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NOS and COX isoforms and abnormal microvessel responses to CO_2 and H^+ in hyperoxia-injured lungs

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ABSTRACT: The aim of the present study was to compare microvessel responses to hypercapnic and isocapnic acidosis in hyperoxia-injured lungs and to assess the role of constitutive and inducible forms of nitric oxide synthase (NOS) and cyclo-oxygenase (COX).

Real-time confocal luminescence microscopy was used to measure changes in the diameter of acinar arterioles, venules and capillaries in response to stimulation with hypercapnic and isocapnic acidosis in isolated rat lungs injured by 90% oxygen exposure for 48 h. Observations were made with and without inhibition of constitutive (endothelial constitutive NOS (ecNOS) and COX-1) and inducible isoforms (iNOS and COX-2) of NOS and COX. Upregulation of NOS was assessed by measuring enzyme levels in lung homogenates by Western blot analysis and enhancement of the COX-related pathway was judged from perfusate concentrations of 6-ketoprostaglandin $F_{1\alpha}$.

ecNOS and COX-1, but not iNOS and COX-2, were upregulated in hyperoxia-injured lungs. The nitric oxide produced by ecNOS attenuated COX-1 activity in injured arterioles and venules, but carbon dioxide enhanced it, leading to paradoxical dilatation of these microvessels under hypercapnic conditions with ecNOS inhibition. Although a high hydrogen ion concentration was unnecessary for excitation of COX-1, venule constriction in response to H^+ was enhanced by COX-1 inhibition.

Constitutive, but not inducible, isoforms of cyclo-oxygenase and nitric oxide synthase play an important role in abnormal microvessel responses to carbon dioxide and hydrogen ions in hyperoxia-injured lungs.

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The vascular reactivity of pulmonary microvessels, especially acinar microvessels, in response to acidosis induced by either alveolar hypercapnia (hypercapnic acidosis (HA)) or metabolic disturbance (isocapnic acidosis (IA)) is one of the major modulators regulating the distribution of pulmonary blood flow [1–4]. An exact knowledge of acinar microvessel behaviour in acidosis is absolutely necessary when treating patients with diseased lungs using controlled ventilation with permissive hypercapnia, which has increasingly assumed an important place in managing acute lung injury [5, 6]. In a previous study, it was demonstrated that HA induces distinct venule dilatation closely associated with vasodilating prostaglandins generated by cyclo-oxygenase (COX), but not by nitric oxide (NO), in intact rat lung [4]. In another study, it was found that this hypercapnia-associated venule dilatation in intact lungs is significantly attenuated in hyperoxia-injured lungs due to inhibition of COX activity by excessive NO [7]. However, these previous studies did not determine the relative contributions of COX (COX-1 and COX-2) and nitric oxide synthase (NOS) isoforms (endothelial constitutive (ecNOS) and inducible NOS (iNOS)) to the abnormal acinar microvessel response to HA [4, 7]. Furthermore, they did not partition the abnormal microvessel response to HA into that caused by a high carbon dioxide concentration and that by a high hydrogen ion concentration, both of which occur under conditions of HA. Therefore, in the present study, the following issues were systematically evaluated in rat lungs injured by sustained exposure to a hyperoxic environment: 1) the relative importance of constitutive (ecNOS and COX-1) and inducible isoforms (iNOS and COX-2) in the impaired response of acinar microvessels to HA; 2) whether the microvessel response to IA differs qualitatively from that to HA; and 3) whether the contributions of NOS- and COX-related products to abnormal microvessel reactions in IA differ qualitatively from those in HA.

For editorial comments see page 6.

Materials and methods

Preparation of isolated perfused rat lungs

Isolated perfused lungs were prepared from pathogen-free 8-week-old male Sprague-Dawley rats

weighing 250–300 g (n=87). The animals were housed in either a normoxic (21% oxygen, group N, n=15) or a hyperoxic (90% O_2, group H, n=72) environment for 48 h. A detailed description of isolated perfused lung preparation has been provided elsewhere [8]. The isolated lung was fixed on its dorsal surface on a microscope stage and perfused with Krebs-Henseleit solution containing 3% bovine serum albumin at a constant recirculating flow rate of 0.2 $mL \cdot s^{-1}$. The perfusate haematocrit was adjusted to 7±1% by adding fresh blood from donor rats. The trachea was ligated in the end-inspiratory position, and gas exchange was maintained with an extracorporeal membrane oxygenator (ECMO; Merasilox-S; Senko, Tokyo, Japan). A gas mixture containing 21% O_2 and 5% CO_2 was used as the control gas flowing into the ECMO, allowing adjustment of perfusate oxygen tension (P_{O_2}) to 18.9±0.7 kPa, carbon dioxide tension (P_{CO_2}) to 4.9±0.5 kPa and pH to 7.43±0.02. A warmed humidified gas mixture containing the same gas composition as used for the ECMO was also supplied to the lung surface. Mean pulmonary artery pressure (P_{pa}) was monitored by force displacement of pressure transducers (TP-400T; Nihon Koden, Tokyo, Japan).

Experimental protocols

HA was produced by altering the gas flowing into the ECMO and blown on to the lung surface from the control gas to a mixture containing 21% O_2 and 15% CO_2, which increased perfusate P_{CO_2} to 12.4±0.4 kPa and decreased pH to 7.08±0.04 without altering P_{O_2}. IA was produced by slow administration of 1 mL 1 M HCl into the perfusion circuit over 5 min. The final concentration of HCl in the perfusate was adjusted to 10 mM, decreasing the perfusate pH to 7.02±0.08, which was not significantly different from the pH obtained in HA. P_{pa} was monitored continuously for 15 min and microvessel diameter measured thereafter. Changes in P_{pa} from baseline under given experimental conditions were used as the measure of overall vascular resistance changes in the pulmonary circulation, including intra-acinar microvessels and large extra-acinar vessels [9].

Changes in P_{pa} and acinar microvessel diameter were measured under conditions in which ecNOS, iNOS, COX-1 and/or COX-2 were inhibited. Various haemodynamic parameters were analysed in the intact animals (group N) in the absence of exogenous agents, and, depending on the agent used, the hyperoxia-injured animals (group H) were divided into eight subgroups for HA and three for IA (table 1). Although the present authors have previously reported the findings observed for the FHA, LHA and IHA groups (defined in table 1) [7], these experiments were repeated in the present study because the data obtained are indispensable for a clear understanding of the relative importance of each isoform of COX and NOS causing abnormal microvessel responses to HA in hyperoxia-injured lungs.

Real-time measurement of acinar microvessel kinetics

Fresh rat blood was centrifuged at 200 × *g* and the buffy coat discarded. The packed erythrocyte solution thus prepared was diluted in phosphate-buffered saline (pH 7.4), and fluorescein isothiocyanate (FITC; Sigma) was added to a final concentration of 0.1 $\mu g \cdot L^{-1}$. FITC-labelled erythrocyte solution (1 mL) was added to the perfusate to enable determination of direction of flow in the pulmonary microcirculation. The microvessels from which FITC-erythrocytes entered the capillary network were defined as "arterioles", and the microvessels into which FITC-erythrocytes flowed from the capillary network as "venules". Microvessel diameter was measured by adding 200 μL 5% FITC-dextran with a molecular mass of 145 kDa (Sigma). The microvessel events in the acinus were analysed using a recently developed real-time confocal laser luminescence microscope [8]. Fluorescent emission from the microvessels (excitation wavelength

Table 1. – Experimental groups studied in hypercapnic (HA) and isocapnic acidosis (IA)

Enzymes inhibited	Agents	Symbol		
		HA	IA	[Ref]
Normoxia-exposed rats (group N)				
No inhibition (control)	None	CHA (8)	CIA (7)	
Hyperoxia-injured rats (group H)				
No inhibition	None	FHA (8)	FIA (6)	
ecNOS, iNOS	L-NAME	LHA (8)	LIA (7)	
iNOS	Aminoguanidine	AMHA (7)		[10, 11]
COX-1, COX-2	Indomethacin	IHA (7)	IIA (5)	
COX-2	NS-398	NSHA (7)		[12, 13]
ecNOS, iNOS, COX-1, COX-2	L-NAME+indomethacin	LIHA (7)		
ecNOS, iNOS, COX-2	L-NAME+aminoguanidine	LNSHA (7)		
NO donor*	SNP	SNPHA (6)		

Data in parentheses are n. Aminoguanidine (5 mM) and indomethacin (20 μM) were from Sigma (St Louis, MO, USA). ecNOS: endothelial constitutive nitric oxide synthase (NOS); iNOS: inducible NOS; L-NAME: N^{ω}-nitro-L-arginine methyl ester (100 μM; Sigma); COX: cyclo-oxygenase; NS-398: *N*-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide (1 μM; Taisho, Tokyo, Japan). *: no enzymes were inhibited; exogenous nitric oxide donor, sodium nitroprusside (SNP, 1 μM; Sigma), administered.

488 nm) was detected by a high-sensitivity charge-coupled-device camera with an image intensifier (EktaPro Intensified Imager VSG; Kodak, San Diego, CA, USA). Confocal units permitted instantaneous images to be obtained at a rate of 1,000 frames·s^{-1}. The resultant field of view was 210×210 μm, corresponding approximately to the diameter of a single pulmonary microvessel adjacent to the terminal bronchiole. Confocal images were recorded at 250 frames·s^{-1} using a high-speed video analyser (EktaPro 1000 Processor; Kodak). The diameter of each microvessel was estimated by processing a confocal video image with a digital image analyser (Quadra 840AV/Image 1.58; Apple, Cupertino, CA, USA).

Determination of prostacyclin (prostaglandin I_2) metabolite in the perfusate

A 2-mL perfusate sample collected into a tube containing a small amount of indomethacin and ethylene diamine tetra-acetic acid was centrifuged and frozen at -80°C until use. The concentration of immunoreactive 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a stable metabolite of prostaglandin I_2, was measured by enzyme-linked immunosorbent assay (EIA kit; Cayman Chemical, Ann Arbor, MI, USA).

Determination of nitric oxide-related metabolites in the perfusate

A 2-mL perfusate sample was collected and frozen at -80°C until use. To measure NO production in the lung, the total concentration of end products of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-), in the perfusate was assessed using the method proposed by GREEN *et al.* [14].

Western blot analysis for nitric oxide synthase proteins in lung homogenates

The ecNOS and iNOS content of crude lung homogenates from groups N and H (both n=3) was determined by Western blot analysis as proposed by NORTH *et al.* [15]. Lung tissue was homogenised on ice in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.5). The homogenate was centrifuged at 12,000 × *g* at 4°C for 10 min to remove cell debris. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was conducted on 100-μg aliquots of homogenate protein. Proteins were transferred to nitrocellulose paper (Immobilon-P; Millipore, Bedford, MA, USA), and the blot was blocked in 20 mM Tris-buffered saline (TBS, pH 7.4), 5% skimmed milk, 0.05% Tween-20 for 24 h at 4°C. The blot was then incubated with a primary antibody directed against ecNOS (immunoglobulin (Ig) G1 monoclonal antibody; BD Biosciences, San Jose, CA, USA) or iNOS (IgG2a monoclonal antibody, BD Biosciences) for 1 h at 24°C. The blot was washed with TBS, 0.05% Tween-20 and then incubated with peroxidase-labelled goat antimouse IgG (containing both heavy and light chains) (Zymed, South San Francisco, CA, USA) for 1 h at 24°C. Protein bands detected by the antibodies were visualised by enhanced chemiluminescence (Amersham, Little Chalfont, UK). Human endothelial lysate (BD Biosciences) was used as the positive control for ecNOS, and mouse macrophage lysate (BD Biosciences) for iNOS.

Statistical analysis

Statistical significance was analysed by one-way analysis of variance followed by multiple comparison Scheffé's analysis. Changes in P_{pa}, microvessel diameter, and NO and 6-keto-$PGF_{1\alpha}$ levels before and after a given stimulation were assessed by paired t-test. P_{pa} increments, changes in microvessel diameter, and changes in NO and 6-keto-$PGF_{1\alpha}$ levels in the perfusate in response to HA and IA during exposure to the same agent were compared by applying an unpaired t-test. Values are expressed as mean±SD, with $p<0.05$ considered significant.

Results

Basic haemodynamics before stimulation with hypercapnic and isocapnic acidosis

Baseline P_{pa} in the agent-free group H were 13.0±0.8 mmHg before HA and 12.4±0.7 mmHg before IA (table 2). These values did not differ significantly from the corresponding P_{pa} in group N (11.8±0.6 mmHg before HA and 10.4±0.5 mmHg before IA).

The baseline diameter of the acinar arterioles and venules in both groups N and H ranged 20–35 μm, with no significant differences among the values. The baseline capillary diameter was 6–7 μm in groups N and H. There was no difference in capillary diameter between the two groups.

Changes in mean pulmonary artery pressure in hypercapnic acidosis

Induction of HA increased P_{pa} by 0.7±0.8 mmHg in group N and 2.1±1.5 mmHg in group H in the absence of any agents, the latter increase being significantly greater than the former ($p<0.05$) (table 2). The enhanced hypercapnia-elicited P_{pa} changes in group H disappeared when N^{ω}-nitro-L-arginine methyl ester (L-NAME, inhibition of ecNOS and iNOS, $p<0.05$ *versus* no agent) but not aminoguanidine (inhibition of iNOS) was administered. The hypercapnia-induced increments in P_{pa} in group H with either indomethacin (inhibition of COX-1 and COX-2) or *N*-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide (NS-398, inhibition of COX-2) did not differ significantly from those in agent-free group H. Administration of indomethacin together with L-NAME restored the decreased hypercapnia-elicited P_{pa} change observed for L-NAME alone to a level comparable to that in agent-free group H ($p<0.05$ *versus*

Table 2. – Increases in mean pulmonary artery pressure (P_{pa}) (ΔP_{pa}) upon hypercapnic and isocapnic acidosis in groups N and H

Agents	Hypercapnic acidosis		Isocapnic acidosis	
	Baseline P_{pa} mmHg	ΔP_{pa} mmHg	Baseline P_{pa} mmHg	ΔPpa mmHg
Normoxia-exposed rats (group N)				
None control	11.8±0.6 (8)	0.7±0.8	10.4±0.5 (7)	0.7±0.6
Hyperoxia-injured rats (group H)				
None	13.0±0.8 (6)	2.1±1.5*	12.4±0.7 (6)	1.9±0.8*
L-NAME	10.5±1.0 (8)	1.0±1.4$^{\#}$	11.3±1.0 (7)	1.8±1.4*,¶
Aminoguanidine	11.5±1.2 (7)	1.7±1.2*		
Indomethacin	12.2±1.1 (7)	2.3±1.6*	13.4±2.0 (5)	1.6±1.0*
NS-398	11.6±1.0 (7)	2.1±1.5*		
L-NAME+indomethacin	11.0±0.6 (6)	2.4±0.7*		
L-NAME+NS-398	13.3±1.7 (7)	0.9±0.2$^{\#}$		
SNP	11.9±1.3 (6)	0.2±1.8$^{\#}$		

Data are presented as mean±SD (n). The enzyme(s) inhibited by the various agents are detailed in table 1. L-NAME: N^{ω}-nitro-L-arginine methyl ester; NS-398: *N*-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide; SNP: sodium nitroprusside. *: p<0.05 *versus* group N; $^{\#}$: p<0.05 *versus* agent-free group H; ¶: p<0.05 *versus* same agent in hypercapnic acidosis.

L-NAME). No such tendency was evident, however, when NS-398 and L-NAME were administered together. Although sodium nitroprusside (SNP, NO donor) did not change the baseline P_{pa}, it significantly suppressed the P_{pa} increase after HA in group H (p<0.05 *versus* agent-free).

Changes in microvessel diameter in hypercapnic acidosis

Acinar arteriole diameter was not altered by HA in either group N or H in the absence of any of the agents, but inhibition of both ecNOS and iNOS by L-NAME unexpectedly dilated the group-H arterioles by 2.6 μm (p<0.01 *versus* agent-free) (fig. 1). This peculiar phenomenon, however, was not observed when only iNOS was inhibited, using aminoguanidine. Neither indomethacin (inhibition of COX-1 and COX-2) nor NS-398 (inhibition of COX-2) changed arteriole diameter under hypercapnic conditions in group H. Addition of indomethacin plus L-NAME significantly inhibited the arteriole dilatation evoked by L-NAME alone (p<0.01). Simultaneous addition of NS-398 and L-NAME, conversely, did not interfere with the L-NAME-elicited arteriole dilatation. SNP did not alter arteriole diameter under hypercapnic conditions.

The acinar venules significantly dilated by 1.8± 1.7 μm in response to HA in group N (p<0.05), but not in agent-free group H (fig. 1). The response of H-group venules to HA was qualitatively the same as that of arterioles under each experimental condition.

HA did not alter capillary diameter in any of the groups.

Changes in mean pulmonary artery pressure in isocapnic acidosis

IA increased P_{pa} by 0.7±0.6 mmHg in group N and 1.9±0.8 mmHg in group H with no agents added, the increase in the latter group being significantly greater than that in the former (p<0.05) (table 2). In contrast to the findings for HA, inhibition of ecNOS and iNOS by L-NAME did not reduce the extent of group-H P_{pa} increases due to IA. The

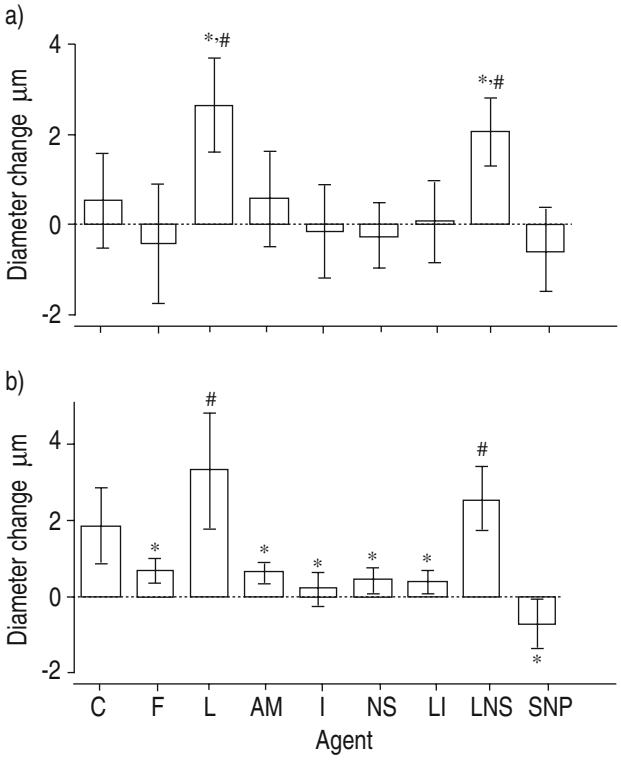

Fig. 1. – Hypercapnia-elicited changes in microvessel diameter in groups N (control) and H (hyperoxia-injured animals): a) acinar arterioles and b) acinar venules. C: control; F: agent-free; L: N^{ω}-nitro-L-arginine methyl ester (L-NAME, inhibition of endothelial constitutive and inducible nitric oxide synthase (iNOS)); AM: aminoguanidine (inhibition of iNOS); I: indomethacin (inhibition of cyclo-oxygenase (COX)-1 and -2); NS: *N*-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide (NS-398, inhibition of COX-2); LI: L-NAME plus indomethacin; LNS: L-NAME plus NS-398; SNP: sodium nitroprusside (nitric oxide donor). *: p<0.05 *versus* group N; $^{\#}$: p<0.05 *versus* agent-free group H.

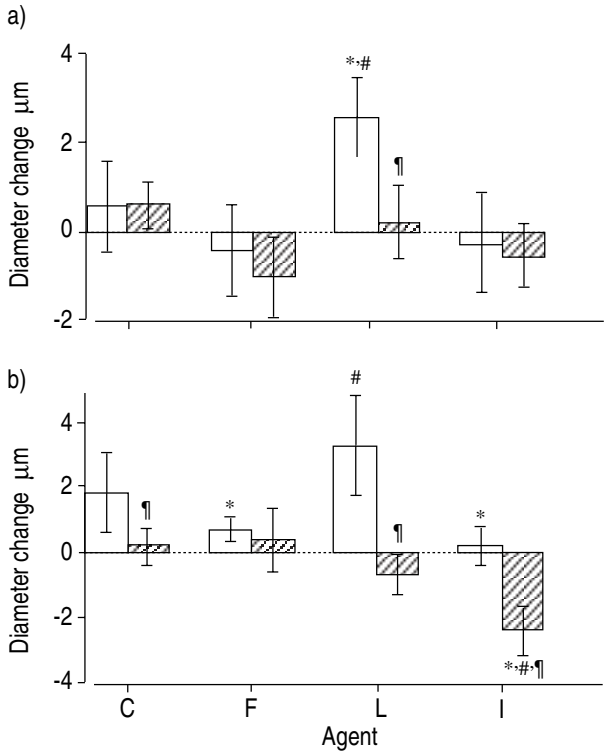

Fig. 2. – Changes in microvessel diameter in isocapnic (IA; ▨) and hypercapnic acidosis (HA; □) in groups N (control) and H (hyperoxia-injured animals): a) acinar arterioles; and b) acinar venules. The HA data are those from figure 1. C: control; F: agent-free; L: N^{ω}-nitro-L-arginine methyl ester (L-NAME, inhibition of endothelial constitutive and inducible nitric oxide synthase); I: indomethacin (inhibition of cyclo-oxygenase-1 and -2). *: $p<0.05$ *versus* group N; #: $p<0.05$ *versus* agent-free group H; ¶: $p<0.05$ *versus* same agent in HA.

group-H P_{pa} increase evoked by IA in the presence of indomethacin (inhibition of COX-1 and COX-2) did not differ from that in the absence of the agent.

Changes in microvessel diameter in isocapnic acidosis

IA did not change either arteriole or venule diameter in group N and agent-free group H (fig. 2). Although arteriole diameter and venule diameter in group H were significantly increased by HA with L-NAME ($p<0.01$ for each microvessel), this phenomenon was not observed in IA with L-NAME. Addition of indomethacin (inhibition of COX-1 and COX-2) under isocapnic conditions did not alter arteriole diameter in group H but significantly constricted group-H venules ($p<0.05$).

Capillary diameter in group H was not altered by isocapnic stimulation in any of the groups.

Perfusate 6-ketoprostaglandin $F_{1\alpha}$ concentrations before hypercapnic acidosis

Although the baseline concentration of perfusate 6-keto-$PGF_{1\alpha}$ before HA in agent-free group H was significantly greater than that in group N ($p<0.05$) (table 3), it significantly decreased in the subgroups treated with indomethacin (inhibition of COX-1 and COX-2) ($p<0.05$). The enhanced baseline production of 6-keto-$PGF_{1\alpha}$ in group H, however, was not inhibited by NS-398 (inhibition of COX-2). Although neither L-NAME (inhibition of ecNOS and iNOS) nor aminoguanidine (inhibition of iNOS) had any effect on baseline production of 6-keto-$PGF_{1\alpha}$, SNP (NO donor) significantly increased it in group H ($p<0.05$).

Perfusate 6-ketoprostaglandin $F_{1\alpha}$ concentrations after hypercapnic acidosis

The hypercapnia-elicited increase in 6-keto-$PGF_{1\alpha}$ production was significantly greater in agent-free group H than in group N ($p<0.05$) (table 3). Indomethacin (inhibition of COX-1 and COX-2), but not NS-398 (inhibition of COX-2), partially inhibited the

Table 3. – Increases in perfusate 6-ketoprostaglandin $F_{1\alpha}$ (Δ6-keto-$PGF_{1\alpha}$) upon hypercapnic and isocapnic acidosis in groups N and H

Agents	Hypercapnic acidosis		Isocapnic acidosis	
	Baseline 6-keto-$PGF_{1\alpha}$ pg·mL^{-1}	Δ6-keto-$PGF_{1\alpha}$ pg·mL^{-1}	Baseline 6-keto-$PGF_{1\alpha}$ pg·mL^{-1}	Δ6-keto-$PGF_{1\alpha}$ pg·mL^{-1}
Normoxia-exposed rats (group n)				
None control	266±31 (6)	90±59	336±44 (4)	-31±39#
Hyperoxia-injured rats (group H)				
None	536±67 (6)*	144±76*	593±70 (5)*	-2±56¶
L-NAME	532±108 (7)*	-72±87*,#	601±63 (5)*	-80±68
Aminoguanidine	540±41 (5)*	88±49		
Indomethacin	379±33 (5)#	42±84#	454±80 (5)#	-107±74¶
NS-398	543±76 (5)*	131±54		
L-NAME+indomethacin	301±69 (5)#	-43±61*,#		
L-NAME+NS-398	593±149 (5)*	-12±81*,#		
SNP	874±69 (5)*,#	188±69*		

Data are presented as mean±SD n. The enzyme(s) inhibited by the various agent are detailed in table 1; L-NAME: N^{ω}-nitro-L-arginine methyl ester; NS-398: *N*-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide; SNP: sodium nitroprusside. *: $p<0.05$ *versus* group N; #: $p<0.05$ *versus* agent-free group H; ¶: $p<0.05$ *versus* same agent in hypercapnic acidosis.

increase in 6-keto-$PGF_{1\alpha}$ concentration in group H ($p<0.05$). Addition of L-NAME (inhibition of ecNOS and iNOS), but not aminoguanidine (inhibition of iNOS), significantly inhibited the increase in 6-keto-$PGF_{1\alpha}$ concentration after HA in group H ($p<0.01$). In the presence of SNP, hypercapnic stimulation significantly increased the perfusate 6-keto-$PGF_{1\alpha}$ concentration in group H ($p<0.05$ *versus* group N). The SNP-elicited increase in 6-keto-$PGF_{1\alpha}$ was comparable to that observed under agent-free conditions.

Perfusate 6-ketoprostaglandin $F_{1\alpha}$ concentrations before isocapnic acidosis

The baseline concentration of perfusate 6-keto-$PGF_{1\alpha}$ in group H before IA was significantly greater than that in group N ($p<0.05$) (table 3). Baseline production of 6-keto-$PGF_{1\alpha}$ in group H was significantly inhibited by indomethacin (inhibition of COX-1 and COX-2) ($p<0.05$). L-NAME had no effect on baseline 6-keto-$PGF_{1\alpha}$ production.

Perfusate 6-ketoprostaglandin $F_{1\alpha}$ concentrations after isocapnic acidosis

The 6-keto-$PGF_{1\alpha}$ concentration in the perfusate in group N did not increase after IA and neither did it increase in drug-free group H (table 3). Comparison of the data obtained for HA and IA revealed a significantly greater increase in perfusate 6-keto-$PGF_{1\alpha}$ concentration in response to HA than upon IA, both in the absence of any agents and with indomethacin (inhibition of COX-1 and COX-2), in group H ($p<0.05$ for both). There was no significant difference between the increase in 6-keto-$PGF_{1\alpha}$ concentration in HA and IA when L-NAME (inhibition of ecNOS and iNOS) was present in group H.

Nitric oxide concentration in the perfusate

The baseline perfusate concentrations of NO-related metabolites were significantly higher in agent-free group H than in group N ($p<0.05$) (fig. 3). L-NAME (inhibition of ecNOS and iNOS) reduced baseline NO production in group H to a level equivalent to that in group N ($p<0.05$), whereas administration of aminoguanidine (inhibition of iNOS) did not. SNP (NO donor) drastically increased baseline concentrations of NO metabolites in group H ($p<0.001$).

Neither HA nor IA increased NO production in any of the groups studied.

Constitutive and inducible nitric oxide synthase content of lung homogenates

The ecNOS in the positive control was detected at a molecular mass of 140 kDa. Densitometric analysis revealed that the luminescent intensity of ecNOS in group H was, on average, 3.8-times higher than that in group N ($p<0.05$) (fig. 4). Although the monoclonal antibody directed against iNOS detected iNOS in the positive control at a molecular mass of 130 kDa, little iNOS was detected in the lung homogenates harvested from either group N or group H (data not shown).

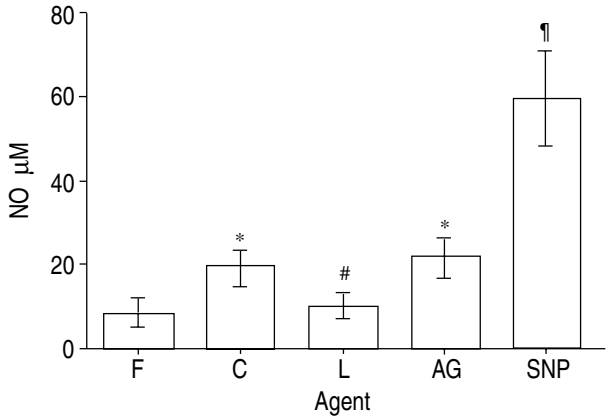

Fig. 3. – Baseline perfusate concentrations of nitric oxide (NO) metabolites in groups N (control) and H (hyperoxia-injured animals). C: control; F: agent-free; L: N^{ω}-nitro-L-arginine methyl ester (L-NAME, inhibition of endothelial constitutive and inducible nitric oxide synthase (iNOS)); AM: aminoguanidine (inhibition of iNOS); SNP: sodium nitroprusside (NO donor). *: $p<0.05$ *versus* group N; #: $p<0.05$ *versus* agent-free group H; ¶: $p<0.05$ *versus* all other conditions.

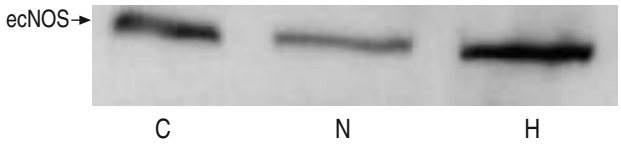

Fig. 4. – Endothelial constitutive nitric oxide synthase (ecNOS) expression on Western blot analysis of lung homogenates. C: positive control; N: normoxia-exposed rats (group N); H: hyperoxia-injured rats (group H).

Discussion

Critique of methods

Many investigators have attempted to assess the effects of HA or IA on vasoconstriction in the pulmonary circulation, mainly based on measurements of P_{pa} changes [16–19]. In the present study, however, a real-time confocal laser scanning luminescence optical microscope, which allows clear discrimination of individual microvessels from their neighbours, was applied [8]. This method, however, has potential limitations since isolated lungs perfused with a buffer containing a small quantity of whole blood were used, and haemodynamic aspects in isolated lungs may be different from those under *in vivo* conditions. Despite these flaws, vasoactive responses of acinar microvessels to acid stimuli were

estimated using real-time confocal images obtained from the isolated lung preparation, which allows reliable separation of microvessels in the acinar microcirculation, *i.e.* the most important issue when attempting to identify the subtle roles of vasoactive substances in modifying the vascular reactivity of acinar microvessels.

Although ecNOS was upregulated in hyperoxia-injured lungs (fig. 4), Western blot analysis did not detect iNOS in tissue homogenates harvested from such lungs. There was also little reduction in perfusate NO concentrations when inhibiting iNOS with aminoguanidine (fig. 3). These results suggest that iNOS expression is not enhanced in lungs exposed to hyperoxia for 48 h. These findings are consistent with those of ARKOVITZ *et al.* [20], who estimated lung iNOS messenger ribonucleic acid (mRNA) levels upon exposure to hyperoxia for 2–5 days by Northern blot analysis. They demonstrated little increase in lung iNOS mRNA levels at any time during exposure to hyperoxia [20].

Importance of nitric oxide-dependent modulation of cyclo-oxygenase-1 activity in hypercapnic acidosis

It was demonstrated in a previous study that hypercapnia-elicited venule dilatation was blunted in hyperoxia-injured lungs and was paradoxically restored by inhibition of NOS isoforms with L-NAME [7]. These findings are qualitatively consistent with those of the present study (fig. 1). The responses of injured arterioles to HA under various experimental conditions were similar to those observed in injured venules, including the unexpected vasodilation elicited by L-NAME (fig. 1). These findings suggest that the essential mechanism(s) mediating the abnormal responses of venules and arterioles in hyperoxia-injured lungs are qualitatively the same. The hypercapnia-evoked microvessel dilatation was not observed in injured lungs under conditions in which iNOS alone was inhibited by aminoguanidine (fig. 1). This indicates that the L-NAME-associated microvessel dilatation was principally mediated by ecNOS, and not iNOS. It was also shown that the hypercapnia-evoked microvessel dilatation observed in the presence of L-NAME was prevented when COX-1 and COX-2 were inhibited with indomethacin in the presence of L-NAME (fig. 1). However, inhibition of COX-2 by NS-398 failed to suppress the L-NAME-associated microvessel dilatation occurring during HA. Taken together, these findings suggest that the hypercapnia-elicited microvessel dilatation in injured lungs is mediated by vasodilator prostaglandins generated by COX-1 but not COX-2, and that COX-1 activity is inhibited by NO produced by ecNOS. The importance of reduction of COX activity by NO was also demonstrated in the experiments in which the perfusate concentrations of NO were artificially increased by SNP. Neither arterioles nor venules exposed to HA dilated despite the presence of excess NO in the circulating medium (fig. 1). This supports the notion that NO, which is a vasodilator in most circumstances, functions as a potent vasoconstrictor in injured microvessels as a result of its inhibitory effects on COX-1 activity.

Several groups of investigators have reported dual effects of NO on COX activity *in vitro* [21–26]. Since COX is a haemoprotein, containing iron, some authors have ascribed the inhibitory effect of NO on the COX activity to the interaction of NO with iron at the active site [22, 24, 26]. KANNER *et al.* [22] and TSAI *et al.* [24] demonstrated that the activity of both COX-1 and COX-2 was maintained by ferric iron and that ferric COX activity was inhibited by NO because NO reduced the ferric enzyme to the ferrous form. LANDINO *et al.* [26], conversely, reported that NO enhanced the activity of both purified COX-1 and COX-2 under conditions in which production of both NO and superoxide was augmented. The NO-dependent COX-1 excitation may be mediated by peroxynitrite, a product of NO and superoxide [26].

Given the present experimental conditions, exposure to hyperoxia may enhance the generation of both reactive oxygen species (ROS) [27–29] and NO (figs. 3 and 4). This suggests that net effects caused by both inhibitory and excitatory actions of NO on the activity of COX isoforms should be taken into account. The finding of restoration of hypercapnia-elicited microvessel dilatation by ecNOS inhibition (fig. 1) is consistent with the idea that the inhibitory effect of NO on COX-1 activity exceeds its excitatory effect, at least in acinar microvessels injured by exposure to hyperoxia. However, negative crosstalk between ecNOS- and COX-1-related pathways may not explain the overall changes in perfusate 6-keto-$PGF_{1\alpha}$ concentration under the various experimental conditions. Measurements of overall perfusate 6-keto-$PGF_{1\alpha}$ concentration under conditions of SNP-increased NO concentration indicate that NO enhances COX-1 activity (table 3). This leads to the conclusion that the inhibitory effect of NO on COX-1 is restricted to acinar microvessels, whereas the excitatory effect of NO on COX-1 predominates in other lung regions. Although it is unclear why COX-1 activity is inhibited by NO in acinar microvessels and augmented in other lung regions, one possibility is that acinar microvessels have a much greater ability to scavenge excess ROS, as suggested by HEFFNER and REPINE [30]. This is inferred from the fact that the flow velocity of erythrocytes, which are rich in antioxidant systems [29], is extremely low in the acinar microcirculation [9], thereby allowing erythrocytes there to act as large sinks scavenging various ROS. If this is true, higher ROS levels in hyperoxia-injured microvessels in acini may be reduced, leading to diminution of peroxynitrite formation, which, in turn, inhibits excitation of COX-1 around acinar microvessels.

The overall changes in P_{pa} elicited by HA in hyperoxia-injured lungs qualitatively paralleled those in microvessel diameter under various experimental conditions (table 2, fig. 1). These findings suggest that changes in microvessel diameter of 2–3 μm have substantial effects on overall vascular resistance in the pulmonary circulation.

Microvessel responses to carbon dioxide and hydrogen ions

In contrast to HA, no dilatation of arterioles or venules was demonstrated in response to IA in the presence of L-NAME (fig. 2). This indicates that a high CO_2 level *per se* is the principal factor causing the enhanced microvessel dilatation during exposure to HA with NOS inhibition. This can be explained if CO_2, but not H^+, is indispensable for activating COX-1. CO_2 can scavenge peroxynitrite mediating the NO-dependent COX-1 excitation [26], indicating that hypercapnia partially reduces COX-1 activity. However, nitrosoperoxycarbonate, a product of CO_2 and peroxynitrite, is a very active substance, and the possibility that nitrosoperoxycarbonate has potential for activating COX-1 cannot be eliminated. Further study is absolutely necessary to clarify the effect of nitrosoperoxycarbonate on COX-1 excitation. Under isocapnic conditions with L-NAME, the activity of COX-1 may be affected by two opposing influences: activation due to diminution of NO production on the one hand, and deactivation by lack of CO_2 on the other. These two factors may be balanced out by each other, resulting in an unchanged microvessel diameter.

IA elicited venule constriction in injured lungs when COX isoforms were suppressed by indomethacin, but no such phenomenon was observed in injured arterioles (fig. 2). These findings indicate that injured venules have a much greater sensitivity to the constrictive effects of H^+ than injured arterioles. Intact venules did not constrict in response to IA in the presence of indomethacin [4]. Taken together, the sensitivity of injured venules to the constrictive effects of H^+ may also be higher than that of intact venules. This H^+-dependent venule constriction in injured lungs was concealed in HA with indomethacin (fig. 2), in which more COX-derived vasodilating prostaglandins may be produced than in IA because of partial excitation of COX by high CO_2 concentrations even in the presence of indomethacin (table 3).

The following conclusions can be drawn. 1) Exposure of the lungs to hyperoxia mainly enhances the expression of constitutive vasoactive enzymes, endothelial constitutive nitric oxide synthase and cyclo-oxygenase-1. 2) Nitric oxide generated by endothelial constitutive nitric oxide synthase inhibits cyclo-oxygenase-1 activity in acinar arterioles and venules. This leads to paradoxical dilatation of both types of microvessel under hypercapnic conditions in the presence of endothelial constitutive nitric oxide synthase inhibition, in which cyclo-oxygenase-1 is potentially activated by high carbon dioxide levels. 3) Cyclo-oxygenase-1 inhibition enhances venule sensitivity to the constrictive effects of hydrogen ions.

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