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EFFECT OF HYPOXIA ON THE CONVERSION OF ANGIOTENSIN I TO II IN CULTURED PORCINE PULMONARY ENDOTHELIAL CELLS*

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Abstract—Earlier studies by other investigators have shown that acute exposure of cultured endothelial cells to hypoxic atmospheres inhibits the activity of the angiotensin converting enzyme *in situ*, resulting in severe but reversible depression of the rate of degradation of bradykinin. We exposed primary cultures of endothelial cells from the pulmonary artery of the pig to a range of hypoxic gas mixtures and measured the activity of the angiotensin converting enzyme *in situ* using angiotensin I as substrate. Each cell flask was exposed in random sequence to both hypoxic gas mixtures (PO_2 29–69 torr) and room air for 40 min in Dulbecco's medium containing angiotensin I at concentrations of 1000 (N = 7), 500 (N = 8) or 100 ng/ml (N = 4). Angiotensin I disappearance rates and angiotensin II generation rates were linear. Recovery of immunoreactive peptide as either angiotensin I or II following 40 min of incubation was 86 ± 17% (S.D.). The rate of increase in angiotensin II concentration in surface medium in room air experiments was 91 ± 51 (S.D.) $ng \cdot ml^{-1} \cdot hr^{-1}$. During hypoxia it was 85 ± 42 $ng \cdot ml^{-1} \cdot hr^{-1}$. The difference in rates was not significant by paired *t* analysis. The results of this study are consistent with earlier observations by the authors which suggest that hypoxia-induced depression of angiotensin I conversion *in vivo* is due to hemodynamic phenomena. Further studies are needed to clarify the role of cellular mechanisms in hypoxia-induced depression of angiotensin metabolism.

Several investigators have reported *in vivo* inhibition of angiotensin converting enzyme activity in the pulmonary microcirculation during exposure to acute hypoxia [1–4]. There is disagreement on the mechanism(s) of this phenomenon. It has been suggested that physiological alterations in the pulmonary circulation attendant on the hemodynamic response to acute hypoxia can account for most if not all of the attenuation in substrate conversion [3, 4]. Stalcup *et al.* [5] recently reported oxygen tension-related complete inhibition of bradykinin degradation in cultured endothelial cells exposed to hypoxic atmospheres. On this basis they proposed that hypoxic inhibition of pulmonary angiotensin converting enzyme activity is primarily an endothelial membrane phenomenon. These observations suggest that there may be a sensitive oxygen chemoreceptor mechanism at the level of the endothelial cell membrane which regulates angiotensin converting enzyme activity. The current study was carried out in order to examine directly the question of whether the conversion of angiotensin I to angiotensin II by pulmonary endothelial cells in culture is influenced by alterations in oxygen tension in the medium.

METHODS

Cell culture. Endothelial cells were derived from the main pulmonary arteries of freshly slaughtered adult pigs by gentle collagenase treatment (Type I, CLS, Worthington Biochemical Corp., Freehold, NJ) essentially as described previously for porcine and bovine aortic endothelial cells [6–8]. Cell suspensions were seeded into modified plastic (Corning) T-25 (25 cm^2) flasks (see below) that had been precoated with human fibronectin. Culture flasks were pre-coated with fibronectin (5 $\mu g/cm^2$ of growth surface) immediately before use as described previously [9]. Cells were seeded and cultured in complete medium consisting of Medium 199 (power medium containing L-glutamine and Earle's Salts, GIBCO, Grand Island, NY), 25 mM HEPES¶ buffer adjusted to pH 7.4, 2 mM fresh glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 20% heat-deactivated (56° for 30 min) fetal calf serum (Rehatiun, F.S., Reheis Chemical Co., Phoenix, AZ), and 150 μg/ml of bovine endothelial cell growth factor (Collaborative Research) [9]. Porcine pulmonary endothelial cells seeded at 1.5 to 2 × 10^5 cells/T-25 flask reached a stable confluent density of about 8 × 10^4 cells/cm^2 of growth surface in 4–6 days with

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¶ HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

an average population doubling time of about 26 hr during the logarithmic phase of growth. Cultures were incubated in a humidified 37° incubator with a 5% CO_2–air atmosphere and refed at 48-hr intervals with fresh complete medium.

Cell numbers were determined by phase-contrast microscopy using a counting reticle (0.5 × 0.5 mm). A minimum of 2500 cells/flask was counted. Cells were identified as endothelial cells by their typical uniform "cobblestone" packing and Factor VIII/von Willebrand factor content (immunofluorescence staining). Immunofluorescence staining was carried out on fixed cells on representative glass cover slips using affinity purified chicken antiporcine von Willebrand factor IgG as described previously [7, 9].

All studies were carried out on post-confluent primary cultures (2–3 days after reaching their stable confluent density) in which at least 95% of the total packed cells in the confluent monolayer stained positively for Factor VIII/von Willebrand factor. Cover slips from representative confluent cultures were fixed and stained.

Culture flasks were modified by insertion of a heated, sawed-off hub of a sterile 16-gauge needle through the wall of each flask. Each hub was cemented on the outside with a drop of Epoxy resin and capped. This needle hub was subsequently used to deliver gas into the culture flask.

Angiotensin I metabolism. The disappearance rates of angiotensin I and appearance rates of angiotensin II in medium were compared for each cell culture flask under two conditions: exposure to air and to hypoxic gas mixtures. Freshly prepared Dulbecco's solution, pH 7.40, was equilibrated with either air (control) or with hypoxic gas using an Instrumentation Laboratory model 137 tonometer (Boston, MA). Hypoxic gas of desired composition was generated by mixing streams of compressed air and nitrogen (Matheson, East Rutherford, NJ) using needle valves and rotameters in parallel. Equilibrium was checked by frequent sampling (Radiometer gas electrode assembly model PHM 71 Mk 2, London Co., Cleveland, OH). Test Dulbecco's solutions contained substrate (1-Asp, 5-Ile angiotensin I) synthesized in our laboratory [10] at concentrations of 1000 ng/ml (N = 7), 500 ng/ml (N = 8) or 100 ng/ml (N = 4). The order of exposure of each flask to air-equilibrated or hypoxic-equilibrated Dulbecco's solution was random. Each flask was studied twice, once under hypoxic conditions and once under normoxic conditions. Culture flasks were rinsed five times with fresh Dulbecco's solution and placed on a wire mesh rack so that the bottoms of the flasks made contact with the surface of a water bath at 38°. The same hypoxic gas mixtures previously used to equilibrate the test medium in the tonometer were then led to the surface atmosphere of the flask through the side wall needle and allowed to exit by loosening the flask cap. Gas equilibrated medium (10 ml) was transferred from the tonometer to the culture flasks using sterile syringes and 0.22 μm filters (Millex, Millipore Corp. Bedford, MA). Aliquots (0.1 ml) of medium were sampled at 10-min intervals for 40 min using an automatic pipet with sterile disposable tips. Immediately before and after each experiment, culture flasks were inspected by phase contrast microscopy to verify that cells had not undergone morphological changes (shape changes and vacuolization) after exposure to hypoxia compared to the appearance of control cultures. There was no evidence of detachment of individual cells or sheets of cells or of vacuolization or rounding up of individual cells. After each run, culture flasks were rinsed twice with Dulbecco's solution, refed with complete M199 medium, and returned to the incubator for 1.5 to 2 hr. Following this period, each flask was restudied using the gas exposure (air or hypoxia) that had not been employed earlier.

To determine whether angiotensin in the medium was being degraded by angiotensinases, four culture flasks containing angiotensin II (50 ng/ml) were incubated for 40 min following equilibration of the medium with air and sampled at 10-min intervals as previously described. To determine whether angiotensin II generation was the result of angiotensin converting enzyme action, a specific competitive inhibitor of converting enzyme (SQ20881, teprotide) was used. Four culture flasks containing angiotensin I (1000 ng/ml) and SQ20881 at 1:200 molar excess were incubated following equilibration of the medium with air as previously described. Angiotensin I and II concentrations were measured by radioimmunoassay according to methods published earlier [11, 12]. Angiotensin I disappearance rates and angiotensin II appearance rates were calculated using linear regressions. The slopes of these regressions during air and hypoxia were compared for each flask by paired *t* analysis using library programs of a pocket calculator (TI 59, Texas Instruments).

RESULTS

Immunofluorescence staining of representative cover slips showed that at least 95% of the cultured cells stained positively for Factor VIII/von Willebrand factor (Fig. 1A). The morphological appearance (shape and vacuolization) of cells examined by phase contrast microscopy prior to and immediately after exposure to the various gas mixtures showed no differences compared to control cells not exposed to abnormal atmospheres (Fig. 1B).

Exposure of culture flasks to hypoxic gas mixtures resulted in a change in PO_2 from room air tensions to levels of 40 ± 11 (S.D.) torr, range 29 to 69 torr. The pH of Dulbecco's medium was adjusted to 7.40 at the beginning of each run. At the end of 40 min the pH averaged 7.38 ± 0.04 (S.D.), range 7.33 to 7.44.

Rates of decline in angiotensin I concentrations and rates of increase in angiotensin II concentrations in supernatant medium were obtained by eye-fitting straight lines through the five data points of each 40-min run (Fig. 2) and were expressed as 1 hr extrapolates. Recoveries of immunoreactive material (angiotensin I and angiotensin II) following 40 min of incubation were 86 ± 17% (S.D.). Rates of rise of angiotensin II concentrations in medium during air and hypoxic runs are compared for individual flasks in Fig. 3. The average rate of rise of angiotensin II concentrations during exposure to room air was 91 ± 51 (S.D.) $ng \cdot ml^{-1} \cdot hr^{-1}$. During exposure to hypoxic atmospheres the average rate

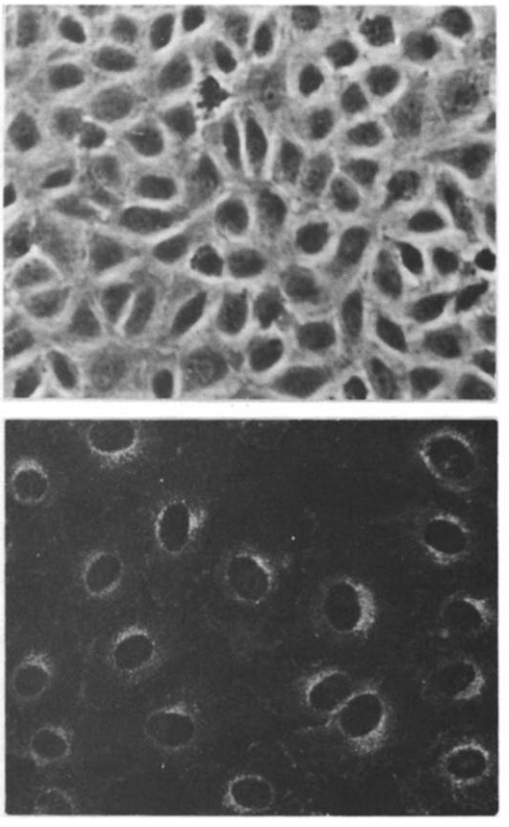

Fig. 1. (A) Immunofluorescence staining of pig endothelial cells (primary cultures) from the main pulmonary artery for Factor VIII/von Willebrand factor using affinity purified chicken antiporcine von Willebrand IgG. Note diffuse staining of the perinuclear region of each cell (×250). (B) Phase contrast photomicrograph of confluent pig endothelial cells (primary cultures) from the main pulmonary artery showing typical polygonal morphology and closely opposed tight packing. This appearance did not change with hypoxia (×200).

was 85 ± 42 ng · ml^{-1} · hr^{-1}. The differences between rates of rise of angiotensin II and rates of decline of angiotensin I for individual cell flasks studied under both hypoxic and normoxic conditions were not statistically significant by paired t analysis. There was no relationship between severity of hypoxia and angiotensin II generation rate.

Incubation of four cell flasks in Dulbecco's medium containing 50 ng/ml angiotensin II for 40 min at 38° resulted in final angiotensin II concentrations of 47.6 ± 1.5 (S.D.) ng/ml, indicating little or no degradation of angiotensin II by angiotensinases and no significant uptake of angiotensin II by endothelial cells.

Incubation of four cell flasks in Dulbecco's medium at 38° containing 1000 ng/ml angiotensin I and SQ20881 at 1:200 molar excess for 40 min resulted in essentially no generation of angiotensin II [0.6 ± 0.5 (S.D.) ng/ml], indicating that the generation of angiotensin II observed in these experiments was entirely due to the activity of converting enzyme.

DISCUSSION

Previous studies using intact anesthetized dogs have demonstrated that the pulmonary conversion of angiotensin I into II and the inactivation of bradykinin are reversibly inhibited during acute hypoxia [1–4]. The mechanism of this hypoxic inhibition is still conjectural. One explanation is that the *in vivo* hypoxic inhibition of angiotensin converting enzyme activity is related to changes in hemodynamic factors occurring in response to acute hypoxia [3, 4]. Studies by Fanburg and Glazier [13] in isolated perfused dog lungs have provided conclusive evidence that intravascular pressures and mean transit times could significantly affect the absolute quantity of angiotensin I converted into angiotensin II in a single passage through the preparation. These studies were performed using saturating concentrations of substrate so as to avoid concentration effects on enzyme kinetics, but it is reasonable to anticipate that the same hemodynamic influences would be operative at lower concentrations of substrate. Thus, Catravas and Gillis [4] demonstrated, during acute hypoxia in intact dogs and rabbits, a direct relationship between mean transit time through the pulmonary circulation and the single passage metabolism of bradykinin and of BPAP (benzoyl-phenylalanyl-alanyl-proline), a synthetic substrate of converting enzyme. Both substrates were administered in less than saturating concentrations. Acute hypoxia *per se* did not alter the pulmonary metabolism of BPAP; the observed changes were interpreted as being secondary to hemodynamic alterations. Oparil, Szidon,

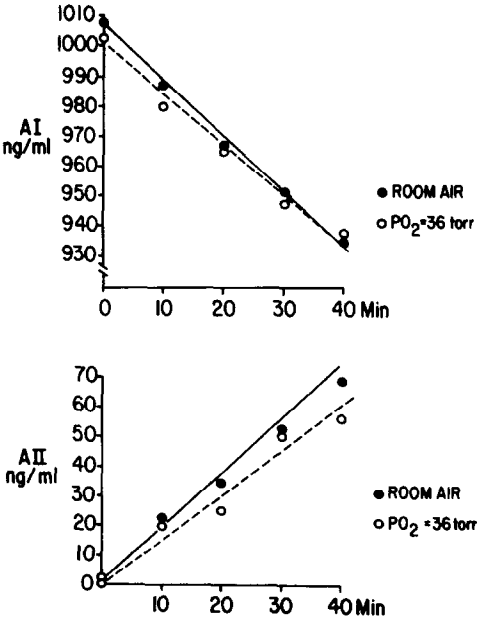

Fig. 2. Representative time course of changes in concentration of angiotensin I (AI) and angiotensin II (AII) in a single culture flask during exposure to room air (●) and, subsequently, during exposure to medium equilibrated with a PO_2 of 36 torr (○). The rates of rise of AII and decline of AI in medium are linear; there is little or no change with hypoxia.

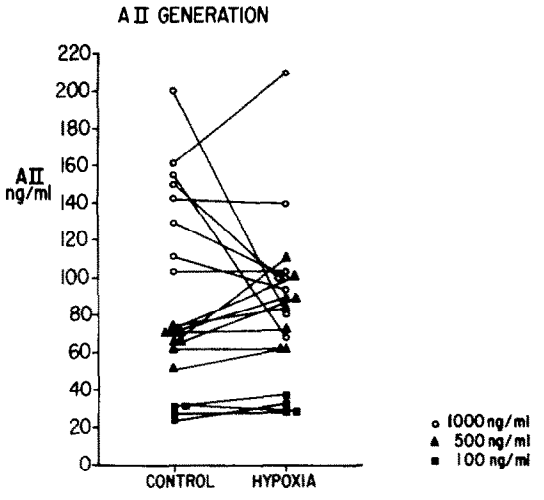

Fig. 3. Comparison of rates of rise of AII concentration in medium during exposure to room air (control) or to hypoxic gas mixtures (hypoxia). Symbols refer to initial concentrations of angiotensin I in medium. There is no significant trend with hypoxia.

and colleagues, in more direct experiments, demonstrated a close exponential relationship between mean transit time and the conversion of boluses of tracer doses of angiotensin I to angiotensin II in single passage experiments using isolated perfused rat lungs. In these experiments changes in transit time were brought about by varying pump flow, and angiotensin I was administered in less than saturating concentrations. Acute hypoxia had no effect on angiotensin metabolism when mean transit time was held constant [14]. Gillis and Catravas [15] have suggested that the measurement of BPAP hydrolysis in single passage through the pulmonary circulation has the potential of becoming a quantitative and sensitive index of damage to the lung microvasculature in man. One major advantage of using this substrate as a metabolic probe in studies of human lung injury is that, in contrast to many other substances metabolized by lung (i.e. serotonin, bradykinin, catecholamines or prostaglandins), BPAP is pharmacologically inert. However, varying degrees of hypoxemia which are relatively unresponsive to increases in inspired oxygen tension occur frequently secondary to acute lung injury in man. If hypoxia can alter directly the metabolism of BPAP, the interpretation of changes in single passage metabolism of BPAP in terms of lung injury will be extremely difficult.

An alternative explanation for hypoxia-induced inhibition of angiotensin converting enzyme activity is that hypoxia has an effect on the endothelial cell membrane on which the enzyme resides. According to the hypothesis of Stalcup and associates [5], acute hypoxia induces a reversible change in the configuration of the luminal side of the endothelial cell membrane so as to render the active portion of the angiotensin converting enzyme inaccessible to substrate. Evidence supporting this hypothesis was provided by the demonstration that the disappearance rate of bradykinin from the medium bathing intact cultured endothelial cells obtained from several different species was profoundly and reversibly depressed by acute hypoxia. The phenomenon could not be shown when cell membrane-free angiotensin converting enzyme was made hypoxic *in vitro*, implying a critical interaction during hypoxia between the endothelial cell membrane and the membrane-bound enzyme [5]. The implication of these experiments has considerable biological significance and transcends the mere question of which factors can modify pulmonary angiotensin metabolism. It suggests that a sensitive oxygen chemoreceptor mechanism exists at the level of the endothelial cell membrane.

In the current study we demonstrated that exposure of primary cultures of endothelial cells harvested from the main pulmonary artery of the pig to levels of hypoxia ranging from 29 to 69 torr did not cause a suppression in the conversion of angiotensin I to II compared to values under normoxic conditions in the same flask. Converting enzyme activity was assessed by measuring generation of angiotensin II and disappearance of angiotensin I in the same culture flasks by radioimmunoassay techniques. Recovery of immunoreactive material in the culture flasks at the end of the incubation period was nearly complete, ruling out a significant effect of angiotensinases in the culture medium. Further, both disappearance of angiotensin I and generation of angiotensin II were blocked by adding SQ20881, a specific peptide inhibitor of converting enzyme. Thus, the metabolic effects observed appear to be the consequences of the action of angiotensin converting enzyme, and the data indicate that alterations in oxygen tension over a wide range (hypoxic to normoxic) do not change angiotension converting enzyme activity in cultured endothelial cells. These results contrast with those of Stalcup and associates [5], who found almost complete suppression of bradykinin degradation at the levels of hypoxia used in our study.

The two sets of experiments have several obvious methodological differences. The salient features of our experiments are (1) we used a uniform source of endothelial cells; (2) we took meticulous care to make sure that intact morphological appearance of cells was verified before and after each intervention; and (3) we used each cell flask as its own control and randomized the order of exposure to air and hypoxia. However, the reasons for the apparent discrepancy between the results of the two sets of experiments remain to be examined further. The findings of the present experiment are consistent with our data in the isolated perfused rat lung [14], and with the experiments of Catravas and Gillis [4] and our own experiments [3] in intact dogs and rabbits, all of which either show a lack of direct effect of hypoxia on the activity of converting enzyme *in situ* or demonstrate effects which can be explained by hemodynamic alterations. However, further studies are needed to assess the role of cellular mechanisms in the relationship between oxygen tension and angiotensin metabolism.

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