Hypoxia reduces endothelin production by rat alveolar type II cells in primary culture

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ABSTRACT: The purpose of the study was to describe endothelin (ET) production and to characterize the effect of hypoxia on preproendothelin-1 (preproET-1) messenger ribonucleic acid (mRNA) expression and ET secretion by rat type II pneumocytes *in vitro*.

Rat type II pneumocytes were incubated in a sealed chamber containing a normoxic (21% O₂) or hypoxic (1% O₂) atmosphere for increasing durations. Immunoreactive ET (irET) was measured in cell supernatants using a radioimmunoassay. Rat preproET-1 mRNA was detected by Northern blot.

Rat type II pneumocytes expressed preproET-1 mRNA, contained irET and secreted irET in a time-dependent manner. ET secretion was dependent on *de novo* ribonucleic acid (RNA) and protein synthesis. Hypoxia decreased irET secretion by 27% and reduced the steady-state level of preproET-1 mRNA by 60% whereas intracellular irET concentration was unchanged. Inhibition was partially reversible with the return to a normoxic atmosphere. Inhibition of nitric oxide synthesis did not prevent the inhibitory effect of hypoxia.

In conclusion, rat type II pneumocytes in primary culture secreted immunoreactive endothelin and expressed preproendothelin-1 messenger ribonucleic acid. Hypoxia reversibly reduced endothelin-1 production through a reduction of the steady-state preproendothelin-1 messenger ribonucleic acid level. Nitric oxide synthesis did not mediate the inhibitory effect of hypoxia.

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Type II alveolar epithelial cells (ATII cells) have been shown to play a key role in the regulation of the alveolar space. ATII cells synthesize and secrete surfactant, control the volume and composition of the epithelial lining fluid and proliferate and differentiate into type I alveolar epithelial cells after injury in order to maintain the integrity of the alveolar wall. Moreover, ATII cells are ideally located to have a role in modulating the activation or proliferation state of macrophages, fibroblasts or endothelial cells, because of the close proximity of the cell types in the alveolar space.

The interactions between the alveolar epithelial cells and the other cells located in the alveoli are brought about by the secretion of mediators such as cytokines [1], but other mediators are probably involved in this phenomenon. Among these mediators, endothelin (ET)-1 a 21 amino acid peptide may play an important role. Indeed, beside its potent effect on the constriction of vascular and bronchial smooth muscle cells, ET-1 acts as a mitogen for different cell types, such as fibroblasts or smooth muscle cells [2]. ET-1 is also involved in the modulation of the inflammatory response through a direct effect on alveolar macrophages [3] or mastocytes [4].

It has been shown that hyperplastic alveolar epithelial cells express preproendothelin-1 (preproET-1) messenger ribonucleic acid (mRNA) and immunoreactive ET (irET)-

1 in the human fibrotic lung [5], although conflicting data exist [6]. Rat ATII cells in primary culture [7], and a cell line derived from rat ATII cells, have been shown to secrete ET-1 *in vitro* [8].

Hypoxia has been identified as an important modulator of ET-1 production by endothelial cells. Hypoxia increases ET-1 production by human umbilical vein endothelial cells [9], but decreases ET-1 production by rat lung endothelial cells [10]. The effect of hypoxia on ET-1 production by rat ATII cells has never been evaluated, despite the fact that ATII cells are potential targets for hypoxia in the alveolar space both in physiological (high altitude) and pathological (e.g. lung parenchyma consolidation) conditions.

Therefore, the aims of this study were: 1) to characterize the regulation of ET-1 production by rat ATII cells in primary culture under low oxygen tension; and 2) to evaluate the influence of nitric oxide on ET-1 production by rat ATII cells.

Materials and methods

Reagents

Tissue culture media and foetal bovine serum were obtained from Gibco BRL Life Technologies (Cergy Pontoise,

France). Tissue culture plastic ware was from Costar (Cambridge, MA, USA). Chemicals were from Sigma (La Verpilliere, France).

Rat alveolar type II cells isolation and culture

ATII cells were isolated from adult pathogen-free male Sprague-Dawley rats (Charles River Breeders, St Aubin les Elbeuf, France) by enzymatic dissociation and purified by differential adherence to plastic as described previously [11]. One million cells were plated in each well of a 24 well cell-culture plate with 1 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 10^5 U·mL⁻¹ penicillin, 100 mg·L⁻¹ streptomycin and 0.25 mg·L⁻¹ amphotericin B (complete DMEM). After a 24 h period, nonadherent cells were removed by gently washing twice with phosphate buffered saline (PBS), fresh complete medium was replaced and the cells were used for the experiments.

In some experiments, ATII cells (5×10^6) were cultured in 10 cm cell culture dishes. At the end of the incubation time, the supernatants were recovered and the cell monolayers were immediately frozen by dipping in liquid nitrogen, and stored at -80°C until ribonucleic acid (RNA) extraction. In some experiments, we measured irET secretion by the A549 cell line, a pulmonary epithelial cell line which has been used as a model of human ATII cells [1]. A549 cells were grown in complete DMEM in 24 well cell-culture plates.

Conditions for achieving hypoxia

ATII cells were exposed for different periods either to normal or low oxygen tensions in humidified air-tight 12 L Lwoff chambers with inflow and outflow valves (Lequeux, Paris, France). The chambers were flushed with pre-analyzed gas mixtures. The chambers were continuously maintained at 37°C. The desired oxygen mixtures (either normoxic: 5% CO₂ 95% air; or hypoxic: 1% O₂, 5% CO₂, balance N₂) were preanalysed (Compagnie Française des Produits de l'Oxygène, Paris, France) and infused into the incubators at a flow rate of 12 L·min⁻¹, initially and at the end of the first 24 h culture period when the cells had to be cultured for more than 24 h. The oxygen fraction was monitored in the gas outflow using an oximeter (Datex OT-101; Datex Instrumentarium Corp., Helsinki, Finland) in order to ascertain that the expected oxygen fraction was achieved in the incubator. Media gas determinations were made at the end of the exposure (ABL 512; Radiometer, Copenhagen, Denmark). The partial pressure of oxygen (PO₂) measured in the cell culture supernatants after 24 h and 48 h incubation was 5.7 ± 0.1 and 5.1 ± 4.0 kPa (43 ± 1 and 38 ± 3 mmHg) with 1% O₂ and 13.8 ± 0.4 and 13.7 ± 0.4 kPa (104 ± 3 and 103 ± 3 mmHg) with 21% O₂ (n=3), respectively. These PO₂ values are in the same range as those measured by other investigators with similar exposure systems [10]. Media pH values were 7.51 and 7.43 with 21% O₂ after 24 and 48 h, respectively, and 7.49 and 7.47 with 1% O₂ after 24 and 48 h, respectively. Cellular viability was assessed after 24 and 48 h of culture using the methyl-thiazol-tetrazolium (MTT) colorimetric assay [12]. At the end of the incubation period, the cell culture super-

natants were recovered, immediately frozen and stored at -80°C until ET assay. Cell monolayers were washed twice with PBS, then sonicated in 500 µL PBS and the cellular protein concentration was measured using the Bradford protein assay [13].

Pharmacological interventions

In some experiments, ATII cells exposed for 24 h in a 1% O₂ atmosphere were transferred to a normoxic atmosphere for a 24 h incubation period in order to assess the reversibility of the action of hypoxia. In other experiments, the cells were cultured with cycloheximide (20 µg·mL⁻¹) in order to inhibit protein synthesis, or with actinomycin D (5 µg·mL⁻¹) in order to inhibit mRNA synthesis.

The effect of NO synthesis inhibition was assessed by culturing the cells in control conditions or supplemented with 0.5 mM N-monomethyl-L-arginine (L-NMMA), (a stereospecific NO synthase inhibitor) or with 5 mM L-arginine (L-Arg) or the combination of L-NMMA and L-Arg. In addition, we evaluated the effect of a nonarginine-based NO synthase inhibitor (S-methyl-isothiouraea sulphate (thiourea), 1 mM) [14] and an NO donor (SIN-1, 0.1 mM) [15] on preproET-1 mRNA content in ATII cells.

Northern blot analysis

Rat preproET-1 mRNA and glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA were detected by a Northern blot analysis and quantified through an electronic autoradiography device (Instant Imager®; Packard, Groningen, the Netherlands) as described previously [7]. The ratio of the preproET-1 mRNA signal and the corresponding GAPDH mRNA signal was calculated for each sample.

ET-1 radioimmunoassay

ET-1 radioimmunoassay was developed in the *laboratoire de biologie hormonale* by two of us (FM, JF). Synthetic peptide rat ET-1 [1–21] was synthesized with an automated peptide synthesizer (model 431 A; Applied Biosystems, Paris, France). Human and rat ET-1 amino acid sequences are identical [16]. ET-1 was iodinated by the chloramine T method. Specific activity of radiolabelled ET-1 was 2,300 Ci·mmol⁻¹.

Immunoreactive ET was assayed in cell culture supernatants without extraction. Briefly, samples (100 µL) and standards (synthetic ET-1) were reconstituted in assay buffer and incubated with rabbit anti-rat ET-1 anti-serum prepared in our laboratory by immunizing a rabbit with synthetic rat peptide ET-1 [1–21]. This was followed by the addition of ¹²⁵I-labelled ET-1 (approximately 10⁴ counts per minute (cpm) per sample). Bound and free radioactivity were separated by the secondary antibody method, and gamma emission from the pelleted anti-body-ET complexes was counted using a gamma counter. Since, the antibody used exhibited a 75% and 60% cross-reactivity with ET-2 and big ET-1, respectively, the results are given as ir-ET rather than ET-1. The intra- and interassay coefficients

of variation were 7 and 13%, respectively. The minimum detectable level is $2.5 \text{ pg}\cdot\text{mL}^{-1}$. Undetectable irET was noted as zero. The irET concentration was normalized for the protein concentration in each dish and expressed as $\text{pg}\cdot\text{mg}^{-1}$ protein. We checked that the foetal bovine serum used in all the experiments did not contain any detectable irET in this assay.

ET immunolabelling

Rat ATII cells grown on glass coverslips were washed with PBS and fixed in acetone for 10 min at room temperature. Endogenous peroxidase was inhibited by incubation with H_2O_2 (3%) for 5 min. Then, the slides were incubated with a normal goat serum for 30 min in order to avoid non-specific binding. Excess serum was tipped off, and cell monolayers were overlaid for 30 min with a polyclonal rabbit anti-ET-1 antiserum (1:100 volume/volume (v/v) in PBS containing 0.1% bovine-serum albumin (BSA)). After several washes in PBS, cell monolayers were incubated for 10 min with a biotinylated goat anti-rabbit antibody, then incubated with streptavidin-peroxidase (Dako, Trappes, France) for 10 min and washed in distilled water. Peroxidase reaction was then revealed by diaminobenzidine (Dako) and H_2O_2 (3%). Anti-irET positive cells appeared brown. Cell monolayers were counterstained with Harris haematoxylin (Ortho Diagnostic Systems, Bucks, UK). Monolayers where the first antibody was omitted or where pre-immune rabbit serum was used instead of the anti-ET-1 antiserum were used as controls in order to assess the specificity of the immunolabelling.

Determination of irET in ATII cells lysates

In order to evaluate intracellular irET, we measured irET in ATII cell lysates according to SUMMARA *et al.* [17]. ATII cells monolayers were washed with PBS and dispersed by trypsinization. After stopping trypsin action with DMEM with 10% foetal bovine serum and centrifugation, 0.5 mL of 0.1% trifluoroacetate and 0.1 mL pepstatin A in 50% acetonitril were added to the cell pellet and the cell suspension was sonicated for 15 s. After centrifugation to remove insoluble materials, the supernatant was recovered and stored at -80°C until irET assay.

Determination of nitrite concentrations in ATII cell supernatants

NO production by rat ATII cells cultured with 21% O_2 was estimated through the production of nitrite in cell culture supernatants using the colorimetric Griess reaction according to GREEN *et al.* [18]. Sodium nitrite resuspended in DMEM supplemented with 10% foetal calf serum (FCS) was used as a standard. One hundred microlitres of Griess reagent was added to 100 μL of sample or standard and incubated in a 96-well plate at room temperature for 10 min. The plate was read at 550 nm using a spectrophotometer. The lower limit of detection of the assay was $0.25 \text{ nmol}\cdot 100 \mu\text{L}^{-1}$. Data are from three different experiments. The assays were performed in duplicate.

Statistical analysis

Data are presented as mean $\pm$ SEM. The nonparametric Kruskal-Wallis test was used for statistical analysis, followed by the nonparametric Mann-Whitney U-test. A p-value of less than 0.05 was considered significant. The results are from 3–8 different experiments performed in triplicate.

Results

Rat alveolar type II cells secrete irET

ET-1 radio immunoassay showed that ATII cells secreted irET in cell culture supernatant in a time-dependent manner (fig. 1). Immunoreactive ET could be detected in cell culture supernatants as soon as 1 h after incubation. ET concentration increased during the 24 h culture period. Immunoreactive ET secretion required mRNA and protein synthesis, since irET was not detected in the supernatant of ATII cells cultured with actinomycin D or cycloheximide.

In order to ascertain that ATII cells were indeed the primary source of ET *in vitro*, immunolabelling was performed on ATII cells cultured for 24 h on glass coverslips. Rat ATII cells demonstrated a strong cytoplasmic immunolabelling, which indicates that *in vitro* ATII cells contain irET (fig. 2).

Change in ET-1 production over time in culture

We measured irET in rat ATII cell supernatants and the preproET-1/GAPDH mRNA ratio at different times after isolation. Rat ATII cells were isolated on day 0, and studied on day 1, day 2 and day 3 after isolation. Fresh medium was placed on the cells on day 1. We observed that the preproET-1/GAPDH mRNA ratio decreased from day

Fig. 1. – Time-dependent secretion of endothelin-1 by rat alveolar type II cells *in vitro*. Immunoreactive endothelin (irET)-1 was measured in the supernatant of rat alveolar type II cells cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum in non-toxic conditions (□). In some experiments, cells were cultured with cycloheximide ($20 \mu\text{g}\cdot\text{mL}^{-1}$; ◇) or actinomycin D ($5 \mu\text{g}\cdot\text{mL}^{-1}$; ■). In these conditions, irET-1 was undetectable and was noted as zero. Results are from 3–6 experiments performed in triplicates.

Fig. 2. – Endothelin immunostaining on rat alveolar type II cells in primary culture. A) rat alveolar type II cells (easily identified with their characteristic intra-cellular lipidic inclusions: arrow) exhibited a strong cytoplasmic immunolabelling; B) a control slide stained in the absence of primary antibody showed no staining. Internal scale bars = 10 μ m.

1 to day 3 (fig. 3). At the same time, the ET concentration in rat ATII cell supernatants increased sharply from day 1 to day 2 and slightly from day 2 to day 3.

Hypoxia decreases the secretion of irET by ATII cells in vitro

Exposure of ATII cells to a hypoxic atmosphere for increasing time periods reduced irET concentration in ATII cells supernatants (fig. 4). The reduction was statistically significant after 24 h (343 ± 29 pg·mg protein⁻¹ with 1% O₂ versus 472 ± 37 pg·mg protein⁻¹ with 21% O₂, $p < 0.05$). A similar decrease of ET concentration was measured in A549 cell supernatants when these cells were cultured for 24 h in hypoxic conditions (512 ± 102 pg·mg protein⁻¹ with 1% O₂ versus 769 ± 89 pg·mg protein⁻¹ with 21% O₂) (fig. 4).

Fig. 3. – Changes in immunoreactive endothelin (irET) in rat alveolar type II (ATII) cell supernatants and preproendothelin-1 (preproET-1)/glyceraldehyde-phosphate dehydrogenase (GAPDH) messenger ribonucleic acid (mRNA) ratio over time in culture. Rat (ATII) cells were isolated on day 0 and studied on days 1, 2 and 3 after isolation. a) representative Northern blot showing preproET-1 mRNA and GAPDH mRNA in rat ATII cells cultured in normoxic conditions. The preproET-1/GAPDH mRNA ratio, expressed as a percentage of the value at day 1, was 94% on day 2 and 60% on day 3. b) representative graph showing variation in irET in rat ATII cell supernatants over time in culture. Data are from one experiment.

Intracellular ET, measured as ET concentration in ATII cell lysates, was not statistically different in ATII cells exposed to 1% O₂ or 21% O₂ for 24 h (35.0 ± 1.3 versus 30.0 ± 2.7 pg·mL⁻¹, respectively) or 48 h (44.0 ± 7.5 versus 38.0 ± 3.0 pg·mL⁻¹, respectively). This indicates that the reduction of ET concentration in ATII cell supernatants was not associated with an intra-cellular accumulation of ET.

Fig. 4. – Hypoxia reduced immunoreactive endothelin (irET) secretion by rat alveolar type II (ATII) cells in primary culture and A549 cells. Cells were cultured for 24 h in either normoxic (21% O₂;) or hypoxic (1% O₂;) atmosphere. *: $p < 0.05$, 21% O₂ versus 1% O₂, Mann Whitney U-test. Values are mean $\pm$ SEM, n=6–8 experiments.

Reversibility of the effect of hypoxia on irET secretion

In order to determine whether the inhibition of ET secretion was reversible, we cultured ATII cells for 24 h in a 1% O₂ atmosphere. The cells were then returned to a 21% O₂ atmosphere for 24 h. The irET concentration in ATII cell supernatants increased with the return to a normoxic atmosphere (fig. 5) and reached the level of irET measured in the supernatant of ATII cells cultured for 24 h with 21% O₂. However, ET concentration remained lower than that measured in the supernatant of cells cultured in a normoxic atmosphere for 48 h.

Effect of hypoxia on ATII cell viability

We explored rat ATII cell viability and A549 cell viability through the use of the colorimetric MTT assay (table 1). No significant alteration of the viability of rat ATII cells could be detected after 24 or 48 h exposure to a hypoxic atmosphere. Similarly, A549 cell viability was not modified after 24 h of hypoxia. However, a significant alteration of A549 cell viability was measured after 48 h of hypoxia.

Fig. 5. – The effect of hypoxia on immunoreactive endothelin (irET) secretion by rat alveolar type II (ATII) cells is reversible. The irET concentration in ATII cell supernatants was measured when cells were exposed to 21% O₂ for 24 h or 48 h, to 1% O₂ for 24 h or 48 h, or to 1% O₂ for 24 h then returned to 21% O₂ for 24 h. Results are from a representative experiment performed in triplicates.

Table 1. – Viability of alveolar type II cells and A549 cells (MTT colorimetric assay)

	Duration of exposure	
	24 h	48 h
ATII cells		
21% O ₂	100 (4)	72.6±5.7 (4)
1% O ₂	91.8±4.3 (4)	78.9±3.3 (4)
p-value	0.21	0.26
A549 cells		
21% O ₂	100 (3)	117.0±2.6 (3)
1% O ₂	104.4±2.3 (3)	73.0±0.8 (3)
p-value	0.3	0.05

Values are viabilities, expressed as a percentage of the control values at 21% O₂ and presented as mean±SEM and number of experiments performed in triplicates in parenthesis. The p-values derive from a Mann Whitney U-test.

Effect of hypoxia on preproET-1 mRNA level

Hypoxia increases the ET-1 secretion by human umbilical vein endothelial cells essentially at a transcriptional level [9]. By contrast, we observed that hypoxia reduced the preproET-1/GAPDH mRNA ratio in ATII cells to 40±6% of the control value (p<0.01) (fig. 6).

Effect of NO on preproET-1 mRNA level

We intended to determine whether the inhibition of ET-1 production by ATII cells could be mediated by NO acting through a paracrine regulatory loop. In the first set of experiments, we attempted to ascertain whether rat ATII cells cultured with 21% O₂ produced NO. The production of NO was estimated through the production of nitrite. Nitrite accumulated in the supernatant of rat ATII cells in control condition (1.0±0.4 nmol·100 µL⁻¹) and was strongly increased with an NO donor (SIN-1) (4.5±1.0 nmol·100 µL⁻¹). Nitrite was undetectable when the cells were cultured with NO synthesis inhibitors (either L-NMMA or S-methylthiourea sulphate). The inhibition induced by L-NMMA was partially corrected with addition of L-Arg (0.5±0.2 nmol·100 µL⁻¹, p<0.05 when compared with control and L-NMMA) whereas nitrite concentration in ATII cell supernatants was not affected by L-Arg treatment (0.9±0.5 nmol·100 µL⁻¹).

Fig. 6. – Nitric oxide does not influence preproET-1 mRNA content. a) typical Northern blots showing preproET-1 mRNA and GAPDH mRNA in rat ATII cells cultured with 21% O₂ (▨) or 1% O₂ (□). The cells were cultured either in control conditions, or with N-monomethyl L-arginine (L-NMMA) (0.5 mM), Thiourea (1 mM), L-arginine (L-Arg; 5 mM) or the combination of L-Arg and L-NMMA, or with an NO donor (SIN-1; 0.1 mM). b) PreproET-1/GAPDH mRNA ratio in rat ATII cells. Values are presented as mean±SEM (n=4 experiments). *: p<0.05 versus normoxic control, Mann Whitney U-test. For definitions, see legend to figure 3.

In a second set of experiments, we observed that neither NO synthesis inhibition nor an NO donor (SIN-1) influenced the preproET-1/GAPDH mRNA ratio in rat ATII cells, either in normoxic or hypoxic conditions (fig. 6). Our results imply that changes in NO synthesis do not account for the hypoxia-induced decrease in irET release.

Discussion

Cultured rat ATII cells in primary culture express preproET-1 mRNA, contain irET in their cytoplasm and secrete high concentrations of irET. ET secretion by rat ATII cells requires mRNA expression and protein synthesis since both cycloheximide or actinomycin D completely inhibited ET secretion. Exposure of rat ATII cells to low oxygen tensions for up to 48 h reduced ET secretion and decreased the steady state level of preproET-1 mRNA without altering cellular viability. The inhibition was not mediated by NO. Hypoxia similarly reduced ET secretion by human A549 cells.

Endothelin secretion by nonvascular cells was demonstrated very early after the discovery of this new family of peptides. In normal human lung, the neuroendocrine cells of the bronchial tree have been shown to be the most important locations of ET-1 immunoreactivity and preproET-1 mRNA detection using *in situ* hybridization [19]. Immunoreactive ET could also be detected in other humans bronchial epithelial cells [19] and bronchial and tracheal epithelial cells have been shown to secrete irET-1 in culture [20]. Moreover, irET could be detected in the bronchoalveolar lavage fluid of normal subjects [21]. In the human lung, expression of irET-1 and preproET-1 mRNA by the hyperplastic alveolar epithelium appears to be a striking finding in interstitial pulmonary fibrosis with or without pulmonary hypertension [5, 22], whereas normal human lung tissue expressed very little ET-1. It must be noted that UGGLON *et al.* [6] found that ET-1 immunoreactivity was virtually confined to small vessel endothelial cells and absent in type II pneumocytes in idiopathic pulmonary fibrosis. The discrepancy between both results is unexplained. However, preproET-1 mRNA expression by hyperplastic alveolar epithelial cells has also been observed in the course of pulmonary vasculitis [4].

Oxygen tension has been shown to modulate ET-1 expression *in vivo*. In rats, normobaric hypoxia for 24 h or 48 h increases the steady-state level of preproET-1 mRNA in the lung, the right atrium and pulmonary artery by twofold [23–25], increases the level of endothelin A (ET_A) receptor steady-state mRNA in the lung [25] and increases the levels of ET-1 in lung homogenates and plasma [25]. However, STEINER *et al.* [26] found that rats exposed to reduced barometric pressure (54.5 kPa (410 mmHg)) for 3 weeks with consequent low PO_2 (10.8 kPa (81 mmHg)) and development of pulmonary hypertension had normal ET-1 content and preproET-1 mRNA expression.

Although endothelins are widely distributed molecules, ET-1 gene transcription and translation into active protein product have been studied most extensively in cultured endothelial cells. In endothelial cells, ET-1 is expressed and released constitutively. Its production is thought to be regulated chiefly at the level of gene tran-

scription and/or translation of the mRNA. Exposure to normobaric hypoxia increases the transcription of the preproET-1 gene and the secretion of ET-1 from cultured human umbilical vein endothelial cells into the media [27]. Bovine coronary endothelial cells also increase ET-1 secretion when exposed to low oxygen tensions [28]. However, BOLL *et al.* [29] have shown that the preproET-1 mRNA content was reduced by hypoxia in Hela cells, in primary heart fibroblasts, in a human hepatoma cell line (HepG2), in normal rat kidney cells and in human embryonic kidney cells. In rat lung endothelial cells, MARINOVIC *et al.* [10] have shown that hypoxia reversibly decreases ET-1 production by 50%. In this cellular model, as we show for rat ATII cells, inhibiting constitutive NO synthesis does not prevent the decrease in ET-1 release caused by hypoxia. Altogether, these data indicate that the regulatory effect of hypoxia on preproET-1 gene expression may vary markedly depending upon the cell type and tissue involved.

Hypoxia may occur in many pathological conditions in which the involvement of NO has been demonstrated, including acute and chronic inflammation. The pathological importance of interaction between NO and ET-1 has been reported in the literature. *In vivo* studies demonstrated the potent participation of NO in mitigating the vasopressor action of ET-1 [30] and several *in vitro* studies indicate that NO reduces the formation of ET-1 via a cyclic guanosine monophosphate (GMP)-dependent mechanism [29]. Since hypoxia has been demonstrated to stimulate NO release [31], and ATII cells are known to secrete NO in some circumstances [32], we attempted to determine whether the inhibition of ET-1 production by ATII cells could be mediated by NO acting through a paracrine regulatory loop. Our results imply that changes in NO synthesis do not account for the hypoxia-induced decrease in ET-1 gene expression because neither NO synthase inhibitors nor NO donors modified preproET-1 mRNA levels compared to control conditions, either in a normoxic or a hypoxic atmosphere. The means by which hypoxia decreased ET-1 production by rat ATII cells remains unresolved. Our results show that the reduction of ET-1 concentration in ATII cell supernatants was not associated with an intracellular accumulation of ET. Whether hypoxia acts directly on the expression of the preproET-1 gene or on the stability of preproET-1 mRNA is unknown. Moreover, whether hypoxia inhibits ET-1 gene expression through the inhibition of secondary secreted mediators, or through interaction with nuclear transcription factors induced by hypoxia, such as cyclic GMP-dependent tyrosin kinase, a negative modulator of ET-1 gene, as already shown [29, 33], is currently unresolved.

We observed that intracellular ET was not different in ATII cells exposed to a hypoxic or a normoxic atmosphere. Perhaps the discrepancy between hypoxia effects on extracellular ET and intracellular ET concentration could be explained if we measured different endothelins in the two samples because of the cross-reactivity of radioimmunoassay between ET-1 and ET-2 or ET-3. If not, the result may only reflect that in endothelial and epithelial cells, the production of ET-1 peptide is dependent on the level of mRNA transcription and that ET-1 secretion occurs through the constitutive pathway without further regulation at the level of exocytosis [34].

ET-1 has been shown to exert some immunoregulatory functions through its effect on immune effector cells. ET-1 is a potent stimulator of human alveolar macrophages via an increase in intracellular calcium [35]. It induces the expression of membrane-associated markers, the release of fibronectin [36] and the secretion of oxygen radicals by normal human alveolar macrophages [35]. ET-1 also acts on the neutrophil to induce its aggregation [37], promote its adhesion to endothelial cells through the expression of membrane adhesion molecules [38] and to enhance its superoxide generation [39]. ET-1 could act also as an immunoregulatory peptide through its action on nonimmune cells. Indeed, ET-1 stimulates 15-lipoxygenase activity in the rat distal lung [3], binds to receptors on airway epithelial cells and initiates the production of a variety of arachidonic acid metabolites that may participate in the regulation of airway inflammatory responses [40]. An autocrine effect of ET-1 on ATII cells is possible since ET-1 stimulates *in vitro* surfactant secretion by rat ATII cells at a concentration of 10 nM [41]. This concentration is probably achieved *in vivo* in the epithelial lining fluid in humans [21].

In conclusion, these broad spectrum properties suggest that in some pathological conditions, endothelin-1 secretion by alveolar epithelial cells could play a significant role in the homeostasis of the alveolar epithelium.

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