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Endogenous endothelins and nitric oxide in hypoxic pulmonary vasoconstriction

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ABSTRACT: The effects of endothelin receptor blockade on the pulmonary circulation have been reported variably, possibly in relation to a more or less important associated release of endogenous nitric oxide (NO). The aim of this study was to test whether endothelin antagonism would inhibit hypoxic pulmonary vasoconstriction, and if it would not, then would it do so after NO synthase inhibition.

Hypoxic pulmonary vasoconstriction (HPV) was evaluated in anesthetised dogs by the increase in the mean pulmonary artery pressure (P_{pa}) minus occluded P_{pa} (P_{pao}) gradient in response to hypoxia (inspiratory oxygen fraction of 0.1) at constant pulmonary blood flow.

Bosentan, an endothelin A and B receptor antagonist, did not affect baseline P_{pa}, P_{pao} or systemic arterial pressure (P_{sa}) and did not alter HPV (n=8). The NO synthase inhibitor N^G-nitro-L-arginine (L-NA) did not affect baseline P_{pa} and P_{pao}, but increased P_{sa} and enhanced HPV (n=12). The addition of bosentan in these dogs did not affect baseline P_{pa} or P_{pao}, but decreased P_{sa} and inhibited HPV. Exhaled NO was decreased by L-NA and by bosentan and abolished by L-NA+bosentan (n=9).

The authors conclude that endogenous nitric oxide is released by, and opposes the vasoconstricting effects of, endothelins *in vivo*, reducing systemic blood pressure and limiting hypoxic pulmonary vasoconstriction.

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Endothelins are potent endothelium-derived vasoconstrictors [1]. The endothelin system has been reported to be activated in various types of experimental or clinical pulmonary hypertension [1]. However, whether endogenous endothelins play a role in the control of systemic and pulmonary vascular tone *in vivo* remains uncertain. Endothelin receptor antagonists have been shown to increase forearm blood flow in humans [2, 3], to reduce brachial artery pressure in patients with essential hypertension [4] and to decrease pulmonary artery pressure in patients with primary pulmonary hypertension [5, 6]. However, some investigators have been unable to demonstrate pulmonary or systemic vascular effects of these compounds *in vivo* [7–17]. The failure to show a vasoconstrictor effect of endogenous endothelins appears related, at least in part, to the release of nitric oxide (NO) by the endothelium, which either counteracts the effect and/or inhibits the synthesis of endothelins [16–19].

Endothelins have been hypothesised to play a role in the mediation of hypoxic pulmonary vasoconstriction (HPV). Hypoxia has been reported to increase the level of immunoreactive endothelin-1 in the plasma and of endothelin-1 messenger ribonucleic acid in the lung and right atrium [12, 20]. However, selective as well as nonselective endothelin receptor blockers have been shown to inhibit HPV in some studies [8–14] but not in others [15, 21–23]. These discrepancies might be explained, at least in part, by variable release of endogenous NO, which has been shown to limit HPV in intact animals [24, 25].

In this study, the effects of endogenous endothelins on hyperoxic and hypoxic vascular tone, with and without NO synthase inhibition by N^G-nitro-L-arginine (L-NA), were examined. The hypothesis was that endothelin antagonism may inhibit HPV, but if not, would do so after NO synthase inhibition. Because endothelins have opposite vascular effects, possibly mediated by different receptors [1], complete blockade of the actions of endogenous endothelins requires antagonism of both endothelin A and endothelin B receptors, as exerted by the combined nonpeptide blocker bosentan [26]. The effects of hypoxia and drugs on vascular tone were evaluated at constant cardiac output (Q') to allow for the discrimination between passive and active tone dependent changes in pulmonary vascular pressure [24, 25].

Methods

The experiments were conducted in agreement with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and were approved by the Committee on the Care and Use

of Animals in Research of the Brussels Free University School of Medicine, Brussels, Belgium.

Animal preparation

Mongrel dogs (16–45 kg) were anaesthetised (pentobarbital 25 $mg \cdot kg^{-1}$ intravenously followed by 2 $mg \cdot kg^{-1}$ hourly), paralysed (pancuronium 0.2 $mg \cdot kg^{-1}$ intravenously followed by 0.2 $mg \cdot kg^{-1}$ hourly), intubated and ventilated (Elema 900 B Servo ventilator; Siemens, Solna, Sweden) with an inspiratory oxygen fraction (F_{I,O_2}) of 0.4. This higher than normal F_{I,O_2} was selected to maintain the lungs above the threshold for HPV [25]. Catheters were placed in the jugular vein, in the femoral artery and in the pulmonary artery as described previously [24, 25]. Cardiac output was measured by thermodilution. A balloon catheter was advanced into the inferior vena cava through a right femoral venotomy and a large-bore cannula was inserted into the left femoral artery and vein to act as an arteriovenous bypass. Stepwise inflations of the balloon catheter or opening of the bypass decreased or increased Q′, respectively [24, 25].

Effect of big endothelin-1 without and with bosentan

Bosentan (Actelion, Allschwil, Switzerland) was diluted in heated distilled water just before the experiments and administered as an intravenous bolus of 3 $mg \cdot kg^{-1}$ followed by a constant infusion of 7 $mg \cdot kg^{-1} \cdot h^{-1}$ [7]. This dose has been shown to provide optimal endothelin receptor blockade in dogs [7]. The ability of this dose of bosentan to antagonise the effects of big endothelin-1 (Novabiochem, Läufelfingen, Switzerland), the precursor of endothelins, was checked. Big endothelin-1 was diluted in sodium chloride 0.9% just before the experiments. After two baseline hypoxic challenges (F_{I,O_2} 0.4–0.1 for 6 min), the dogs received 1 $nmol \cdot kg^{-1}$ of big endothelin-1 alone (n=3) or after bosentan (n=2), and two additional hypoxic challenges were performed thereafter. Q′ was kept constant using the femoral bypass and the inferior vena caval balloon. Mean pulmonary artery pressure (P_{pa}), occluded P_{pa} (P_{pao}), systemic artery pressure (P_{sa}), Q′ and blood gases were measured after 3 min stabilisation.

Effect of bosentan

The response of the P_{pa}–P_{pao} gradient to changes in F_{I,O_2} (0.4, 0.21, 0.12, 0.1) was examined, with Q′ kept constant, before and after bosentan. Each F_{I,O_2} was maintained for at least 6 min to allow stabilisation.

Effect of bosentan after N^G-nitro-L-arginine

Two hypoxic challenges (F_{I,O_2} 0.4–0.1) were performed at baseline, after L-NA (Sigma-Aldrich, Bornem, Belgium) 5 $mg \cdot kg^{-1}$ intravenous bolus followed by a constant infusion of 5 $mg \cdot kg^{-1} \cdot h^{-1}$, and again after bosentan. This dose of L-NA has been shown to enhance canine HPV [24, 25]. Q′ was kept constant as described above.

Effect of N^G-nitro-L-arginine and bosentan on exhaled nitric oxide

The concentration of NO was continuously monitored at the tip of the endotracheal tube using a chemiluminescence analyser (Model LR2000; Logan Research, Rochester, UK) [27]. Exhaled NO concentrations were read in triplicate at the maximum value of expired carbon dioxide. These measurements were performed after bosentan alone (n=4), after L-NA (n=5) and after bosentan given after L-NA (n=4).

Analysis of the data

Results are expressed as means±SEM. A two-factor analysis of variance for multiple measurements and modified t-tests were used as appropriate [24, 25, 28]. Values of $p<0.05$ were considered as significant.

Results

Effects of big endothelin-1 without and with bosentan

As designed in the protocol, Q′ was kept constant in all experiments.

Big endothelin-1 alone. At F_{I,O_2} 0.4, big endothelin-1 increased P_{sa} from 104 to 132 mmHg and had no effect on P_{pa}–P_{pao} which was 8 mmHg before and after big endothelin-1. The increase in P_{pa}–P_{pao} in response to hypoxia was 2 mmHg before and 4 mmHg after big endothelin-1 (n=3).

Bosentan followed by big endothelin-1. At F_{I,O_2} 0.4, bosentan slightly reduced P_{sa} from 118 to 109 mmHg. Big endothelin-1 given after bosentan had no effect on P_{sa}, which remained at 105 mmHg. P_{pa}–P_{pao} remained at 8 mmHg at baseline, 8 mmHg after bosentan and 8 mmHg after big endothelin-1. Hypoxia-induced increases in P_{pa}–P_{pao} were 11 mmHg at baseline, 11 mmHg after bosentan and 10 mmHg after big endothelin-1 (n=2).

Effects of bosentan alone

Hypoxia increased the P_{pa}–P_{pao} gradient measured at constant Q′ (table 1 and fig. 1). Administration of bosentan did not affect P_{pa}–P_{pao} in hyperoxia, normoxia and hypoxia (fig. 1). Neither hypoxia nor bosentan significantly affected P_{sa} (table 1) (n=8).

Effects of bosentan after N^G-nitro-L-arginine

At constant Q′, bosentan inhibited the L-NA-induced increase in HPV (fig. 2) (n=12). Bosentan

Table 1. – Effects of bosentan at different inspiratory oxygen fractions (F_{I,O_2}) in eight dogs

	F_{I,O_2}				p-value
	0.4	0.21	0.12	0.1	
Q′ L·min^{-1}·m^{-2}					
Baseline	4.9±0.5	4.8±0.5	4.8±0.5	4.8±0.5	NS
Bosentan	5.0±0.5	4.8±0.5	4.8±0.5	4.8±0.5	NS
P_{pa} mmHg					
Baseline	12±1	11±1	13±1	16±1	<0.01
Bosentan	11±1	11±1	13±1	15±1	<0.01
P_{pao} mmHg					
Baseline	3±1	2±1	2±1	2±1	NS
Bosentan	2±1	2±1	1±1	2±1	NS
P_{sa} mmHg					
Baseline	109±10	114±12	123±15	122±17	NS
Bosentan	105±9	104±14	108±14	104±11	NS
f_c beats·min^{-1}					
Baseline	152±4	152±7	160±8	157±8	NS
Bosentan	154±7	155±9	166±7	162±6	NS
pH_a					
Baseline	7.32±0.03	7.33±0.01	7.35±0.01	7.34±0.02	NS
Bosentan	7.34±0.01	7.34±0.02	7.36±0.02	7.37±0.02	NS
P_{a,O_2} mmHg					
Baseline	204±11	99±5	46±2	35±2	<0.01
Bosentan	213±16	98±8	47±4	35±3	<0.01
P_{a,CO_2} mmHg					
Baseline	45±2	41±2	41±2	40±3	NS
Bosentan	39±3	37±2	37±3	38±3	NS
P_{v,O_2} mmHg					
Baseline	62±4	51±3	32±2	26±2	<0.01
Bosentan	59±4	52±4	34±4	27±3	<0.01

Data are presented as mean±SEM. p-value: level of significance of the F-ratio of the analysis of variance; Q′: cardiac index; P_{pa}: mean pulmonary artery pressure; P_{pao}: pulmonary artery occluded pressure; P_{sa}: mean systemic artery pressure; f_c: cardiac frequency; pH_a: arterial pH; P_{a,O_2}: oxygen tension in arterial blood; P_{a,CO_2}: carbon dioxide tension in arterial blood; P_{v,O_2}: oxygen tension in mixed venous blood.

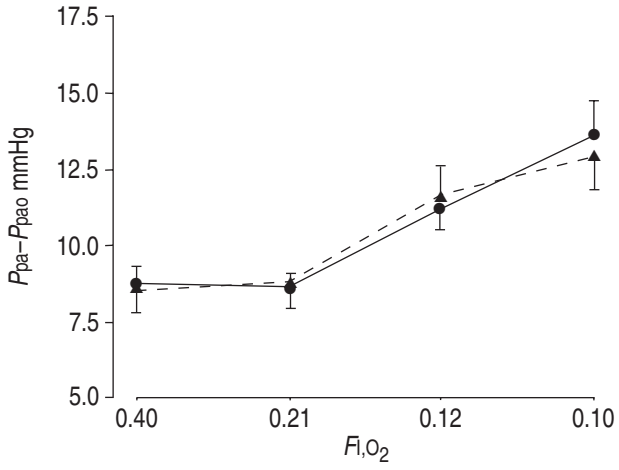

Fig. 1. – Mean pulmonary artery pressure (P_{pa}) minus occluded P_{pa} (P_{pao}) at constant cardiac output in eight dogs as the inspiratory oxygen fraction (F_{I,O_2}) was decreased from 0.4 to 0.1, before (●) and after (▲) the administration of bosentan. Data are presented as mean±SEM. Bosentan had no effect on hypoxic pulmonary vasoconstriction assessed by constant flow hypoxia-induced increase in P_{pa}–P_{pao}.

also reduced the systemic arterial hypertension induced by L-NA in hyperoxia and in hypoxia (table 2).

Effects on exhaled nitric oxide

Exhaled NO was 0.96±0.12 parts per billion (ppb) in hyperoxia and 1.01±0.13 ppb in hypoxia (p=NS, n=9). L-NA decreased exhaled NO to 0.42±0.16 ppb in hyperoxia and 0.39±0.17 in hypoxia (p<0.01 compared to baseline at the same F_{I,O_2}; p=NS hyperoxia compared to hypoxia, n=5). Bosentan alone decreased exhaled NO to 0.17±0.05 ppb in hyperoxia and 0.14±0.07 ppb in hypoxia (p<0.01 compared to baseline at the same F_{I,O_2}, n=4). L-NA followed by bosentan decreased exhaled NO to 0.05±0.03 ppb in hyperoxia and in hypoxia (p<0.01 compared to baseline, n=4).

Discussion

The present results show that endothelin receptor antagonism by bosentan has no effects on pulmonary or systemic haemodynamics in normoxic nor in hypoxic conditions, but that it inhibits the enhancement of acute HPV and the increase in systemic blood pressure observed after NO synthase inhibition. As bosentan decreased exhaled NO, it appears that endothelins inhibit their own vasoconstricting effect by generating NO. Since a combined endothelin A and B receptor antagonist were used, the results

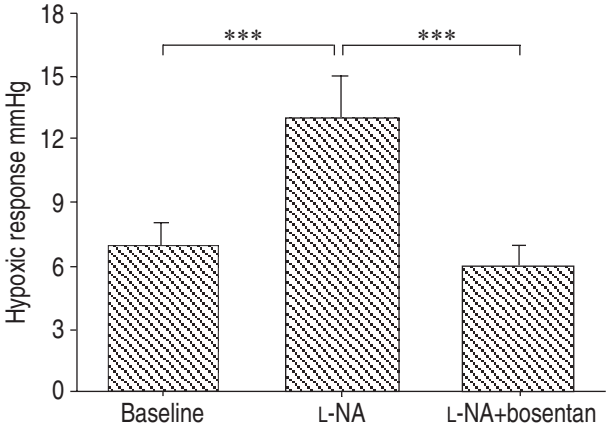

Fig. 2. – Hypoxic response defined as the increase in the gradient between mean pulmonary artery pressure and occluded pulmonary artery pressure measured at constant cardiac output in response to a reduction in the fraction of inspiratory oxygen from 0.4 to 0.1 at baseline, after administration of N^G-nitro-L-arginine (L-NA), and after the addition of bosentan to L-NA in 12 dogs. Data are presented as mean±SEM. The L-NA-induced enhancement of hypoxic pulmonary vasoconstriction was reversed by bosentan. ***: $p<0.001$.

cannot identify the respective effects of these receptors on HPV and exhaled NO.

Pulmonary and systemic vascular tone during hyperoxia and normoxia

The present results are in agreement with a previous observation that combined endothelin A and B receptor blockade by bosentan does not affect pulmonary nor systemic haemodynamics in well-oxygenated dogs [7], and may suggest that endogenous endothelins do not contribute to basal vascular control. However, an alternative explanation could be that a tonic release of endothelins occurs without demonstrable vasoconstriction because of antagonistic release of NO or other endogenous vasodilator mediators [16–19]. The current data suggest that this is the case for the systemic but not the pulmonary circulation, since inhibition of NO synthesis did not affect pulmonary vascular resistance but was associated with a bosentan-reversible increase in systemic vascular resistance [17].

Hypoxic pulmonary vasoconstriction

In the present study, HPV was not affected by bosentan, given at a dose verified to block the vascular effects of the endothelin precursor big endothelin-1. It is of interest that big endothelin-1 alone markedly increased systemic vascular resistance but had no effect on hyperoxic or hypoxic pulmonary vascular resistance. Other authors have also been unable to inhibit HPV with endothelin receptor antagonists, including bosentan [15, 21–23]. Variable results of effects of endothelin receptor blockers on HPV in different experimental models may be explained by variable antagonism of tonic effects of endothelins by circulating or endothelium-derived vasodilating mediators.

Bosentan after nitric oxide inhibition

The current authors previously reported that inhibition of NO synthase enhances HPV in intact anesthetised dogs [24, 25]. Therefore, the effects of endothelin receptor blockade by bosentan after inhibition of NO synthase were tested. The present results confirm that NO synthase inhibition does not affect pulmonary vascular tone in well oxygenated dogs, but that it increases HPV [24, 25]. They additionally show that NO synthase inhibition unmasks a pulmonary vasoconstrictor effect of endothelins but only in acute hypoxia. This result is in agreement with a previous observation that combined endothelin A and B receptor antagonism inhibits the pulmonary vasoconstrictor response to L-NA in isolated perfused lungs from rats with chronic hypoxic pulmonary hypertension [19]. The hypertensive foetal pulmonary circulation also dilates in response to the administration of endothelin receptor antagonists [29, 30]. These studies indicate that pulmonary hypertension may

Table 2. – Effects of bosentan after N^G-nitro-L-arginine (L-NA) in 12 dogs

	Baseline		L-NA		L-NA+bosentan	
	F_{I,O_2} 0.4	F_{I,O_2} 0.1	F_{I,O_2} 0.4	F_{I,O_2} 0.1	F_{I,O_2} 0.4	F_{I,O_2} 0.1
Q' L·min^{-1}·m^{-2}	3.3±0.3	3.4±0.3	3.2±0.3	3.2±0.3	3.1±0.3	3.3±0.3
P_{pa} mmHg	12±1	18±2*	19±2¶	27±3*,¶	18±2¶	22±3$^{\#}$
P_{pao} mmHg	4±1	3±1	10±2¶	6±1	8±1$^{\#,¶}$	5±1
P_{sa} mmHg	113±7	109±7	144±8¶	144±7¶	125±7$^{\#,¶}$	130±8$^{\#,¶}$
f_c beats·min^{-1}	149±8	157±8	115±6¶	145±8	121±7¶	145±8
pHa	7.33±0.01	7.35±0.01	7.36±0.01	7.37±0.01	7.37±0.01	7.39±0.02
P_{a,O_2} mmHg	195±12	36±1*	201±7	35±1*	160±14$^{\#,¶}$	32±2*
P_{a,CO_2} mmHg	37±1	33±1*	35±1	34±1	34±1	33±2
P_{v,O_2} mmHg	51±2	24±2*	46±1	22±2*	43±3¶	22±1*

Data are presented as mean±SEM. F_{I,O_2}: inspiratory oxygen fraction; Q': cardiac index; P_{pa}: mean pulmonary artery pressure; P_{pao}: pulmonary artery occluded pressure; P_{sa}: mean systemic artery pressure; f_c: cardiac frequency; pHa: arterial pH; P_{a,O_2}: oxygen tension in arterial blood; P_{a,CO_2}: carbon dioxide tension in arterial blood; P_{v,O_2}: oxygen tension in mixed venous blood. *: $p<0.05$ compared with F_{I,O_2} 0.4, same drug condition; $^{\#}$: $p<0.05$ compared with L-NA, same F_{I,O_2}; ¶: $p<0.05$ compared with baseline, same F_{I,O_2}.

result from an imbalance of endothelin-1 receptor activation favouring pulmonary vasoconstriction [29], or from a combination of enhanced endothelin A receptor-mediated vasoconstriction together with a decreased NO synthase activity [30].

In the present study, bosentan reduced L-NA-induced systemic hypertension. In anesthetised rats, mixed endothelin A and endothelin B receptor antagonism and selective endothelin A receptor antagonism did not affect P_{sa} when given alone, but decreased it after NO synthase inhibition [16, 17]. Blockade of NO synthase also increased the plasma levels of endothelin-1 [17]. Compared to normotensive controls, hypertensive patients had larger vasodilation in response to endothelin antagonism and smaller vasoconstriction in response to NO synthase inhibition, both responses being inversely correlated [2]. In accordance with these results, the present data suggest that the systemic hypertensive effect of NO synthase inhibition could be endothelin-mediated.

Exhaled nitric oxide

As expected, a decrease in exhaled NO was observed after NO synthase inhibition. The current results additionally show, for the first time, that bosentan also reduces exhaled NO. This observation suggests that the vasoconstrictive effects of endothelins are self-limited by an endothelin-induced synthesis of endogenous NO. Although it is generally proposed that endothelial NO synthesis is related to endothelin B receptor stimulation [1, 31, 32], a recent study shows that activation of endothelin A receptors also can enhance NO production in the vasculature [33]. Patients with acute respiratory distress syndrome have arterial hypoxaemia partially due to inefficient HPV, possibly related to the release of NO [34]. The current results show that bosentan does not inhibit HPV and decreases NO release. It could be speculated that bosentan might be given to reduce pulmonary hypertension in patients with ventilation-to-perfusion impairment, without further deteriorating gas exchange, as usually observed after systemic administration of vasodilators. This hypothesis could be tested in experimental models of lung injury.

Clinical relevance and conclusions

The current authors propose that endothelins stimulate nitric oxide production in pulmonary vessels, which counteracts their own constricting effect, and that the vasoconstricting action of endothelins can appear only when endogenous nitric oxide synthesis is deficient. The present results are in keeping with the concept that increased pulmonary vascular tone may result from an imbalance between the antagonistic effects of nitric oxide and endothelins, and that pharmacological manipulation of this imbalance offers therapeutic possibilities in pulmonary hypertension. The lungs of patients with severe pulmonary hypertension show a reduced expression of endothelial nitric oxide synthase [35] and an increased expression of endothelin-1 [36]. Endothelin receptor antagonism with bosentan has been reported recently to decrease pulmonary vascular resistance and to improve functional class and exercise capacity in patients with primary pulmonary hypertension [5, 6].

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