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Alteration of endothelium-mediated vasodilator response in the rat hindlimb vasculature consecutive to chronic hypoxic stress: NO and EDHF involvement

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ABSTRACT

The previously documented impairment of hindlimb blood flow consecutive to chronic hypoxia might be related to endothelial vasomotor dysfunction. The aim of this study was to assess *in-vivo* the effect of chronic hypoxic stress on endothelium-mediated vasodilator response of hindlimb vascular bed, especially as regards to endothelium-derived hyperpolarizing factor (EDHF) and nitric oxide (NO) pathway contribution. Dark Agouti rats were randomly assigned to live at barometric pressure ≈760 mmHg (N rats) or ≈550 mmHg (CH rats). Under anesthesia, catheters were placed in the carotid artery for arterial pressure measurement, and in the saphenous vein and iliac artery for drug delivery. Hindlimb blood flow (HBF) was measured by transit-time ultrasound flowmetry, at baseline and during endothelium-dependent vasodilator response induced by intra-arterial injection of acetylcholine (0.75 ng and 7.5 ng) with and without specific blockers of NOS (L-NAME) and EDHF (Charybdotoxin + Apamin). HBF and hindlimb vascular conductance changes in response to ACh infusion were significantly lower in CH than in N rats. The mechanisms responsible for this blunted response involved impairment in both NO pathway and EDHF. The chronic hypoxia-induced alteration of NO pathway was mainly related to the bioavailability of its substrate L-Arginine, since the infusion of L-Arginine restored the endothelial response to ACh in CH rats to the level of N rats. These results demonstrate that the impairment in endothelium-mediated vasodilator response of the hindlimb vascular tree induced by chronic hypoxic stress involves both NO and EDHF.

1. Introduction

Chronic hypoxic stress in rat has consistently been shown to be associated with impaired endothelium-dependent relaxation properties in large conduit arteries (Trang et al., 2001; Reboul et al., 2005a). In addition, Tahawi et al. (2001) have previously reported in arteriolar of cremaster muscles in rats that relaxation to acetylcholine (ACh) was significantly attenuated following prolonged exposure to hypoxic stress (8 h/day for 35 days). Endothelium-dependent relaxation along the vascular tree involves mainly prostacyclin, nitric oxide (NO) and an endothelium-derived hyperpolarizing factor (EDHF). The relative contribution of these endothelium-derived relaxing factors appears to be largely dependent on species, organs and vessels. Previous *in-vivo* experiments have demonstrated that ACh-mediated vasodilatation of the hindlimb beds of rats is dominated by NO and EDHF, with prostacyclin playing a minor role (Parkington et al., 2002; Fitzgerald et al., 2007). The aforementioned depressed endothelium-dependent vasorelaxation after chronic hypoxia in isolated aortic rings has been attributed to impairment of the NO pathway, implying partly L-Arginine (L-Arg) bioavailability (Reboul et al., 2005a). The effect of chronic hypoxia exposure on the rat hindlimb blood flow (HBF) regulation is poorly documented, and controversial results have been obtained (Kuwahira et al., 1993a,b; Smith and Marshall 1999). Whether and how chronic hypoxic stress affects the vasodilator function of hindlimb resistance vessels remains currently unknown. Interestingly, previous studies of the effects of prolonged hypoxic stress in human have shown reduced exercise leg blood flow and vascular conductance (Bender et al., 1988; Calbet et al., 2003; Lundby et al., 2006), whose control during exercise involves a complex interplay between endothelium-dependent vasodilators, including NO, EDHF and prostacyclin.

Inhibitors of NO Synthase (NOS) and cyclooxygenase have been available for *in-vivo* use for many years, and although the mechanisms by which endothelium induce hyperpolarization of vascular smooth muscle cells remain unclear, several authors (Parkington et al., 2002; Fitzgerald et al., 2007) recently reported that the EDHF-attributed response is suppressed by a combination of Charybdotoxin (Chtx) and Apamin, which blocks big-, intermediate- and small-conductance K_{Ca}

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channels. Therefore, the aim of the present study was to investigate *in-vivo* in a rodent model the effect of chronic hypoxic stress on endothelium-mediated vasodilator response of hindlimb vascular bed. For this purpose, we evaluated, in normoxic and chronically hypoxic rats, hindlimb blood flow and conductance changes after endothelial stimulation by ACh, in the presence and absence of specific blockers, allowing distinguishing the contribution of NO from that of EDHF. We hypothesized that chronic hypoxia would result in endothelium-dependent vasodilator dysfunction as a result of impairment in NO pathway and/or EDHF release.

2. Methods

2.1. Animals

Ten-week-old Dark Agouti male rats (Harlan laboratories, France) were randomly assigned to live continuously for 5 weeks in hypobaric hypoxia (barometric pressure ≈ 550 mmHg; CH rats) or in normoxia (barometric pressure ≈ 760 mmHg; N rats). The corresponding environments were created in steel chambers with a vacuum pump as previously described (Reboul et al., 2005b). CH rats were fed *ad libitum*. Because of hypoxic impact on food intake and consequently on animal growth, a pair feed model was applied to N rats (Reboul et al., 2005b), resulting at time of final measurements in similar body weight between the 2 groups (N rats: 250.4 ± 2.9 g; CH rats: 249.5 ± 3.5 g). All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and with the approval of the French Ministry of Agriculture.

2.2. Experimental procedure

2.2.1. Surgical procedure

All experiments were performed in anesthetized animals (Thiobarbital sodium, Inactin®, 120 mg kg^{-1} i.p.) within the 4 h following the end of experimental exposure. The trachea was cannulated to maintain airway patency, and the animals breathed normoxic room air spontaneously. Body temperature was maintained at approximately 37 °C using a heating pad controlled by rectal temperature.

Hindlimb blood flow (HBF) was measured by a transit-time ultrasound flow probe (model 1.5PSL519, Transonic Systems Inc, NY, USA) placed around the abdominal aorta 1–2 mm proximal to the iliac bifurcation. Mean arterial pressure (MAP) was measured by a Baxter Uniflow gauge pressure transducer (Baxter Healthcare, Irvine, CA, USA) connected to a polyethylene cannula (PE-50, A-M systems, Carlsborg, USA) filled with heparinized solution (50 IU/ml) and inserted into the left carotid artery. Hindlimb vascular conductance (HVC) was calculated as [HVC = HBF / MAP]. Basal MAP, heart rate (HR) and HBF reached a steady state within 5–10 min of completing the preliminary surgical procedures. Hemodynamic measurements, including HBF, MAP and HR, were then recorded digitally at 500 Hz (MP100, Biopac, Goleta, Santa-Barbara, USA) and continuously displayed by a data acquisition and treatment software (AcqKnowledge, Biopac, Goleta, Santa-Barbara, USA).

A catheter was inserted in the right femoral artery and advanced to the iliac bifurcation to allow for direct arterial injection into the left hindlimb circulation. Because cyclooxygenase providing prostacyclin-mediated dilation has been shown *in-vivo* to have minor influence on the control of basal HBF and HBF response to ACh (Parkington et al., 2002; Fitzgerald et al., 2007), cyclooxygenase was inhibited throughout the entire experiment. Thus, indomethacin saline solution was infused (10 mg kg^{-1} h^{-1}) via an intravenous catheter (saphenous vein) throughout the entire experiment at 40 µl/min. It is of note to highlight that in the present experiment, no differences concerning basal MAP, HR, HBF and HVC were reported before and after (equilibration period of 20 min) intravenous infusion of indomethacin in both N and CH rats.

The endothelium was stimulated by bolus administration of ACh directly into the left hindlimb circulation. As previously described by Parkington et al. (2002), care was taken so that the dose of ACh infused was kept below the level at which changes MAP or HR may occur, thus ensuring no or minimal overflow into general circulation.

2.2.2. Effects of L-NAME on hindlimb vascular responses (Fig. 1A, experimental procedure)

We evaluated the effect of the NOS inhibitor N^{ω}-nitro-L-Arginine methyl ester (L-NAME) on the vasodilator response to ACh of hindlimb vessels. After control responses to ACh (0.75 and 7.5 ng i.a.) were obtained (standard conditions, i.e. in presence of indomethacin), L-NAME (25 mg kg^{-1}) was administered intravenously. Because variations in vascular tone may complicate the analysis of responses, sodium nitroprusside (SNP), a NO-donor, was then infused (10–30 µg min^{-1} i.v.) to palliate for the loss of basal NO production and restore MAP to stable pre-L-NAME levels, after which responses to ACh were once again obtained. No differences between N and CH rats were observed as regards to the dose of SNP infused (N: 22 ± 4; CH: 20 ± 5 µg min^{-1}).

2.2.3. Effects of Charybdotoxin and Apamin on hindlimb vascular responses (Fig. 1B, experimental procedure)

After control responses to ACh (0.75 and 7.5 ng i.a.) were obtained (standard conditions), Charybdotoxin (Chtx) and Apamin were nfused to the hindlimb via the aortic catheter (Chtx 0.9 µg min^{-1} i.a.; Apamin 3.5 µg min^{-1} i.a.), and responses to ACh were again obtained 5–10 min after the infusion of Chtx and Apamin was started. This time was previously used in the literature to reach a steady state after the infusion of Chtx and Apamin (Parkington et al., 2002; Dabisch et al., 2004a,b).

2.2.4. Effects of L-Arginine on NO-dependent hindlimb vascular responses (Fig. 1C, experimental procedure)

After control responses to ACh (0.75 and 7.5 ng i.a.) were obtained (standard conditions), the NOS substrate L-Arginine (L-Arg) was injected locally (in the presence of Chtx 0.9 µg min^{-1} i.a. and Apamin 3.5 µg min^{-1} i.a.) to the hindlimb circulation via the aortic catheter (30 mg kg^{-1}), and responses to ACh were again obtained.

2.3. Statistical analysis

All values are reported as mean ± S.E.M. The effects of drugs and hypoxic stress on cardiovascular variables were assessed by a two-way ANOVA with repeated measures using Statview software (Abacus Concept, Berkeley, CA). In case of interaction between the main factors, *post-hoc* tests were applied. MAP, HBF (basal, peak, delta changes, area and duration of the response) and HVC were recorded under basal and Ach-induced endothelium stimulation conditions. The duration of the blood flow response was defined as the time from the injection of ACh to the return of blood flow to pre-injection baseline. The area under blood flow vs time tracing was calculated by integration using Biopac AcqKnowledge software. A p-value of <0.05 was used as the criterion for statistical significance.

3. Results

3.1. Mean arterial pressure (Table 1)

Basal MAP measured under standard conditions did not differ between CH and N rats. L-NAME infusion increased MAP significantly and to the same extent in N and CH rats (Table 1). Infusion of SNP during L-NAME treatment restored MAP to basal levels in both groups (Table 1; Fig. 1A). Irrespective of groups, Chtx and Apamin did not