we did not evaluate possible changes in plasma MR-proADM levels before and after specific management of concomitant cardiovascular diseases or before or after reduction of excessive blood volume during the interval between haemodialysis therapies. Therefore, further investigations should be conducted that will examine these additional factors.

In conclusion, the present results suggest that plasma MR-proADM concentrations may increase in association with cardiac dysfunction, excessive blood volume and systemic inflammation. These levels may provide a possible index of these conditions in haemodialysis patients with concomitant cardiovascular disease. A large prospective population-based study will be necessary to confirm these preliminary observations.

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Conflict of interest statement. None declared.

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Possible role of adrenomedullin in the regulation of Fontan circulation: Mature form of plasma adrenomedullin is extracted in the lung in patients with Fontan procedure

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Abstract

Objective: We investigated the pathophysiological significance of molecular forms of adrenomedullin (AM) in patients after the Fontan procedure.
Methods: Plasma concentrations of mature AM (AM-m), an active form, glycine-extended AM (AM-Gly), an inactive form, and total AM (AM-T: AM-m+AM-Gly) were measured by specific immunoradiometric assay in the femoral vein, pulmonary artery and femoral artery of 29 consecutive patients after the Fontan procedure. The eleven patients who had history of Kawasaki disease and have normal coronary and hemodynamics served as control.
Results: Patients who underwent Fontan procedure had significantly higher venous concentrations of AM-T, AM-Gly, and AM-m than age-matched normal controls (AM-T, 12.0±3.3 vs. 9.6±2.0; AM-Gly, 10.4±3.0 vs. 8.5±1.6; AM-m, 1.6±0.7 vs. 1.0±0.6 pmol/l, each $p<0.05$). In patients with Fontan procedure, there were no differences in plasma AM-T, AM-Gly or AM-m levels between the femoral vein and pulmonary artery, however, there was a significant step-down in the AM-m levels, but not in plasma AM-T or AM-Gly levels, between the pulmonary artery and femoral artery (1.3±0.6 to 1.0±0.6, $p<0.05$). The venous concentrations of AM-m correlated negatively with systemic blood flow (cardiac output) ($r=-0.46$, $p<0.05$).
Conclusions: Results suggest that in Fontan circulation plasma AM-m is increased in parallel with those of AM-T and AM-Gly and that AM-m is extracted in the lung. Extracted AM-m may be involved in the regulation of pulmonary arterial tonus, although further studies are necessary to elucidate the exact role of AM in Fontan circulation.

Keywords: Adrenomedullin; Mature form of adrenomedullin; Fontan procedure

1. Introduction

The patients who underwent the Fontan procedure have a unique circulation, so-called the Fontan circulation, which is lacking in the right heart pump. In the Fontan circulation, central venous pressure is elevated and cardiac output is reduced. Fontan circulation is not simple systemic pump failure; because systemic ventricular filling pressure is not high and systemic ventricular volume is not increased. Fontan circulation has reduced cardiac output and high pulmonary vascular tone for lack of right heart pump.

Adrenomedullin (AM) is a hypotensive 52-amino-acid peptide that was originally discovered in acid extracts from human

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pheochromocytoma [1]. AM mRNA is reported to be highly expressed in the adrenal gland, lung, kidney, heart and vascular walls [1–3]. In addition, AM binding sites are highly expressed in rat lung [4]. Recent studies have demonstrated that AM receptor mRNA is abundantly expressed in human lung tissue [5]. These results suggest an importance of AM in the pulmonary arterial tonus. Indeed, AM has been shown to preferentially dilate the pulmonary vessels and increase pulmonary blood flow [6,7]. In addition, high plasma AM levels have been reported in rats with experimentally induced pulmonary hypertension and in patients with primary and secondary pulmonary hypertension [8–10]. These findings suggest the possibility that circulating AM may participate in the control of pulmonary vascular tone in pathological condition.

The human AM precursor consists of 185 amino acids, including a putative signal peptide [11]. AM is produced from the AM precursor by a two-step enzymatic reaction. First the AM precursor is converted into C-terminally glycine-extended AM (AM-Gly), a 53-amino-acid peptide that represents an intermediate, inactive form of AM. Subsequently, inactive AM-Gly is converted into the mature, active form of AM (AM-m), a 52-amino-acid peptide with a C-terminal amide structure, by enzymatic amidation. Kitamura et al. previously reported that both AM-m and AM-Gly circulate in human blood [12]. However, the ratio of these two molecular forms of plasma AM, as well as their production and clearance sites and their pathophysiological significance in patients after the Fontan procedure, remains unknown.

The present study aimed to explore the production and clearance sites and the pathophysiological significance of these two molecular forms of AM in patients with the Fontan procedure. Our hypothesis is that plasma AM-m is increased in the Fontan circulation and plays a role in this condition. To investigate this hypothesis, we measured plasma AM-m and total AM (AM-T; AM-T = AM-m + AM-Gly) concentrations in blood samples obtained from the femoral vein, pulmonary artery and femoral artery of 29 consecutive patients after the Fontan procedure, using a specific immunoradiometric assay [13,14].

2. Methods

This protocol was approved by the institutional ethical committee.

2.1. Patients

We studied 29 consecutive patients after the Fontan procedure (15 females and 14 males aged 1.4 to 22.6 years; mean age of 9.7±5.9 years) who underwent follow-up cardiac catheterization under local anesthesia. Their diagnoses were all functionally univentricular hearts. The techniques of the Fontan procedure were as follows: atrio-pulmonary connection in four patients, total cavo-pulmonary connection with intra-atrial rerouting in six patients, total cavo-pulmonary connection with intra-atrial grafting in nine patients, total cavo-pulmonary connection with-extra-cardiac grafting in ten patients. Mean age at the Fontan procedure was 3.8±2.4 years, mean age at the examination was 9.7±5.9 years, and mean period between the Fontan procedure and the examination was 5.8±4.9 years. We selected as age- and gender-matched control subjects 11 patients with a history of Kawasaki disease without coronary artery dilation (5 females and 6 males aged 0.9 to 17.3 years; mean age 7.8±5.5 years) who had normal hemodynamics and cardiac function. They had no other abnormality. Cardiac catheterization was performed, mean 2016 days (61 days to 5732 days, median 1938 days) after activity of Kawasaki disease was relieved. No inflammatory response was observed in any patients. In our institution, we have been doing cardiac catheterization only in patients with Kawasaki disease who had coronary artery dilatation in acute phase, because some of them develop coronary artery stenosis. As regard to the blood sampling, informed consent was obtained from the participants and/or their parents prior to initiation of the study.

2.2. Cardiac catheterization

All patients underwent right- and left-sided heart catheterization. The procedure included: (1) measurement of mean central venous pressure, mean pulmonary arterial pressure, mean pulmonary capillary wedge pressure, systemic ventricular end-diastolic pressure, and mean systemic arterial pressure, (2) measurement of oxygen saturation in pulmonary artery, systemic ventricle, systemic artery, and the determination of mixed vein saturation by using superior and inferior vena cava, (3) calculation of pulmonary blood flow, systemic blood flow (cardiac output) by using the oxymetric principle of Fick [15], and pulmonary vascular resistance, and (4) volummetry of systemic ventricular end-diastolic volume and systemic ventricular end-systolic volume by using Shimpson's method, and calculation of systemic ventricular ejection fraction.

Table 1
Haemodynamic variables in patients after the Fontan procedure and controls

Variable	Fontan	Control	*p* Value
CVP (mm Hg)	12.0 (2.2)	3.5 (1.9)	<0.0001
mPAP (mm Hg)	11.6 (2.7)	13.4 (1.6)	<0.05
PCWP (mm Hg)	7.5 (2.5)	7.2 (2.6)	NS
SVEDP (mm Hg)	9.0 (3.7)	10.2 (3.8)	NS
mSAP (mm Hg)	85.3 (9.8)	82.8 (6.9)	NS
MVsat (%)	64.3 (6.9)	74.9 (2.7)	<0.0001
SA sat (%)	93.7 (3.7)	97.7 (1.2)	<0.005
Hb (mg/dl)	13.9 (2.0)	13.2 (2.1)	NS
Qs (l/min/m^2)	2.8 (0.7)	3.8 (0.9)	<0.001
Rp (units·m^2)	1.6 (0.5)	1.6 (0.5)	NS
SVEDV (percent of normal)	88.7 (27.0)	81.8 (15.5)	NS
SVEF	0.50 (0.10)	0.62 (0.06)	<0.005
ANP (pg/ml)	39.9 (47.8)	18.5 (12.5)	NS

Values are means (SD) unless otherwise stated.
ANP, atrial natriuretic peptide; CVP, central venous pressure; Hb, haemogrobin; mPAP, mean pulmonary arterial pressure; mSAP, mean systemic arterial pressure; MVsat, oxygen saturation in mixed vein; NS, not significant; PCWP, pulmonary capillary wedge pressure; Qs, systemic blood flow; Rp, pulmonary vascular resistance; SAsat, oxygen saturation in systemic artery; SVEDP, systemic ventricular end-diastolic pressure; SVEDV, systemic ventricular end-diastolic volume; SVEF, systemic ventricular ejection fraction.

2.3. Blood sampling

Blood samples were obtained via catheters from the femoral vein, pulmonary artery and femoral artery of patients after the Fontan procedure and controls. The blood was transferred immediately into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) for the measurement of plasma concentrations of AM-m, AM-T (=AM-m+AM-Gly) and atrial natriuretic peptide. The blood was centrifuged immediately at 4 °C, and the plasma was frozen and stored at −80 °C until assayed.

2.4. Assays

Both AM-m and AM-T were measured using recently developed specific immunoradiometric assay kits (AM mature RIA and AM total RIA; Shionogi Co., Osaka, Japan) [13,14].

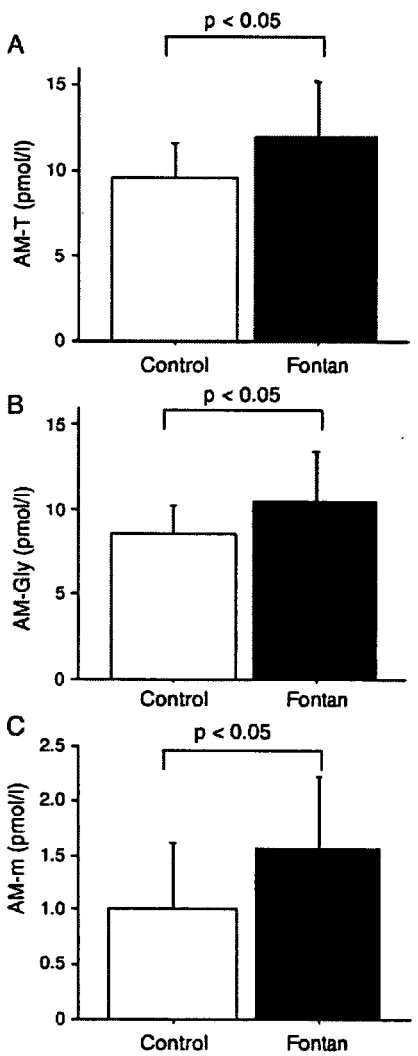

Fig. 1. Plasma concentrations of total adrenomedullin (AM-T) (A), glycine-extended adrenomedullin (AM-Gly) (B), and mature adrenomedullin (AM-m) in femoral vein in normal controls (control) and patients with the Fontan procedure (Fontan). Values are means±SD.

Table 2
Plasma concentrations of total adrenomedullin (AM-T), glycine-extended adrenomedullin (AM-Gly) and mature adrenomedullin (AM-m) in the femoral vein, pulmonary artery and femoral artery in patients after the Fontan procedure

Vessel	Plasma concentration (pmol/l)		
	AM-T	AM-Gly	AM-m
Femoral vein	11.7(3.4)	10.3(3.1)	1.4(0.5)
Pulmonary artery	11.5(4.2)	10.0(3.6)	1.3(0.6)
Femoral artery	11.7(5.1)	10.7(4.9)	1.0(0.6)*,†

Data are expressed as means(SD). Significance of differences: *$p<0.05$ compared with femoral vein; †$p<0.05$ compared with pulmonary artery.

These assay systems use two monoclonal antibodies against human AM, one recognizing a ring structure of human AM (both kits) and the other recognizing either the C-terminal sequence (AM-m kit) or AM-(25–36) (AM-T kit). The assay measures human AM-m or AM-T by sandwiching it between the two antibodies without the extraction of plasma. The minimum quantity of human AM-m or AM-T detectable using these assays is 0.5 pmol/l (both kits). The plasma AM-Gly concentration was calculated using the following formula: AM-Gly=AM-T−AM-m. Plasma concentrations of atrial natriuretic peptide were measured using the Shiono RIA ANP assay kit (Shionogi Co.), as reported previously [16].

2.5. Statistical analysis

All data are expressed as means±standard deviation. Student's unpaired *t*-test was used to evaluate differences between the controls and the patients with the Fontan procedure. The comparisons of plasma AM concentrations between the femoral vein, pulmonary artery and femoral artery were done by nested ANOVA followed by Scheffé's test. The correlation coefficients were calculated by linear regression analysis. Multiple regression analysis was applied to analyze dependence between variables. A value of $p<0.05$ was considered statistically significant.

Table 3
Correlation coefficients between AM levels and hemodynamic variables

Variables	AM-T(FV)		AM-m(FV)		AM-Gly(FV)	
	r	*p* Value	*r*	*p* Value	*r*	*p* Value
CVP (mm Hg)	−0.248	NS	0.051	NS	−0.286	NS
mPAP (mm Hg)	−0.028	NS	0.215	NS	−0.08	NS
PCWP (mm Hg)	−0.147	NS	0.162	NS	−0.2	NS
SVEDP (mm Hg)	−0.334	NS	0.004	NS	−0.372	NS
mSAP (mm Hg)	−0.101	NS	−0.155	NS	−0.077	NS
MVsat (%)	**−0.53**	**<0.005**	−0.201	NS	**−0.54**	**<0.005**
SA sat (%)	−0.165	NS	0.128	NS	−0.21	NS
Hb (mg/dl)	−0.093	NS	0.107	NS	−0.125	NS
Qp (l/min/m^2)	−0.133	NS	−0.343	NS	−0.071	NS
Qs (l/min/m^2)	−0.291	NS	**−0.46**	**<0.05**	−0.129	NS
Rp (units·m^2)	0.261	NS	0.344	NS	0.212	NS
SVEDV (percent of normal)	0.062	NS	−0.104	NS	0.093	NS
SVEF	−0.093	NS	−0.276	NS	−0.038	NS
ANP (pg/ml)	0.027	NS	0.292	NS	−0.034	NS

NS, not significant, Other abbreviations as in Table 1.

3. Results

3.1. Hemodynamic characteristics

Hemodynamic variables in patients after the Fontan procedure and controls are presented in Table 1. These patients after the Fontan procedure are characterized by higher mean central venous pressure, and a lower mean pulmonary arterial pressure, oxygen saturation in mixed vein, oxygen saturation in systemic artery, systemic blood flow and systemic ventricular ejection fraction. Thus, these patients had characteristics of Fontan hemodynamics.

3.2. Plasma AM concentrations

The venous plasma concentrations of AM-m, AM-Gly and AM-T in the control subjects and in the patients after the Fontan procedure are shown in Fig. 1. Venous plasma AM-m, AM-Gly, and AM-T levels in the patients with the Fontan procedure were higher than those in the age-matched controls; the extent of the increase was similar for all forms (all $p<0.05$). There was a good relationship between the levels of AM-m and AM-T ($r=0.54$, $p<0.005$). There were no differences in the concentrations of AM-m between the femoral vein and pulmonary artery (Table 2). However, the concentrations of AM-m were significantly ($p<0.05$) lowered in the femoral artery compared with the pulmonary artery and femoral vein (Table 2). In contrast, there were no differences in plasma AM-T and AM-Gly levels between any of the sample sites (Table 2).

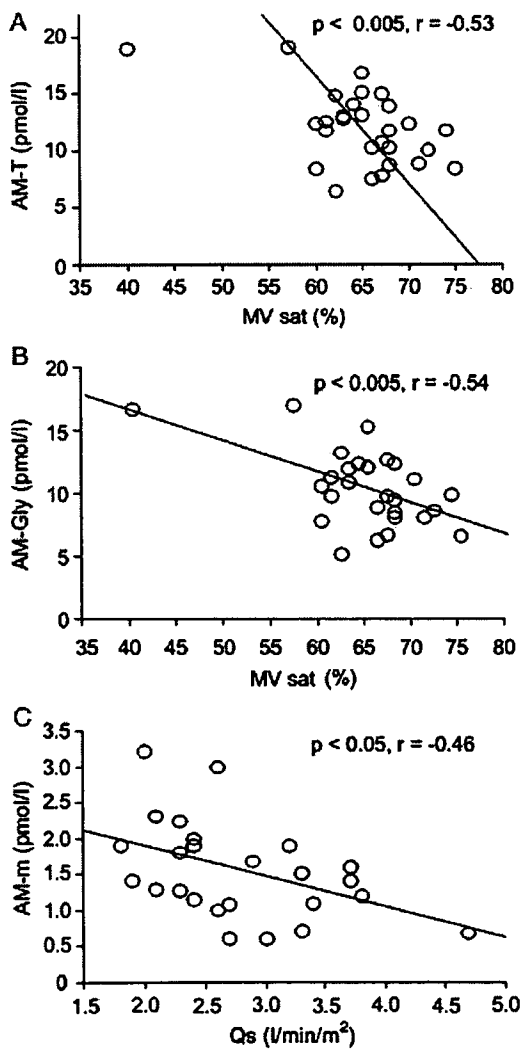

Fig. 2. Relationships between plasma concentrations of total adrenomedullin (AM-T) and oxygen saturation in mixed vein (MVsat) (A), glycine-extended adrenomedullin (AM-Gly) and oxygen saturation in mixed vein (MVsat) (B), mature adrenomedullin (AM-m) and systemic blood flow (Qs) (C) in femoral vein in patients after the Fontan procedure.

Table 4
Multiple regression analysis

Variable	AM-T (FV)	AM-Gly (FV)	AM-m (FV)
	R squad	*R* squad	*R* squad
	0.328	0.357	0.301
	p Value	*p* Value	*p* Value
CVP	NS	NS	NS
mSAP	NS	NS	NS
SVEDP	NS	NS	NS
MVsat	<0.05	<0.05	NS
Qs	NS	NS	<0.05

NS, not significant, Other abbreviations as in Table 1.

3.3. Relationships of plasma AM-m, AM-Gly and AM-T levels with hemodynamic variables

Correlations between the venous concentrations of AM and the hemodynamic variables are shown in Table 3 and Fig. 2. Plasma AM-T and AM-Gly levels negatively correlated with oxygen saturation of mixed vein (AM-T: $r=-0.53$, $p<0.005$; AM-Gly: $r=-0.54$, $p<0.005$), whereas plasma AM-m levels correlated negatively with systemic blood flow ($r=-0.46$, $p<0.05$) (Fig. 2). Multiple regression analysis also showed that plasma AM-T and AM-Gly correlated MVsat, whereas AM-m was correlated with Qs (Table 4).

4. Discussion

According to the study of Kitamura et al. [12], plasma AM levels reported previously were levels of AM-T, being the sum of AM-m and AM-Gly, because the radioimmunoassay system used polyclonal antibodies, which could not distinguish the structures with or without a C-terminal amide. For this reason, in order to measure the molecular forms of plasma AM, Kitamura et al. used two kinds of radioimmunoassay systems after the extraction of large amounts of plasma [12]. A one-step direct immunoradiometric assay system for the measurement of AM-m and AM-T using different monoclonal antibodies has been developed recently [13,14], which enables us to measure AM-m and AM-T specifically in a small sample without the prior extraction of plasma. The immunoradiometric assay for AM-m has no cross-reactivity with AM-Gly or other inactive metabolites of AM, and the immunoradiometric assay for AM-T

recognizes both AM-m and AM-Gly. In the present study, using this immunoradiometric assay for AM-T, we showed that plasma AM-T levels were increased in patients with the Fontan procedure compared with normal controls. We also showed that plasma AM-m and AM-Gly levels were increased significantly in patients with the Fontan procedure compared with normal controls. In addition, plasma AM-Gly and AM-T levels showed similar negative correlations with oxygen saturation of mixed vein, whereas plasma AM-m levels showed negative correlations with systemic blood flow. These findings indicate that the production of AM-m and AM-Gly is regulated similarly under pathological conditions such as the Fontan circulation.

AM-m represented approximately 13% of AM-T in the present study, suggesting that the major circulating form of AM is AM-Gly. This finding is consistent with a previous report [12]. The low AM-m/AM-T ratio in plasma may be explained by a low level of secretion of AM-m from tissues, and/or by a longer half-life of AM-Gly. Cultured endothelial cells and vascular smooth muscle cells produce large amounts of AM-m and small amounts of AM-Gly [2,3,17]. However, as AM acts as an autocrine and/or paracrine factor, AM-m produced in the tissues may be consumed almost entirely via receptor binding, and only small amounts of AM-m may be released into the circulation. In contrast, AM-Gly, an inactive form, cannot bind to the receptors, and therefore most of the AM-Gly produced may be released into the circulation. Furthermore, the low biological activity of AM-Gly suggests that the half-life of AM-Gly is longer than that of AM-m. This expected longer half-life of AM-Gly may, at least in part, be responsible for the low AM-m/AM-T ratio in human plasma.

In the present study, the plasma AM-m levels were markedly lower in the femoral artery than in the pulmonary artery, although there were no differences in plasma AM-Gly and AM-T levels between the femoral artery and pulmonary artery. An autoradiographic study showed that an intravenous injection of ^{125}I-AM was strongly taken up by the lung, suggesting that the lung has abundant binding sites for AM [18]. In addition, a previous study reported that AM binding sites were highly concentrated in the lung [4]. Furthermore, a recent study revealed that AM receptor mRNA is highly expressed in human lung tissue [5]. Indeed, several studies have shown that plasma AM was partially metabolized in the pulmonary circulation of patients with primary and secondary pulmonary hypertension and ischemic heart disease [10,19,20]. The present study demonstrated that this observed extraction of plasma AM in the lung is due to the extraction of AM-m. A study by Hirayama et al. showed that plasma AM-m levels were lower in the pulmonary capillary wedge portion than in the pulmonary artery portion in patients with ischemic heart disease [17], which is in good agreement with the present study. As regards the action of AM, previous studies have demonstrated that AM preferentially and strongly dilates pulmonary vessels over systemic vessels in animals [6,7,21,22]. Furthermore, a recent study reported that the pulmonary vasodilatory activity of AM is much more potent than that of acetylcholine or ATP on a molar basis in patients with pulmonary hypertension [23]. Many neurohumoral factors including nitric oxide, endothelin, norepinephrine, atrial natriuretic peptide, brain natriuretic peptide and renin–angiotensin are reported to be activated in Fontan circulation [24] and it is possible that these neurohumoral factors may modulate the pulmonary arterial tonus in this condition. The balance between vasoconstricting factors and vasodilating factors are important determinant for pulmonary vascular resistance. Indeed, a recent study demonstrated that imbalance between increased ET-1 and relatively decreased AM contributed to dominant effects of ET-1, leading pulmonary vasoconstriction after the Fontan procedure [25]. In addition, endothelin antagonist significantly reduced pulmonary artery resistance in Fontan circulation [26]. Moreover, inhalation of nitric oxide significantly attenuated pulmonary artery resistance in this condition [27]. Thus, many neurohumoral factors modulate the pulmonary artery resistance in Fontan circulation and AM also may be involved in the regulation of pulmonary vascular resistance in this condition.

In conclusion, the major molecular form of circulating AM is AM-Gly in normal subjects and in patients with the Fontan procedure, and plasma AM-m levels increase in parallel with those of AM-Gly in the Fontan circulation. AM-m is produced in the peripheral circulation, and the main site for clearance of circulating AM-m is the lung. Extracted AM-m may be involved in the regulation of pulmonary arterial tonus, although further studies are necessary to elucidate the exact role of AM in Fontan circulation.

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PULMONARY VASCULAR REACTIVITY OF SPONTANEOUSLY HYPERTENSIVE RATS IS EXACERBATED IN RESPONSE TO THE CENTRAL ADMINISTRATION OF EXOGENOUS NITRIC OXIDE

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SUMMARY

1. Centrally, nitric oxide (NO) is a sympathoinhibitory substance. Spontaneously hypertensive rats (SHR) have an impaired central nitroxidergic system and, consequently, NO-mediated decrease in sympathetic activity is exacerbated in SHR compared with Wistar-Kyoto (WKY) rats. We have demonstrated previously that acute hypoxic pulmonary vasoconstriction (HPV) is enhanced by central NO administration. Therefore, in the present study, we hypothesized that accentuation of the HPV by NO would be exacerbated in SHR compared with WKY rats.

2. Mean pulmonary arterial pressure, systemic mean arterial blood pressure, cardiac output and heart rate were measured in pentobarbitone-anaesthetized, artificially ventilated, male SHR and WKY rats. The brief, transient response to a bolus intracerebroventricular (i.c.v.) dose of N^G-nitro-L-arginine methyl ester (L-NAME; 150 μg in 10 μL) was recorded in all rats. Upon recovery, rats were exposed to acute hypoxia (10% O_2 for 4 min) before and after the i.c.v. administration of the NO donor 3-[4-morpholinyl]-sydnonimine-hydrochloride (SIN-1; 100 μg in 10 μL).

3. In WKY rats, central inhibition of NO synthesis by L-NAME caused a mild increase in tonic pulmonary vascular tone and induced a large systemic pressor response. These responses were not observed in SHR. In contrast, SIN-1 failed to alter tonic pulmonary vascular tone, although it enhanced the HPV in WKY rats and, significantly more so, in SHR.

4. These results confirm that accentuation of the HPV by NO is exacerbated in SHR compared with WKY rats. The mechanism(s) by which the HPV is accentuated by central NO remains to be fully elucidated, but is likely to be associated with the sympathoinhibitory effects of NO and, if so, supports the idea that the nitroxidergic system of the SHR is impaired. Further electrophysiological studies are essential to confirm these assumptions.

Key words: hypertensive rats, hypoxic pulmonary vasoconstriction, nitric oxide, sympathetic nervous system.

INTRODUCTION

Ever since its discovery, nitric oxide (NO) has been implicated in a diverse range of physiological functions. In the past decade, several studies have implicated NO as an important central neuromodulator involved in the neural regulation of cardiovascular function, independent of its direct effects on vascular smooth muscle cells.[1] Centrally, NO is produced within various parts of the brain, including the nucleus tractus solitarius (NTS) of the medulla oblongata and the ventrolateral medulla, important cardiovascular regulatory centres.[2–4] Central blockade of NO using L-arginine analogues, such as N^G-nitro-L-arginine methyl ester (L-NAME), enhances sympathetic outflow and, subsequently, arterial blood pressure (ABP).[3,5–7] Conversely, central administration of exogenous NO has been shown to decrease sympathetic outflow and ABP.[8,9] A review by Patel *et al.*[10] concluded that the general consensus in the literature was that NO acts as a sympathoinhibitory substance within the central nervous system.

Spontaneously hypertensive rats (SHR) are characterized by a high ABP and enhanced systemic vascular reactivity to various vasoconstrictor agonists[11] compared with normotensive Wistar Kyoto (WKY) rats, the normotensive control for SHR. There is accumulating evidence in the literature that implicates an elevated sympathetic outflow as a contributing factor to the hypertension of SHR.[12–14] Furthermore, impairment of the central nitroxidergic system has been linked to the enhanced sympathetic tone in SHR.[14–17] Cabrera *et al.*[18] demonstrated that the pressor response to central NO inhibition was considerably attenuated in SHR compared with WKY rats. Moreover, the systemic depressor response to an increase in central NO levels is significantly exacerbated in SHR compared with WKY rats.[15,18,19]

Although an impaired central nitroxidergic system in SHR has been implicated as a contributing factor for systemic hypertension, there have not been any studies describing the central role of NO in the modulation and reactivity of the pulmonary vasculature in SHR. Evidence in the literature suggests that SHR have mild pulmonary arterial hypertension compared with that of WKY rats,[20,21] that acute hypoxic pulmonary vasoconstriction (HPV) is attenuated[20] and that chronic hypoxia-induced pulmonary hypertension is accentuated.[22] Whether impairment of the central nitroxidergic system in SHR is associated with dysfunction of the pulmonary vasculature is currently unknown.

We have demonstrated previously that the central administration of exogenous NO enhances acute HPV, although central endogenous NO does not appear to have a significant role in the tonic modulation of pulmonary arterial pressure (PAP).[23] Even though the underlying

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mechanisms remain to be fully elucidated, enhancement of the HPV is likely to involve the modulation of sympathetic outflow by central NO. In the present study, we aimed to ascertain the significance of the central nitroxidergic system in modulating baseline pulmonary vascular tone in SHR and, furthermore, to establish whether enhancement of the hypoxic pulmonary vasoconstrictor response by exogenous central NO is exacerbated in SHR compared with WKY rats.

METHODS

Animals

Experiments were conducted on age-matched 8–10-week-old male WKY rats ($n = 6$, bodyweight approximately 250 g) and SHR ($n = 6$; bodyweight approximately 230 g). All rats were maintained on a 12 h light/dark cycle at 25 ± 1°C and were provided with food and water *ad libitum*. All experiments were approved by the local Animal Ethics Committee and conducted in accordance with the guidelines of the Physiological Society of Japan.

Anaesthesia and surgery

Rats were initially anaesthetized with pentobarbitone sodium (45 mg/kg, i.p.) and supplementary doses of anaesthetic were administered periodically throughout the experimental protocol (15–30 mg/kg per h, i.p.) so as to maintain a constant surgical level of anaesthesia (assessed by using the limb withdrawal reflex test). Rectal temperature was maintained at 38°C using a rectal thermistor coupled to a thermostatically controlled heating pad.

Using a stereotaxic frame, the blunt tip of a 27 gauge stainless-steel cannula was positioned in the right lateral cerebral ventricle based on the coordinates of Paxinos and Watson[23] (0.8 mm posterior to the bregma, 1.5 mm lateral to the midline and 5.0 mm ventral to the skull surface). The distal end of the cannula was connected to a 0.5 mL syringe for subsequent drug administration. Correct positioning of the i.c.v. catheter was confirmed after each experiment by staining with Evans blue dye (10 μL).

The trachea was cannulated and the lungs ventilated with a rodent ventilator (SN-480-7; Shinano, Tokyo, Japan). The inspirate gas was enriched with O_2 (approximately 50% O_2) and the ventilator settings were adjusted (tidal volume (V_T) approximately 3.5 mL; frequency approximately 80 /min) to maintain normocapnic arterial P_{CO_2}. The femoral artery was cannulated for measurement of systemic arterial blood pressure. A right thoracotomy was made between the second and third ribs and the conus of the right ventricle was exposed. A 23 gauge needle was used to pierce the ventricle wall and then the gel-filled sensing catheter of a telemetric transmitter (model TA11PA-C20; Data Sciences International, St Paul, MN, USA) was inserted anteriorly into the right ventricle and advanced into the pulmonary artery. The catheter was fixed in position with a 7.0 prolene suture to the pericardium. The aorta was blunt-dissected free from the pulmonary artery and a transonic perivascular flowprobe (model 2RB; Transonic, Ithaca, NY, USA) was positioned around the ascending aorta for continuous measurement of cardiac output.

Drugs

Artificial cerebrospinal fluid (a-CSF) was used as the vehicle for administering the NO synthase (NOS) inhibitor L-NAME (Sigma, St Louis, MO, USA) and the slow-releasing NO donor, 3-[4-morpholinyl]-sydnonimine-hydrochloride (SIN-1; Tocris Cookson, Avonmouth, UK). The composition of the a-CSF (pH 7.36–7.43) was (in mmol/L): NaCl 150; KCl 3.0; $MgCl_2$ 0.8; $CaCl_2$ 1.4; Na phosphate 1.0. Intracerebroventricular (i.c.v.) injections were given as a 10 μL bolus, over a 30 s period.

Experimental protocols

Once all variables had stabilized after surgery (approximately 20 min), baseline values were recorded for 10 min in response to the i.c.v. administration of a-CSF (10 μL). Rats then received a bolus i.c.v. injection of L-NAME (150 μg). The transient response to L-NAME was of a short duration, such that all cardiovascular variables had returned to pre-injection values within approximately 4 min. Therefore, it was not possible to assess the effects of central NOS inhibition on the cardiovascular responses to acute hypoxia. We have reported previously that acute central NOS inhibition does not modify the cardiovascular responses to acute hypoxia.[24] After fully recovering from the bolus dose of L-NAME (> 10 min), rats were given a bolus i.c.v. injection of vehicle and then exposed to hypoxia (10% O_2 balanced by N_2) for 4 min. Once all variables had recovered from hypoxia, rats received a bolus i.c.v. injection of SIN-1 (100 μg). All cardiovascular variables reached a new steady state within 10 min, after which the acute hypoxic test was repeated. Preliminary experiments indicated that the cardiovascular responses to SIN-1 were not modified by pretreatment with L-NAME in either SHR or WKY rats.

Data acquisition and analysis

Pulmonary arterial pressure was measured using telemetry. The signal from the implanted transmitter (model TA11PA-C20; Data Sciences) was calibrated in reference to an input from an ambient-pressure monitor (C11PR; Data Sciences) and subsequently relayed to a personal computer. Cardiac output (CO) was measured continuously from the ascending aorta using a transonic small animal blood flowmeter (model T206; Transonic Systems) with a flow probe (model 2RB). Arterial blood pressure (ABP) was measured continuously with a Deltran pressure transducer (Utah Medical Products, Midvale, UT, USA) and the signal was relayed to a Powerlab bridge amplifier (ML117; ADInstruments, Tokyo, Japan).

The signals for ABP, CO and PAP were relayed from their respective units and sampled continuously at 200 Hz with an eight-channel MacLab/8s interface hardware system (ADInstruments) and recorded on a Macintosh Power Book G4 (Apple, Cupertino, CA, USA) using Chart (v. 5.0.1; AD Instruments). Heart rate (HR) was derived from the arterial systolic peaks. Cardiac output was normalized (off-line) to 100 g bodyweight. Total systemic vascular resistance (SVR) and total pulmonary vascular resistance (PVR) were calculated by dividing mean ABP (MABP) and mean PAP (MPAP) by the normalized CO. A 10 s block of data was analysed –60 and –30 s and immediately before (time '0') acute hypoxic exposure and then after 20, 40, 60, 90, 120, 180 and 240 s of exposure to 10% O_2. Normoxic data for individual rats in each group were averaged from values acquired at –60 and –30 s and 0 time. An arterial blood sample (0.1 mL) was extracted 5 min after the completion of surgery for analyses of arterial P_{CO_2} using an ABL 605 blood gas analyser (Radiometer, Copenhagen, Denmark).

Statistical analysis

All statistical analyses were conducted using Statview (v. 5.01; SAS Institute, Cary, NC, USA). All results are presented as the mean ± SEM. Two-way ANOVA (repeated measures) was used to test significance for: (i) changes in each variable in response to 4 min of acute hypoxia; and (ii) whether the hypoxic response was altered by drug administration (i.c.v.).

One-way ANOVA (factorial) was used to test for differences in baseline values for WKY rats and SHR. Where statistical significance was reached, post hoc analyses were incorporated using the paired or unpaired *t*-test with Dunnet's correction for multiple comparisons. $P \leq 0.05$ was predetermined as the level of significance for all statistical analyses.

RESULTS

Baseline cardiovascular data for WKY rats and SHR are presented in Table 1. Pulmonary arterial pressure and PVR of SHR were significantly greater ($P < 0.001$) than those of WKY rats. As expected, both systemic arterial pressure (MABP) and SVR were significantly higher in SHR compared with WKY rats. There was no significant difference in CO or HR between WKY rats and SHR.

Table 1 Baseline cardiovascular variables of Wistar-Kyoto and spontaneously hypertensive rats

	WKY rats ($n = 6$)	SHR ($n = 6$)
MPAP (mmHg)	16.3 ± 0.7	19.2 ± 0.7*
MABP (mmHg)	91 ± 5	138 ± 7**
CO (mL/min per 100 g)	12.6 ± 1.0	11.2 ± 0.6
HR (b.p.m.)	377 ± 10	389 ± 13
SVR (mmHg/mL per min per 100 g)	7.62 ± 0.70	12.59 ± 1.0**
PVR (mmHg/mL per min per 100 g)	1.35 ± 0.13	1.74 ± 0.10*

Data are the mean±SEM. *$P < 0.05$, **$P < 0.01$ compared with Wistar-Kyoto (WKY) rats.

MPAP, mean pulmonary arterial pressure; MABP, mean arterial blood pressure; CO, cardiac output; HR, heart rate; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance; SHR, spontaneously hypertensive rats.

Administration of L-NAME

The cardiovascular response to a single bolus dose of L-NAME (150 μg in 10 μL, i.c.v.) was brief and transient, such that a peak response was achieved after 20–40 s before returning to pre-injection values within 4 min (consequently, we could not test acute hypoxia). The 'maximum' cardiovascular responses to L-NAME are shown in Fig. 1. In WKY rats, L-NAME caused a small, albeit significant, 8 ± 3% increase in PAP ($P < 0.05$), which was due to pulmonary vasoconstriction (9 ± 3% increase in PVR) because there was no significant change in CO. Similarly, L-NAME significantly increased MABP (34 ± 4%) in WKY rats, solely attributed to systemic vasoconstriction (37 ± 8% increase in SVR). Despite the increase in MABP, there was no baroreflex decrease in HR. In SHR, L-NAME failed to have any effect on either the systemic or pulmonary vasculature (Fig. 1).

Responses to acute hypoxia

In WKY rats, acute exposure to 10% O_2 provoked a significant 53 ± 7% increase in MPAP by 4 min (see Fig. 2a), in spite of a decline in CO (26 ± 10%), reflecting substantial pulmonary vasoconstriction (134 ± 39% increase in PVR). In SHR, the magnitude of the HPV, as indicated by an increase in PVR (109 ± 13% increase), was similar to that of WKY rats (see Fig. 2b). However, because the decline in CO was comparatively smaller for SHR (17 ± 5% decline) the increase in MPAP during acute hypoxia was significantly greater for SHR (68 ± 8% increase) compared with WKY rats (Fig. 2a).

Acute hypoxia also provoked a 56 ± 3% decrease in MABP by the end of 4 min in WKY rats, which was due to the decline in CO as well as systemic vasodilatation (34 ± 9% decrease in SVR; see Fig. 2a,b). Spontaneously hypertensive rats had a higher baseline MABP and SVR so that even though the actual decline in MABP and SHR was somewhat larger than that of WKY rats (Fig. 2), the magnitude of the response to acute hypoxia (53 ± 8% decrease in MABP; 42 ± 9% decrease in SVR) was similar to that in WKY rats (Fig. 2b). Hypoxia did not significantly alter HR in either WKY rats or SHR.

Administration of SIN-1

Administration of SIN-1 (100 μg in 10 μL, i.c.v.) induced a significant ($P < 0.05$) and sustained reduction in MPAP, similar for both WKY rats (18 ± 3% decrease) and SHR (21 ± 2% decrease). However, the reduction in MPAP was entirely due to a significant decline in CO (approximately 26% decrease), because PVR was not significantly altered by SIN-1 (Table 2). In both WKY rats and SHR, SIN-1 also caused a 45–50% decrease in MABP, which was attributable to both the SIN-1-induced decline in CO and systemic vasodilatation (approximately 25% decrease, $P < 0.05$). In addition, SIN-1 caused a mild bradycardia in both WKY rats and SHR (Table 2).

The magnitude of the acute HPV response was accentuated by SIN-1 in WKY rats and, significantly more so, in SHR ($P < 0.05$; Fig. 2b). Administration of SIN-1 accentuated the decline in CO during acute hypoxia for both WKY rats and SHR. Consequently, SIN-1 did not significantly alter the magnitude of the MPAP response to hypoxia for either WKY rats or SHR, although the MPAP response for SHR was still significantly larger than that of WKY rats (Fig. 2a). In both WKY rats and SHR, the magnitude of MABP and SVR responses to hypoxia were attenuated in the presence of SIN-1 ($P < 0.05$). However, this attenuation was due to a lower prehypoxic baseline after SIN-1 treatment (described above), because the absolute MABP (and SVR) values during hypoxia were similar before and after SIN-1 administration.

DISCUSSION

The primary findings of the present study indicate that: (i) compared with normotensive WKY rats, SHR have an enhanced baseline pulmonary tone; (ii) HPV is enhanced by the central administration of exogenous NO (i.e. SIN-1) in WKY rats and SHR; and (iii) accentuation of HPV by NO is noticeably exaggerated in SHR.

Nitric oxide acts as a sympathoinhibitory substance within the cardiovascular control centres.[10] Accordingly, it has been well documented that central NO inhibition increases MABP[8,25,26] by increasing sympathetic nerve activity.[7,27] In the present study, the central administration of L-NAME (150 μg) elicited an increase in systemic ABP for WKY rats, but failed to increase ABP in SHR. Other studies have also reported an attenuated pressor response to central NO inhibition in SHR.[13,16] Cabrera *et al.*[18] also demonstrated that central NOS activation (by centrally administering Ca^{2+}) induced a depressor response in WKY rats, which was considerably attenuated in SHR. Collectively, these observations indicate that central NOS activity in SHR is reduced compared with that of WKY rats.

This difference in NOS activity for WKY rats and SHR is unlikely to be due to a difference in the central expression of NOS because Kishi *et al.*[19] showed that NOS expression was not different, at least within the rostral ventrolateral medulla (RVLM), between SHR and WKY rats. Kishi *et al.*[19] further demonstrated that increasing NO production, induced by the overexpression of NOS in the RVLM, decreased blood pressure, HR and sympathetic nerve activity in conscious rats[14] and that this effect was further exacerbated in SHR.[19] The decrease in MABP following the central administration of exogenous NO has been attributed to a decrease in sympathetic activity.[8,9] Collectively, these results indicate that the nitroxidergic system of the SHR is impaired and is a major contributing factor to the hypertensive state.

It has been reported that SHR not only have systemic hypertension, but also pulmonary hypertension.[20,21] Accordingly, the PAP of SHR (19.2 mmHg) in the present study was significantly higher than that of WKY rats (16.3 mmHg). These results are very similar to those of Janssens *et al.*,[20] who also reported that the MPAP of SHR

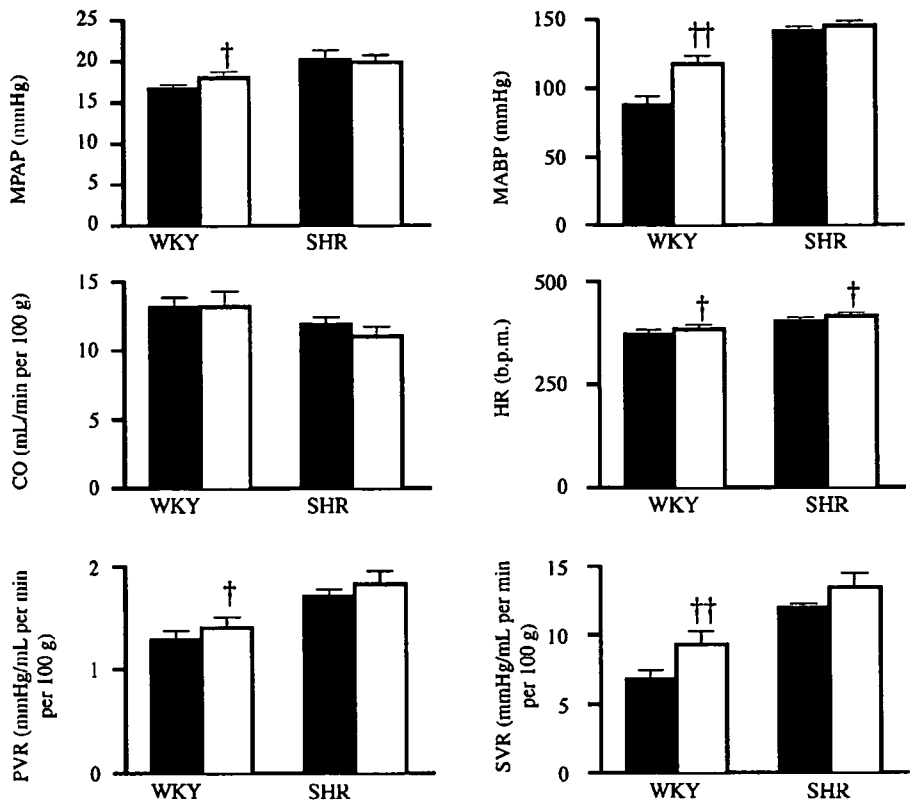

Fig. 1 Maximal cardiovascular responses to intracerebroventricular N^G-nitro-L-arginine methyl ester (L-NAME; 150 μg in 10 μL; □) in Wistar-Kyoto (WKY) rats ($n = 6$) and spontaneously hypertensive rats (SHR; $n = 6$). MPAP, mean pulmonary arterial pressure; MABP, mean arterial blood pressure; CO, cardiac output; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance; HR, heart rate. Data are the mean±SEM. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.05$ compared with control (■).

(20 mmHg) was higher than that of WKY rats (17 mmHg). Furthermore, both the present study and that of Janssens *et al.*[20] observed that the PVR of SHR was significantly higher than that of WKY rats.

Because an impaired central nitroxidergic system has been implicated as a significant factor for systemic hypertension in SHR, we hypothesized that it may also contribute to the pulmonary hypertension reported for SHR. We demonstrated that central NOS inhibition (L-NAME, 150 μg, i.c.v.) caused a mild and brief increase in MPAP and PVR in WKY rats, yet had no effect in SHR. These results are somewhat surprising because we have demonstrated previously that central L-NAME does not alter pulmonary vascular tone in Sprague-Dawley rats.[24] The reasons for the discrepancy are uncertain. Although the present results may implicate the importance of central NO in modulating tonic pulmonary vascular tone (i.e. PVR), the present study also showed that NO donation (SIN-1, 100 μg, i.c.v.) failed to alter PVR in either SHR or WKY rats. The SIN-1-induced reduction in PAP was brought about by a decrease in CO, in which the decrease in HR had a significant role, without altering PVR. These results concur with those of a previous report,[24] indicating that the central nitroxidergic system may only have a somewhat limited role in modulating 'tonic' pulmonary vascular tone, particularly in the hypertensive state.

Although the pulmonary vasculature is innervated by sympathetic fibres, neural control of 'tonic' pulmonary vascular tone is less prominent than that of the systemic vasculature. However, modulation of the pulmonary vasculature by the sympathetic nervous system becomes critically important under stressful conditions, such as hypoxia. For example, Shirai *et al.*[28] demonstrated that pulmonary β-adrenoreceptor blockade had only a minor effect on baseline pulmonary vascular tone in normoxia, but it significantly accentuated acute HPV. They concluded that β-adrenoreceptor-mediated vasodilation of the pulmonary vasculature was a vital homeostatic mechanism to limit the magnitude of vasoconstriction during acute hypoxia.

In the present study, the PAP response to acute hypoxia appeared to be larger for SHR compared with WKY rats, although this difference was related to CO, because the magnitude of the HPV (i.e. increase in PVR) was similar for SHR and WKY rats. These results suggest that pulmonary vasoreactivity to hypoxia for SHR and WKY rats is similar. In contrast, Janssens *et al.*[20] reported that the HPV of SHR was attenuated compared with WKY rats. Although the reasons for the discrepancy between studies is uncertain, it is likely related to the changes in CO during hypoxia. The present study used an anaesthetized preparation and reported a decrease in CO during hypoxia, whereas Janssens *et al.*[20] used a conscious model and reported an increase in CO in response to acute hypoxia.

In an earlier report, we demonstrated that the central administration of exogenous NO enhances HPV in normal rats.[24] In the present study, similar observations were recorded for WKY rats, but the unique result of the present study is the fact that accentuation of the HPV by central NO was considerably larger for SHR. Although the underlying mechanism(s) by which NO enhances HPV remains to be confirmed, we postulate that a central NO-induced decrease in sympathetic activity is likely to be a primary factor. Even though the pulmonary vascular is innervated by α- and β-adrenoreceptors, it is a predominant β-adrenoceptor-mediated vasodilatory response that is activated during hypoxia in order to limit the local vasoconstrictor effects of hypoxia.[28,29] Therefore, we speculate that attenuation of the β-adrenoceptor-mediated vasodilatory component of the

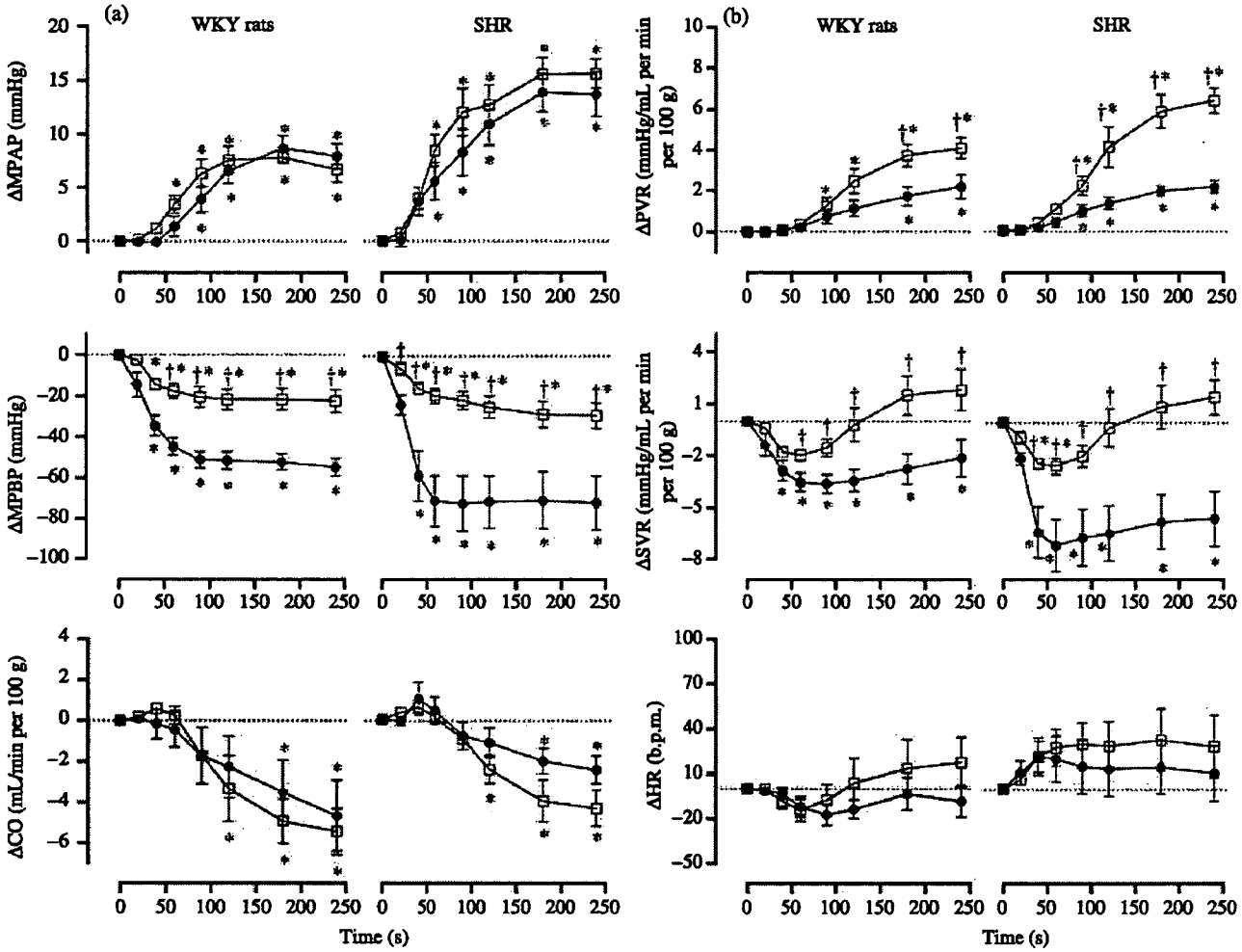

Fig. 2 Effect of 3-[4-morpholinyl]-sydnonimine-hydrochloride (SIN-1; □) on the (a) mean pulmonary arterial pressure (MPAP), (b) mean arterial blood pressure (MABP) and cardiac output (CO) responses and (b) pulmonary vascular resistance (PVR), systemic vascular resistance (SVR) and heart rate (HR) responses to acute hypoxia (10% O_2 for 4 min) in Wistar-Kyoto (WKY) rats ($n = 6$) and spontaneously hypertensive rats (SHR; $n = 6$). (●), control (artificial cerebrospinal fluid). Data are the mean±SEM. $^*P < 0.05$ comapred with pre-acute hypoxia values; $^\dagger P < 0.05$, compared with control.

Table 2 Steady state responses to the intracerebroventricular administration of 3-[4-morpholinyl]-sydnonimine-hydrochloride (100 μg in 10 μL) in Wistar-Kyoto and spontaneously hypertensive rats

	WKY rats ($n = 6$)		SHR ($n = 6$)	
	Control	SIN-1	Control	SIN-1
MPAP (mmHg)	16.3 ± 0.7	13.3 ± 0.4**	19.2 ± 0.7	15.1 ± 0.4**
MABP (mmHg)	91 ± 5	51 ± 5**	138 ± 7	68 ± 8**
CO (mL/min per 100 g)	12.6 ± 1.0	9.2 ± 1.0**	11.2 ± 0.6	8.24 ± 1.1*
HR (b.p.m.)	377 ± 10	356 ± 9*	389 ± 13	348 ± 10**
SVR (mmHg/mL per min per 100 g)	7.62 ± 0.70	5.62 ± 0.36*	12.59 ± 1.0	8.77 ± 1.24*
PVR (mmHg/mL per min per 100 g)	1.35 ± 0.13	1.54 ± 0.17	1.74 ± 0.10	1.99 ± 0.25

Data are the mean±SEM. $^*P < 0.05$, $^{**}P < 0.01$ compared with control.

MPAP, mean pulmonary arterial pressure; MABP, mean arterial blood pressure; CO, cardiac output; HR, heart rate; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance; WKY rats, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

pulmonary vasculature during acute hypoxia is exacerbated in SHR, because sympathoinhibition is greater in SHR.[19]

One of the primary limitations of the present study is the fact that we did not record sympathetic nerve activity during the central administration of SIN-1. Indeed, recording nerve activity would be the only sure way to conclusively state that the NO-induced modulation of HPV is attributable to changes in sympathetic activity. However, all previous papers that have described the central role of NO on sympathetic nerve activity have recorded nerve activity from the renal sympathetic nerve and have correlated changes in activity with changes in systemic ABP. Yet, changes in sympathetic activity to functionally different organs are quantitatively non-uniform.[30] Indeed, Shirai *et al.*[31] demonstrated in the cat that acute hypoxia provoked an increase in pulmonary sympathetic activity that was twofold larger in magnitude than that for renal sympathetic activity. Therefore, in future studies it will be essential to record pulmonary sympathetic nerve activity in the rat, a task that has not yet been achieved.

Although the central administration of NO in general has been shown to decrease MABP, Nurminen and Vapaatalo[32] showed that the central administration of SIN-1 (approximately 600 μg/kg) increased MABP. At high doses, SIN-1 can cogenerate NO and superoxide anions.[33,34] Increases in the central generation of superoxide anions have been reported to increase sympathetic activity and, consequently, ABP.[35] Therefore, the increase in MABP in the study of Nurminen and Vapaatalo[32] may be attributed to the formation of superoxide anions. At low doses, the superoxide-associated effects of SIN-1 are significantly outweighed by the actions of NO.[33] Therefore, because the present study used a comparatively low dose of SIN-1 (100 μg), we interpret the decrease in MABP to indicate that, at this dose, SIN-1 was acting solely as an NO-donor.

In summary, we have shown that central NO may have a small, although limited, role in modulating tonic MPAP in WKY rats, but not in SHR. Furthermore, exogenous NO enhanced the acute HPV in WKY rats and considerably more so in SHR. Whether central NO acts to inhibit β-adrenoceptor-mediated vasodilation of the acute HPV is an area that warrants further investigation.

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CARDIOVASCULAR SYSTEM

α_2-Adrenoreceptor mediated sympathoinhibition of heart rate during acute hypoxia is diminished in conscious prostacyclin synthase deficient mice

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Abstract Acute hypoxia increases ventilatory drive in conscious animals, resulting in tachycardia. Sustained hypoxia changes the initial chemoreflex ventilatory increase to secondary ventilatory depression, which then evokes a gradual secondary heart rate (HR) reduction. Prostacyclin (PGI_2) release is known to potentiate α_2-adrenoreceptor (α_2-AR) mediated inhibition of sympathoactivation during ischaemia and hypoxia. We examined whether α_2-AR mediated sympathoinhibition was responsible for limiting hypoxic heart rate increases during initial sympathoactivation, and subsequent secondary HR depression, and if PGI_2 is required for sympathoinhibition of HR. The responses of unrestrained PGI_2 synthase deficient (PGID) and wild type (WT) mice to acute hypoxia (10% O_2 for 30 min) were investigated by simultaneous telemetry, whole body plethysmography and open-flow respirometry. PGID mice exhibited potentiated $\dot{V}_E$ ($p<0.007$) after intraperitoneal vehicle injection ($n=8$), but not so HR responses compared to WT mice during sustained hypoxia. Idazoxan (α_2-AR antagonist, i.p. bolus 3 mg/kg) pretreatment did not change hypoxic ventilatory response in either group, but significantly elevated hypoxic HR in WT mice only ($p<0.013$). Sodium meclofenamate (cyclooxygenase inhibition, i.p. bolus 25 mg/kg) pretreatment eliminated the potentiated $\dot{V}_E$ of PGID and caused significant basal hypotension that led to a transient hypertensive response to hypoxia. From these results, we suggest that α_2-AR activation is required for coupling HR to central inspiratory drive during acute

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hypoxia, and that PGI_2 is required to enhance the inhibition of sympathoactivation.

Keywords Acute hypoxia · α_2-Adrenergic receptor · Sympathetic nervous system · Prostaglandins · Transgenic mouse · Respiratory control

Introduction

Autonomic control of heart rate (HR) is coupled to the central inspiratory drive, enabling homeostasis of pH and respiratory gas transport. In conscious and spontaneously breathing adult mammals, acute exposure to moderate hypoxia evokes significant ventilatory increases in addition to a brief tachycardia due to sympathoactivation [1]. Both of these responses are potentiated by defense responses [2, 3] because the activation of the amygdala and hypothalamus with behavioural arousal or anxiety results in cardiovascular preparation for "fight or flight". If hypoxic exposure is sustained for more than a few minutes, central inspiratory drive decreases and both tidal volume (V_T) and ventilation rate decline (often referred to as hypoxic ventilatory depression), and secondary bradycardia and significant hypotension develop. Changes in HR are highly correlated with changes in V_T, especially during chemoreceptor stimulation, suggesting that HR is neurally coupled to central inspiratory drive [4, 5]. The latter studies show that in anaethetised animals at least, the chemoreceptor reflex evokes bradycardia (as opposed to tachycardia in conscious animals) that is mediated in part by vagal efferent activity. The contribution of vagal stimulation to the progressive bradycardia in conscious mice is considered in a separate study using muscarinic acetylcholine receptor knockout mice (manuscript in preparation). Whether the sympathetic cardiac efferent pathway is involved in the secondary HR reduction during hypoxic ventilatory depression of conscious animals is yet to be determined.

In this study, we suggest that the HR decline evoked during sustained hypoxia might involve α_2-adrenoreceptor (α_2-AR) mediated inhibition of sympathoactivation, independent of changes in vagal activity. Evidence for this concept can be derived from the α_2-AR distribution in the brainstem and spinal cord and the actions of noradrenaline (NA) during hypoxia: (1) neurons in the nucleus tractus solitarius (NTS) and the C1 adrenergic group in the rostral ventrolateral medulla (RVLM) contain the highest densities of α_2-AR and are critical to the integration of chemoreceptor reflexes; (2) NA release in the brainstem was shown to attenuate ventilation [6], which would facilitate HR decreases; (3) serotonin mediated facilitation of ventilation by serotonergic neurons in the caudal raphe complex is attenuated by NA during hypoxia, which is likely to provide a possible means of fine control of ventilation during acute and intermittent exposure to hypoxia (reviewed in [7]); (4) presynaptic activation of α_2-AR in the preganglionic neurons of the medulla and spinal cord is largely responsible for regulating the amplification of cardiac and vasomotor sympathetic outflow [8–11]; and furthermore, (5) Prabhakar and Kou [12] have already demonstrated that sympathetic efferent innervation of the carotid bodies (from the superior cervical ganglion) inhibits ventilation during sustained hypoxia, and therefore might be in part responsible for hypoxic ventilatory depression.

Here, it should be noted that aside from the secondary depression of the central inspiratory drive, it is likely that pulmonary vagal afferent C-fibres are activated by increased lung inflation and pulmonary blood flow during sustained hypoxia [5]. Hence, the modulation by at least two afferent pathways would lead to increased central sympathoinhibition. We hypothesised that α_2-AR mediated inhibition of sympathoactivation might be involved in the coupling of ventilatory depression and HR attenuation during sustained hypoxia. This attenuation could be evoked by α_2-AR mediated inhibition of chemoreflex through sympathetic innervation of the carotid bodies and/or α_2-AR mediated inhibition of sympathetic cardiac efferents. Furthermore, we considered that α_2-AR mediated inhibition of HR during hypoxic sympathoactivation might be enhanced by endogenous prostacyclin (PGI_2) generation.

Prostaglandin (PG) release from the terminal endings of sympathetic postganglionic fibres is thought to be an important regulator of α_2-AR mediated inhibition of neural NA release. Prostaglandin E_2 (PGE_2) and PGI_2 production from postganglionic fibres is increased by sympathetic stimulation via an action on α_2-AR [13]. Wennmalm et al. [14] first demonstrated that PGI_2 mediated inhibition of neurotransmitter release in the heart and therefore might be an important feedback mechanism in vivo. Furthermore, numerous studies show that in many animal models, the cardiac muscle release of PGE_2 and PGI_2 is significant during ischaemia and hypoxia [15–20]. On the other hand, no study was able to demonstrate if PG release in the central and peripheral nervous systems modulate cardiorespiratory regulation, although receptors for both PGI_2 and PGE_2 are distributed in unique regions of the brainstem and sensory vagal afferents that are important for cardiovascular regulation [21]. It is noteworthy that PG are important modulators of carotid body catecholamine release during hypoxia [22].

We used telemetry and whole body plethysmography with analyses of excurrent gas to examine the effects of α_2-AR antagonism (idazoxan) on the hypoxic responses in unrestrained, conscious wild type (WT) mice and mice deficient in PGI_2 synthase ($PGI_2^{-/-}$, PGID mice) in a transgenic model described in an earlier study [23]. Our results suggest that α_2-AR regulation of HR is important for the maintenance of

coupling between the inspiratory drive and HR during sustained moderate hypoxia. Further, the synergistic actions of PGI_2 and α_2-AR play an important role in reducing sympathoactivation in conscious mice.

Materials and methods

Animals and housing

Experiments were conducted with male WT (CB57BL/6; Japan SLC, Shizuoka, Japan) and PGID mice (PGI_2 synthase$^{-/-}$ mouse colony maintained at the National Cardiovascular Center) that were 9–17 weeks old (body mass 21–31 g). Mice were maintained under a 12:12 h light–dark cycle with lights on between 7 A.M. and 7 P.M. Experimental and surgical procedures were conducted in accordance with the guidelines of the Physiological Society of Japan.

Telemetric device implantation

A telemetric system (Data Sciences International, St. Paul, MN, USA) was used to record left carotid arterial blood pressure from blood pressure transducer units (PA-C20) after a correction for changes in ambient pressure [24]. PA-C20 transmitter and catheter implantation were performed under isoflurane anaesthesia (4% induction followed by 1.5–2.0%, sufficient to eliminate limb withdrawal reflexes) similar to the procedure of Butz and Davisson [24] with the exception that the transmitter body was placed in a subcutaneous pouch on the back to avoid undesirable effects on ventilation (likely with abdominal implantation). Continuous arterial pressure recordings were made from the time of surgery for 1 week. We confirmed in all mice that normal circadian rhythms in HR and arterial pressure were obtained within this time period. The first hypoxia test was conducted 7–10 days post implantation.

Open-flow respirometry system and measurements

Mice were placed in plethysmograph chambers (model PLY3211; Buxco Electronics, Sharon CT, USA) during the daylight phase for simultaneous open-flow respirometry and ventilatory measurements (see "Acute hypoxia protocol and pretreatments" section). Compressed air was pulled into the chambers through inlet ports by a bias flow regulator (Buxco), downstream of the gas analysers at 300 ml/min. Differential pressure transducers (TRD5100, Buxco) continuously compared (in AC mode) the animal and reference sides of the chambers and their outputs were connected to strain gauge preamplifiers (Max II, Buxco). In DC mode, the voltage output difference between closing and re-opening a 3-way valve was then set to the maximum flow change, after correcting for chamber temperature and water vapour influences [25]. Ventilation rate (f, per min) was calculated directly from ventilation-induced pressure fluctuations. Tidal volume (V_T, μl/g) was integrated from the area under the flow signal of each breath. Minute ventilation ($\dot{V}_E$, ml/[g min]) was determined from the product of f and V_T. Inspiratory flow was calculated as V_T/inspiration time (μl/[10 g body mass min]). HR (bpm) was determined from the systolic arterial pressure interval.

Air from the mouse chambers was pulled through Peltier condensers (PC-1, Sable Systems International, Las Vegas NV, USA) to remove water vapour before entering independent channels of a differential O_2 analyzer (Oxilla, Sable Systems International) and then CO_2 analyzers (CA-2A, Sable Systems International) using the bias flow regulator. Analyser calibration was performed using external air (20.95% O_2), pure nitrogen and a certified gas (5% CO_2, 15% O_2 and remainder N_2). Mice were not in a postabsorptive state during measurements. All experiments were performed at a room temperature of 26°C (chamber ~28°C) to minimize the possibility of significant hypothermia during hypoxia. Analog outputs from the analyzers and pressure transducers were recorded on a personal computer via an A/D converter using the HEM 3.4 software (Notocord, France), simultaneous with the telemetry signals derived from another computer after signal conversion and barometric compensation using Dataquest A.R.T. software (version 1.0.0, Data Sciences International). O_2 consumption and CO_2 production were calculated according to Withers with iterative fitting assuming an initial respiratory exchange ratio (CO_2/O_2) of 0.8 [26].

Acute hypoxia protocol and pretreatments

Mice were lightly anaethetised (isoflurane ~10 s) to minimise the stress induced by intraperitoneal (i.p.) injections, and immediately placed in the chambers for normoxic measurements. Normoxic (21% O_2, 0% CO_2) measurements of pretreated mice were made for 30 min (equilibration time), and then the gas was changed to hypoxia (10% O_2, 0% CO_2) for 30 min.

The effects of pretreatment with the α_2-AR antagonist, idazoxan hydrochloride (Sigma, 3 mg/kg i.p. bolus, $n=5$ in both mouse groups) or vehicle (0.9% saline, $n=8$), on acute hypoxic responses was examined in WT and PGID mice. Since PGE_2 and thromboxane are both upregulated in PGID mice [23], we also examined the effect of total PG inhibition on hypoxic responses of both groups by pretreatment with sodium meclofenamate (cyclooxygenase inhibitor, Sigma, 25 mg/kg i.p.) on a different experiment day (WT $n=7$ and PGID $n=5$). Selection of drug doses was assumed to achieve maximal or near maximal receptor

antagonism [27, 28] and cyclooxygenase inhibition [29–31], respectively.

Analyses and statistics

Resting normoxia baseline values of all parameters for each mouse were determined from the 20 s-mean interval corresponding to the minimum resting $\dot{V}_E$ during the last 20-min period before hypoxia, and thus least likely to be affected by activity. Resting state was confirmed from tidal breathing in the ventilation recordings and stable arterial pressure recordings. The mean of all 20 s-intervals for each 5-min interval of hypoxia (from the time chamber O_2 content decreased below 10%) was determined. Means were compared by ANOVA with repeated measures followed by post hoc Student–Newman–Keuls tests. $p<0.05$ was considered to be statistically significant.

Results

Resting normoxic baselines and vehicle hypoxic responses

In a preliminary investigation, the effect of the implanted transmitters on ventilation was determined using the same protocol described above. Basal minimum $\dot{V}_E$ of WT mice with no transmitters ($n=5$) was essentially the same as that of implanted mice, however, peak hypoxic $\dot{V}_E$ was significantly higher (4.91±0.48 (SEM) vs 3.81±0.26 $ml \cdot g^{-1} \cdot min^{-1}$, $p<0.049$). Hence, compliance of the thorax may have been affected at high ventilations by the mass of the transmitter even when placed on the back. It is unlikely that diminished cerebral blood flow resulting from the carotid cannulation was responsible for the lower peak response as the peak hypoxic $\dot{V}_E$ of WT mice implanted with ECG transmitters (DSI) was not significantly different from the WT mice in this study ($n=5$, 4.17±0.28 $ml \cdot g^{-1} \cdot min^{-1}$, unpublished data of Pearson and Shirai). Therefore, it should be realized that the results concerning ventilation in implanted mice presented hereafter do not necessarily reflect that of non-instrumented animals.

Haemodynamic function of the PGID mice did not differ from WT mice during the 20-min period before hypoxia (Table 1). Only V_T and $\dot{V}_E$ were significantly elevated on average in PGID mice during this period. In all mice, the haemodynamic variables at the $\dot{V}_E$ minima were slightly lower than the mean of the whole baseline interval. Even under resting conditions, the V_T and CO_2 production of PGID mice remained significantly larger than WT in normoxia after vehicle pretreatment ($p<0.05$). Therefore, we also confirm that endogenous PGI_2 is not essential for normal cardiovascular and ventilatory functions in normoxia [23, 32, 33].

In another pilot study, mice with transmitters were not subjected to injection pretreatments or brief isoflurane during our hypoxia protocol. These mice responded to acute hypoxia exposure with behavioural arousal that was evident as changes in ventilation pattern and initial increases in HR and arterial pressure, followed by rapid hypotension and gradual bradycardia (Fig. 1). The same response patterns for all variables were evident in mice after vehicle treatment. Similar time courses for the transient increases in ventilation [2, 34, 35] and HR [2, 3, 36] were described for rats in response to moderate hypoxia. However, the responses of conscious mice to sustained hypoxia (>5 min duration) were not described until now. In both groups f, V_T and $\dot{V}_E$ peaked within 5 min of hypoxic exposure and decreased significantly with exposure time thereafter (Fig. 2a–c,

Table 1 Haemodynamic and ventilatory functions[a] of conscious WT and PGID mice after vehicle pretreatment under resting conditions in normoxia

	20-min baseline mean		Mean values at lowest[b] basal $\dot{V}_E$	
($n=8$ per group)	WT	PGID	WT	PGID
Body mass[c] (g)	26.2±0.9	24.8±1.1		
MHR (bpm)	422.2±16.0	436.4±17.2	361.2±16.1	362.0±16.9
MAP (mmHg)	97.0±2.8	105.1±2.7	85.2±2.3	95.3±3.4
Pulse pressure (mmHg)	22.7±1.5	26.5±3.3	19.5±1.2	22.5±3.3
V_T ($\mu l\ g^{-1}$)	10.3±0.5	14.6±1.1**	5.84±0.49	7.47±0.56*
Ventilation rate (min^{-1})	270±33	380±60	148±9	141±5
$\dot{V}_E$ ($ml\ g^{-1}\ min^{-1}$)	2.49±0.38	4.35±0.64*	1.15±0.08	1.31±0.17
O_2 consumption ($\mu l\ g^{-1}\ min^{-1}$)	56±5	63±5	39±2	46±3
CO_2 production ($\mu l\ g^{-1}\ min^{-1}$)	56±5	64±6*	38±2	45±3*

Mean (±SEM) of male mice implanted with blood pressure transmitters (*$p<0.05$, **$p<0.001$ different from WT).
[a] Mean (SEM) of male mice implanted with blood pressure transmitters.
[b] The lowest 20 s-mean interval in the 20-min before hypoxia.
[c] Excludes mass of transmitter unit.

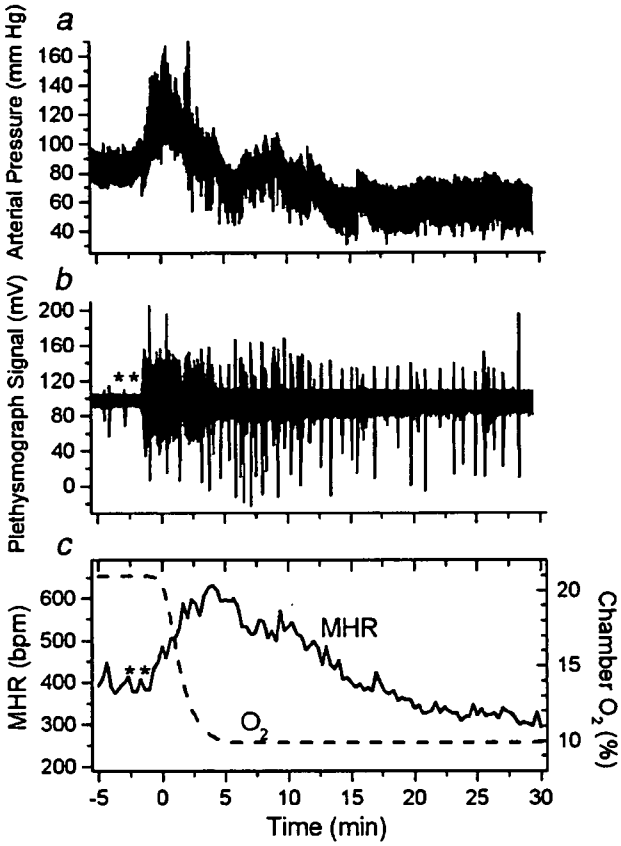

Fig. 1 Typical recordings of arterial pressure, mean heart rate (MHR) and ventilatory signal in a conscious WT mouse during acute hypoxia (10% O_2) exposure. Acute hypoxia protocol evoked arousal resulting in increased ventilatory pressure fluctuations and a sudden increase in arterial pressure followed by gradual tachycardia and hypotension as chamber O_2 decreased. *Asterisks* indicate periods of stable ventilation when the mouse was resting during normoxia

$p<0.001$). The exaggerated hypoxic ventilatory responses of PGID mice were significant ($\dot{V}_E$, group $p<0.007$), as the elevated V_T in PGID mice did not decrease significantly with time (NS in PGID only, Fig. 2b). Inspiratory fraction did not differ significantly between groups, remaining ca. 45% during hypoxia (Fig. 2e).

Significant reductions in MAP of 20–25 mmHg occurred with exposure time after the first 5-min of hypoxia in both groups (time $p<0.001$) (Fig. 2f), while pulse pressure remained stable in WT mice and decreased significantly in PGID mice (<5 mmHg reduction, $p<0.001$). The MHR increase peaked during the first 5-min interval and was not significantly different between groups (Fig. 2d). O_2 consumption and CO_2 production were not depressed during hypoxia, remaining between 96% and 105% of the pre-hypoxic baseline in both groups. These results suggest that HR was coupled to the decline in the central inspiratory drive, but HR depression occurred to a lesser extent during sustained hypoxia in the absence of PGI_2 when other endogenous PG were present as V_T and $\dot{V}_E$ did not decrease significantly.

Effects of idazoxan and sodium meclofenamate

Idazoxan pretreatment did not change pulse pressure, but increased the normoxic MHR of PGID ($p<0.028$) and the MAP of WT mice (Fig. 3, $p<0.029$). Normoxic $\dot{V}_E$ baseline was not changed significantly by idazoxan. Sodium meclofenamate pretreatment did not significantly change MAP, MHR or the $\dot{V}_E$ resting normoxic baselines in either group (Fig. 4). However, sodium meclofenamate eliminated the potentiated hypoxic $\dot{V}_E$ of PGID mice, and was without effect on WT mice. Sodium meclofenamate pretreatment did not change the peak hypoxic MHR (first 5-min) increase in PGID mice (Fig. 4d). Therefore, a cyclo-oxygenase product other than PGI_2 was responsible for secondarily potentiating the hypoxic ventilatory responses in PGID mice during sustained hypoxia. To compare hypoxic HR responsitivity of the mice to treatments, the change in MHR from the baseline level was determined for each mouse (Fig. 5). Idazoxan potentiated the peak MHR increase (~110 bpm) of WT mice relative to the vehicle (Fig. 5, $p<0.013$), but did not significantly change either the MAP decrease or $\dot{V}_E$ increase during hypoxia. In contrast to the WT mice, the peak MHR increase at the onset of hypoxia was not significantly changed by idazoxan pretreatment in PGID mice, indicating that the PGID acute HR response was attenuated (Fig. 4). Idazoxan did not change the MAP decrease and $\dot{V}_E$ increase during hypoxia in PGID mice (data not shown).

Although hypoxic $\dot{V}_E$ was elevated in PGID mice relative to WT after vehicle pretreatment (Fig. 6), the same curvilinear relation between hypoxic MHR and inspiratory flow described both groups. In contrast, idazoxan pretreatment caused a disproportionate increase in the MHR of WT mice relative to the inspiratory flow, but was attenuated in PGID mice. This suggests that endogenous PGI_2 facilitated an enhanced chemoreflex response in WT mice during inhibition of α_2-AR.

Discussion

This study shows for the first time that the α_2-AR is an important regulator of sympathetic control of HR in conscious mice during sympathoactivation induced during acute hypoxia, by limiting both the peak HR increase during the initial chemoreflex and by reducing HR during sustained hypoxia exposure. In contrast, α_2-AR did not play a significant role in the modulation of hypoxic ventilatory responses, although idazoxan pretreatment

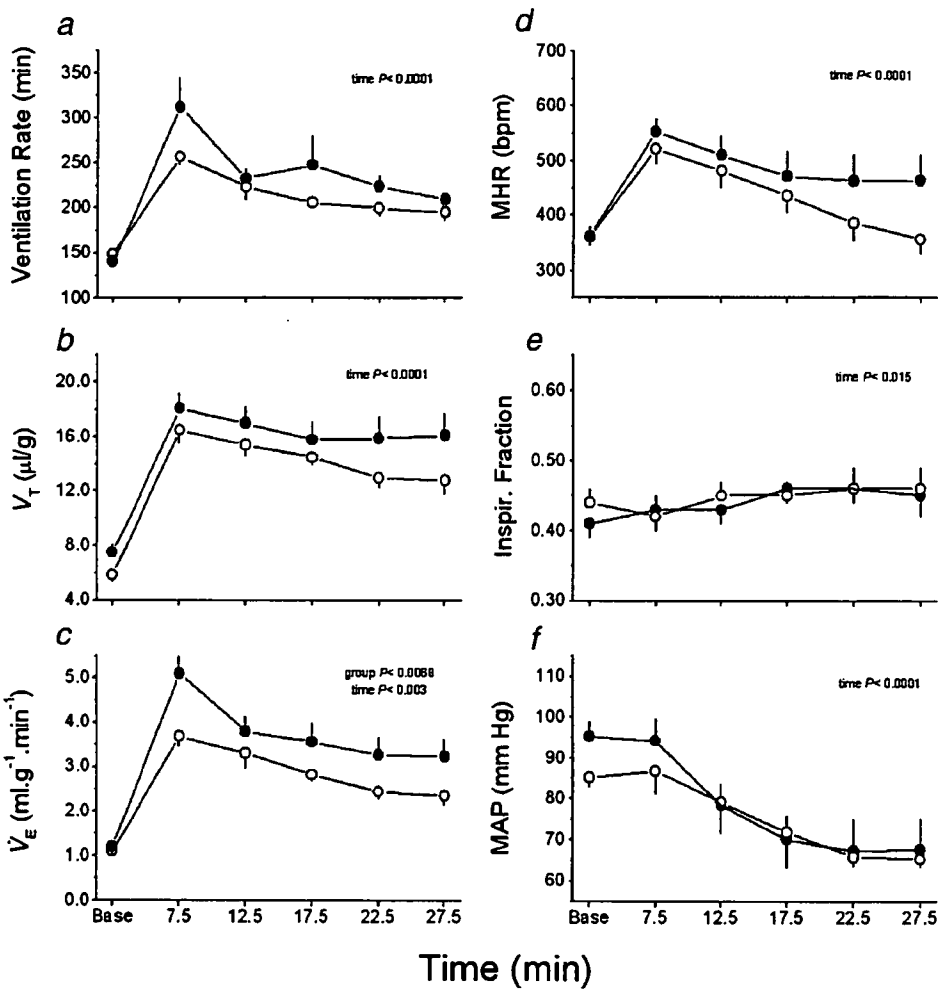

Fig. 2 Mean values of haemodynamic and ventilatory functions (±SEM) during exposure to acute hypoxia (10% O_2) after i.p. pretreatment with 0.9% saline vehicle injection in WT (*open symbols*) and PGID (*closed symbols*) mice. Mean values of 5-min intervals are presented in relation to median time. Significant differences by ANOVA between WT and PGID groups are indicated in each panel

increased central inspiratory drive during hypoxia. Our results also suggest that endogenous release of PGI_2 is required for normal α_2-AR mediated attenuation of chemoreflex induced cardiac sympathetic activation.

Our findings neither support nor dismiss a possible role for α_2-AR in the regulation of arterial pressure decreases during hypoxia. Idazoxan pretreated mice had elevated resting baseline MAP (ca. 20 mmHg), which suggests that α_2-AR inhibition resulted in a modest increase in tonic sympathetic outflow to the peripheral vasculature. This finding is expected based on the many studies of the tonic cardiovascular effects of α_2-AR agonists and antagonists [37]. However, the decrease in MAP during acute hypoxia in this study was not changed by idazoxan pretreatment, suggesting that other factors might be responsible for facilitating hypoxic vasodilatation.

Origins of acute responses to hypoxia

Sympathoactivation is induced by acute hypoxia in spontaneously breathing mammals, evoking ventilatory and cardiovascular changes that comprise the initial chemoreceptor reflex, mediated by peripheral chemoreceptors located in the carotid bodies [1, 34]. In the conscious animal, defense responses initiated within the amygdala and hypothalamus by behavioural arousal or anxiety potentiate the ventilatory and HR increases evoked by acute hypoxia [2, 3]. Thus acute hypoxia evokes initial increases in ventilation and HR in many conscious animals, including mice as we confirmed in this study (Figs. 1 and 2). The direct inhibitory effect of hypoxia on the heart (sinoatrial node) [1, 3] is opposed by its effect on the central nervous system, which augments cardiac sympathoactivation [38]. The CO_2 production of spontaneously breathing mice in this study was maintained during hypoxia (although mild hypocapnia occurred subsequently) and no significant change in inspiratory time or inspiration fraction was noted during the initial hyperventilation. Thus, an increased central inspiratory drive and increased negative feedback on cardiac vagal motor neurones due to sustained pulmonary stretch receptor stimulation are the most likely causes of the tachycardia, although hypocapnia can also contribute to tachycardia (see [1]).

Hypoxic pulmonary vasoconstriction and a decline in central inspiratory drive both potentially contribute to the