum. The RV and LV were subsequently flash frozen in liquid nitrogen and stored at -80°C .

Protein Analyses

The respective ventricles were placed in homogenization buffer containing 20mM HEPES, 250mM sucrose, 100mM NaCl, 0.2mM EDTA, 200 μM PMSF, 0.5 mM DTT, 1 μM leupeptin, 1 μM aprotinin and phosphatase inhibitor cocktail II and IV (EMD Chemicals Inc., San Diego, CA). The ventricles were homogenized while on ice followed by centrifugation at 10,000g for 10min at 4°C . Supernatant was used for total protein analysis. Proteins were resolved using SDS-PAGE. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes and immunoblot analysis was performed using appropriate antibodies for PKC- α , PKC- δ , PKC- ϵ , and procollagen I (Santa Cruz Biotechnologies, CA). Some of the homogenate was further processed for subcellular fractionation using a 1 hour 100,000g ultracentrifugation. The supernatant was considered the cytosolic fraction, while, the pellet was considered the membranous fraction. Ponceau S staining was subsequently performed on the membrane to normalize for equal protein loading. Densitometric analyses were performed using ImageQuant software (GE Healthcare Biosciences, Pittsburg, PA).

RV Collagen Content Measurement

RV collagen content measurements were made using Sircol collagen dye binding assay (Biocolor Lifescience assays, UK) following manufacturer's instructions (16). Briefly, 1ml of Sircol dye reagent was mixed to equal amounts of protein from homogenized RV samples. The collagen dye complex was centrifuged at 12,000g for 10 minutes to firmly pack the collagen-dye complex at the bottom of the tube. After draining the unbound dye, ice-cold acid wash (750 μl) was added to further remove unbound dye and tubes were centrifuged at 12,000g $\times$ 10 minutes and drained again. Bound dye was released and dissolved by addition of alkali agent and vortexed $\times$ 10 minutes. Released dye was measured spectrophotometrically at 555nm.

Statistics

Values are presented as mean $\pm$ SEM. Differences in mean values between experimental groups were analyzed by two-way ANOVA using Stata (StataCorp LP, College Station TX). Where significant differences were found, differences between individual groups were assessed by Fisher-Hayter post hoc comparison or 2-tailed t-test. Differences at $P < 0.05$ were considered statistically significant.

Results

3.1 Pulmonary Hemodynamic Responses to Hypoxia and Bosentan

Bosentan had no effect on RVSP under normoxic conditions. Hypoxia increased RVSP in vehicle and bosentan treated animals. There was no significant difference in RVSP between bosentan- and vehicle-treated animals exposed to hypoxia (Hypoxia/Vehicle: 72.1 ± 4.3 vs. Hypoxia/Bosentan: 64.5 ± 5.0 mmHg, $p = 0.26$, $n = 13$ and 12 , respectively; Table 1). Mean carotid arterial pressures, and right atrial pressures were not significantly altered by either exposure to chronic hypoxia or treatment with bosentan (Table 1).

RV mass as assessed by RV/body weight (RV/BW) and RV/left ventricle + septum weight (RV/(LV+S)) was not affected by bosentan under normoxic conditions. Exposure to chronic hypoxia markedly increased RV/BW and RV/(LV+S). Treatment with bosentan significantly attenuated the increase in the RV mass in animals exposed to hypoxia. (RV/LV +S: Normoxia/Vehicle- 0.26 ± 0.01 , Hypoxia/Vehicle- 0.50 ± 0.02 , Hypoxia/Bosentan- 0.43 ± 0.02 ; $n = 14$; Figure 1).

3.2 Echocardiographic Parameters

Chronic hypoxia significantly increased RV free wall thickness compared to normoxia/vehicle treated animals (Table 2). Both TAPSE, a measure of RV systolic function, and PAT, an inverse measure of degree of pulmonary hypertension, were significantly reduced in hypoxic animals compared to normoxic animals consistent with impaired RV contractility and higher pulmonary pressure in the hypoxia-adapted animals (Table 2, Supplementary Figure 1). Bosentan did not significantly improve RV systolic function or pulmonary pressure assessed by PAT.

3.3 Cardiac Fibrosis

Three weeks of hypoxia significantly increased procollagen I expression in the RV compared to normoxic controls. Bosentan had no effect on procollagen I expression in rats kept under normoxic conditions, but significantly reduced procollagen I expression in hypoxia-adapted rats (Figure 2a).

Chronic hypoxia also increased total collagen content in the RV of vehicle treated animals. Again, bosentan had no effect on total RV collagen under normoxic conditions but completely abrogated the rise in RV total collagen content after exposure to chronic hypoxia (Figure 2b).

3.5 PKC Isoform Expression

Under normoxic conditions treatment with bosentan had no effect on the expression of total PKC- α or - ϵ isoforms in the RV (Figure 3a & c), but RV expression of PKC- δ was increased (Figure 3b). Compared to normoxic controls, 3 weeks of hypoxia increased right ventricular expression of PKC- δ , but had no effect on the expression of PKC- α , and PKC- ϵ . Bosentan did not attenuate the hypoxia-induced increase in RV PKC- δ expression (Figure

3b) Also, there was no change in the expression of PKC- α , PKC- δ , or PKC- ϵ in the LV of animals exposed to hypoxia (Supplementary Figure 2).

Subcellular fractionation revealed an alteration in RV PKC- δ localization in the hypoxic rats treated with bosentan (Table 3). While hypoxia caused an increase in cytosolic PKC- δ in the RV of vehicle treated animals; there was a significant increase in membranous PKC- δ in the hypoxia-adapted rats treated with bosentan. The subcellular distribution of PKC- α or - ϵ did not change significantly in the RV or LV of either vehicle or bosentan treated animals.

Discussion

Cardiac remodeling is important in the pathophysiology of cardiac dysfunction secondary to pulmonary hypertension and is characterized by myocyte hypertrophy and changes in extracellular matrix. The extracellular matrix provides a supportive scaffold for cardiac myocytes and blood vessels of the heart (17). However, abnormal increases in collagen synthesis can eventually lead to cardiac dysfunction by replacing contractile cells with proteins that have greater tensile strength leading to RV systolic dysfunction and failure.

In the present study, we examined the role of endothelin receptor blockade on RV hypertrophic responses to chronic hypoxia model of PAH. Endothelin-1 is increased during chronic hypoxia and acts both as a paracrine and autocrine factor in cardiac hypertrophy (18) and induces fibroblast proliferation leading to increased collagen synthesis (19). We observed a marked increase in RV mass and a significant decrease in RV function in rats exposed to chronic hypoxia. LV function and size (ejection fraction, stroke index, LV mass) did not differ significantly between the 4 groups of animals, suggesting effects of chronic hypoxia are limited to changes in the RV. Treatment with the nonselective endothelin receptor antagonist, bosentan, at a dose that has been shown to maximally attenuate the effect of endothelin in animal models (20), significantly attenuated the hypoxia-induced increase in RV mass, despite only minor changes if any in RV pressure. Although the effect of bosentan on RV mass was not associated with significant improvement in RV systolic function as assessed by the TAPSE index, there were trends toward smaller RV diastolic diameter, RV free wall thickness and increased CO in the hypoxia-adapted animals treated with bosentan. While these trends are promising and consistent with a beneficial effect of bosentan on RV function, they will need to be confirmed in a larger sample and human studies. Furthermore, bosentan inhibited the hypoxia-induced increase in RV procollagen 1 and total collagen expression. Our finding that bosentan significantly reduces RV weight/total body weight is similar to prior studies in hypoxic rats (7). Taken together, the results of the present study suggest that the inhibition of hypoxia-induced RVH by bosentan is not entirely dependent on reduction in RVSP and may be due, in part, to attenuation of maladaptive hypertrophic responses in the RV that are mediated by ET-1.

In the present study, we saw no significant inhibition of the hypoxia-induced increase in RVSP in rats treated with bosentan. Although previous studies have reported a significant reduction in RVSP with bosentan in experimental models of pulmonary hypertension, this response has not been uniform. For example, Hill et al reported no significant reduction in RVSP by bosentan in rats with monocrotaline-induced pulmonary hypertension (8). Interestingly, bosentan was found to reduce RV mass in the monocrotaline treated animals in that study despite the lack of hemodynamic improvement, similar to our observations in the present study. However, their investigation was focused on evaluating the effect of bosentan on pulmonary vasculature, inflammation and injury and did not address the pathophysiological changes in the right ventricle. In contrast, our study evaluated the effect of bosentan on the right ventricle hypertrophy, fibrosis and possible underlying mechanism. The monocrotaline model of PAH is a more acute toxic model of vascular injury

associated with endothelial cell injury, (21, 22), inflammation (23, 24) and impaired fibrinolysis (25). The hypoxic model may be a more relevant model for pulmonary hypertension associated with obstructive pulmonary disease (COPD), sleep disordered breathing, and lung parenchymal diseases (26). Exposure to chronic hypoxia and increased RV afterload was associated with an increase in pro-collagen I expression and total collagen content in the RV and this effect was attenuated by treatment with bosentan. Changes in the extracellular matrix such as increased cardiac fibrosis are important in the pathophysiology of RV remodeling in pulmonary hypertension. Interstitial collagens are synthesized and secreted out of cells as procollagen. During the secretion, carboxy- and amino terminal propeptides are cleaved off from the parent molecule by specific proteases (27) releasing the collagen molecule. A balance between synthesis and degradation by matrix metalloproteases determines collagen content in the ventricles (28, 29). Previous studies have shown that ET increases collagen synthesis (30). ET-1 has also been noted to increase cardiac fibroblast DNA synthesis suggesting an increase in fibroblast proliferation (19). Hence, inhibition of procollagen-1 synthesis leading to decreased collagen content with bosentan would be consistent with prior observations of the role of endothelin-1 in fibrosis.

In addition to increasing cardiac fibrosis, ET-1 increases diacylglycerol synthesis, the primary upstream activator of Protein kinase C (PKC). Activation of PKC by diacylglycerol results in phosphorylation and translocation of PKC from the cytosol to the cell membrane (31). However, we found that exposure to chronic hypoxia increases the expression of PKC- δ predominantly in the cytosol, suggesting decreased translocation. This observation is similar to earlier reports of RVH induced by hypoxia and PA banding (6, 15). Paradoxically, endothelin receptor blockade with bosentan appeared to increase translocation of PKC- δ as suggested by the lower levels of cytosolic and higher levels of membranous PKC- δ in the hypoxic rats. Interestingly, we also observed an increased expression of PKC- δ in bosentan treated normoxic rats. This could represent a compensatory increase in the downstream signaling pathway and/or activation of other neurohormonal mediators/G-protein coupled signaling in response to decreased endothelin receptor activation. Whether or not the antihypertrophic effects of bosentan in the model of RVH utilized in the present study are mediated by PKC is unclear. Further studies will be needed to determine if translocation of PKC- δ in RV myocytes or fibroblasts contributes to hypertrophic and fibrotic responses. Our results pertaining to the changes in PKC expression in response to bosentan treatment are contrary to those reported by Jiang et. al. in a streptozotocin diabetic rat model at 11 weeks (32). This discrepancy is likely due to nature of the hypertrophic stimuli between the models. While the current study and others have reported an increase in PKC- δ expression with increased RV afterload (6, 15), studies have shown conflicting observations regarding the effect of diabetes on the myocardial PKC isoform expression (32–34).

Even though exposure to 3 weeks of hypoxia was sufficient to produce RV hypertrophy and increase in RV hypertrophy, we did not find any significant change in PKC- α or - ϵ protein expression at 3 weeks. Our results are consistent with a recent study showing no change in either cytosolic or membranous PKC- α despite an increase in PKC- α mRNA expression at 3 weeks (15). Also, while PKC- ϵ has been shown to be important in left ventricular hypertrophy (35, 36); there appears to be limited change in its expression with right ventricular hypertrophy (6, 15). Further investigation is needed to study if longer duration of hypoxia may result in significant changes in the RV remodeling and/or signaling mechanisms involved.

In summary, we demonstrate that hypoxic pulmonary hypertension is associated with decreased RV function in the setting of increased RV mass and increased RV expression of procollagen-1, total collagen and PKC- δ . Treatment with bosentan blunted the increase in RV mass and collagen expression and increased membranous levels of PKC- δ . Alterations

in RV PKC- δ expression and activity may contribute to bosentan induced decreases in RV collagen expression and inhibition of RV hypertrophic responses to pulmonary hypertension. RV dysfunction and failure in patients with PAH is an important contributor of increased morbidity and mortality. Also, RV failure is the primary reason that patients with PAH undergo lung and heart/lung transplantation. Though, there are several evidence-based therapies available for left ventricular dysfunction, none have been rigorously evaluated for right ventricular failure. Further studies are needed to determine if the changes noted in this study contribute to the mechanisms by which endothelin receptor antagonists have been shown to improve functional capacity in patients with PAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CO	Cardiac output
ET	Endothelin
PAH	Pulmonary arterial hypertension
PAT	Pulmonary acceleration time
PKC	Protein kinase C
RAP	Right atrial pressure
RV	Right ventricle
RVOT	Right ventricular outflow tract
RVSP	Right ventricular systolic pressure
SEM	Standard error of mean
TAPSE	Tricuspid annular plane systolic excursion
VTI	Velocity time integral

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Figure 1. Right ventricular hypertrophy

Adult Sprague Dawley rats were exposed to 3 weeks of normoxia or 10% FiO₂ and treated with either vehicle or 100 mg/kg bosentan by oral gavage daily. Right ventricle was dissected from the left ventricle and septum. Bar graphs representing the ratio of (a) RV wt. to LV+Septum wt. (b) RV wt. to body weight. (Mean $\pm$ SEM, n= 14–15, * p<0.05 compared to normoxia/vehicle, # p<0.05 compared to hypoxia/vehicle)

Figure 2. Right ventricular fibrosis

(a) Right ventricular tissue lysates from rats exposed to three weeks of normoxia or normobaric hypoxia (10% FiO₂) and treated with vehicle or 100mg/kg/day bosentan were resolved on SDS-PAGE and probed for procollagen-1. Ponceau staining was performed to ensure equal loading. Bars represent mean densitometry of procollagen-1 bands normalized to Ponceau. (b) Equal amounts of RV lysate were used in the Sircol collagen dye binding assay and total collagen content was calculated. (Mean ± SEM, n= 3, * p<0.05 compared to normoxia/vehicle, # p<0.05 compared to hypoxia/vehicle)

Figure 3. Protein Kinase C isoform Expression

Equal amounts of protein from ventricular tissue lysates of rats exposed to 3 weeks of normoxia or normobaric hypoxia (10% FiO₂) were resolved on SDS-PAGE, and immunoblotted for PKC- α (a), - δ (b), or - ϵ (c). Bars represent mean densitometry of PKC isoforms normalized to Ponceau. (Mean $\pm$ SEM, n= 3–6, * p<0.05 compared to normoxia/vehicle).

Table 1**Hemodynamic Data**

	Normoxia		Hypoxia	
	Vehicle (n)	Bosentan (n)	Vehicle (n)	Bosentan (n)
Mean Carotid Arterial Pressure (mmHg)	100.6 ± 5.7 (8)	86.2 ± 4.9 (9)	116.2 ± 12.4 (3)	106.1 ± 3.8 (9)
Right Atrial pressure (mmHg)	2.1 ± 0.7 (7)	3.4 ± 1.2 (7)	4.3 ± 1.2 (5)	3.5 ± 1.6 (5)
Mean RVSP (mmHg)	25.1 ± 1.8 (12)	32.4 ± 2.9 (15)	72.1 ± 4.3 [*] (13)	64.5 ± 5.0 [*] (12)
Body Weight (g)	357.0 ± 5.0 (17)	356.2 ± 7.1 (18)	311.3 ± 7.2 [*] (18)	312.9 ± 7.6 [*] (16)

Data is mean ± SEM

^{*}
p < 0.05 compared to normoxia vehicle

Table 2

Echocardiographic data

	Normoxia		Hypoxia	
	Vehicle	Bosentan	Vehicle	Bosentan
Heart Rate (bpm)	380.2 ± 13.6	350.8 ± 22.7	349.4 ± 17.3	388.1 ± 18.1
LVEF (%)	77.99 ± 3.34	78.89 ± 3.29	82.21 ± 3.21	83.45 ± 1.75
Fractional Shortening	48.53 ± 3.37	49.49 ± 3.22	52.77 ± 3.62	53.56 ± 2.21
LV Mass	968 ± 112	801 ± 115	672 ± 46	735 ± 126
RV Diastolic Dimension (cm)	2.13 ± 0.25	2.11 ± 0.23	2.41 ± 0.15	2.08 ± 0.17
RV free wall thickness (mm)	0.85 ± 0.05	1.16 ± 0.13	1.55 ± 0.09 [*]	1.32 ± 0.07 [*]
TAPSE (mm)	3.48 ± 0.18	3.22 ± 0.40	2.23 ± 0.42 [*]	2.19 ± 0.06 [*]
PAT(ms)	36.62 ± 2.24	35.11 ± 1.92	25.69 ± 1.09 [*]	25.78 ± 3.08 [*]
SV (ul)	3006 ± 486	2860 ± 454	2263 ± 239	3002 ± 750
CO (ml/min)	1150 ± 206	983 ± 136	795 ± 102	1117 ± 234
Stroke Index (μl/g)	8.49 ± 1.51	8.19 ± 1.19	6.65 ± 0.64	9.74 ± 2.61

Data is mean ± SEM. N= 6 for all parameters except TAPSE (n=3);

^{*}
p < 0.05 compared to normoxia vehicle

Table 3

Change in expression of PKC isoforms in the cytosolic and membranous fractions of the right ventricle in animals treated with vehicle or bosentan and exposed to hypoxia. Densitometry normalized to ponceau and normoxic controls.

	Cytosolic		Membranous	
	Vehicle	Bosentan	Vehicle	Bosentan
PKC α	1.1 $\pm$ 0.4	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	2.0 $\pm$ 0.4
PKC δ	3.4 $\pm$ 0.7 [*]	1.7 $\pm$ 0.3	1.2 $\pm$ 0.3	2.5 $\pm$ 0.3 [#]
PKC ϵ	1.1 $\pm$ 0.2	1.1 $\pm$ 0.1	0.8 $\pm$ 0.1	1.1 $\pm$ 0.0

N=3–6, mean $\pm$ SEM,

^{*} p<0.05 compared to normoxia/vehicle;

[#] p<0.05 compared to normoxia/bosentan