In vitro hypoxia impairs β2-adrenergic receptor signaling in primary rat alveolar epithelial cells

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Baloğlu E, Ke A, Abu-Taha IH, Bärtsc P, Mairbäurl H. In vitro hypoxia impairs β2-adrenergic receptor signaling in primary rat alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 296: L500–L509, 2009. First published December 19, 2008; doi:10.1152/ajplung.90390.2008.—Hypoxia inhibits β2-adrenergic receptor (β2-AR) signaling in a variety of tissues, but effects in alveolar epithelium are unclear. We therefore examined the effect of 24 h of hypoxia on β2-AR function in primary rat alveolar epithelial [alveolar type II (ATII)] cells. ATII cells were isolated, cultured to confluence, and incubated in normoxia or hypoxia (3% O2) for 24 h. Hypoxia decreased maximal terbutaline-stimulated cAMP production by 37%; potency of terbutaline was not affected. Reoxygenation (3 h) reversed this effect. Density of β2-AR assessed by (−)-[125I]iodocyanopindolol binding was decreased in hypoxia (−22%). Hypoxia did not affect terbutaline binding affinity to β2-AR. Hypoxia decreased Gs protein levels by 27%, whereas no change was observed in Gi1/2, Gi3, and Gβ subunits. Forskolin-stimulated cAMP production was not inhibited by hypoxia. Pertussis toxin (PTX; 0.5 μg/ml, 2 h), an inhibitor of Gi/Go proteins, restored terbutaline-stimulated cAMP production of hypoxic ATII cells to normoxic control values. Cholera toxin (CTX)-stimulated Gi protein activity did not change in hypoxia. Hypoxia increased the sensitivity of β2-AR to desensitization. These results indicate that despite the decrease in Gi protein level, Gi protein was still functional and that hypoxia impairs β2-AR signaling due to an increased activity of Gi/Go proteins. Although the lung is the region in the body exposed to the highest levels of oxygen, it appears particularly sensitive to hypoxia. Many lung diseases such as acute respiratory distress syndrome (ARDS), acute lung injury (ALI), and chronic obstructive pulmonary disease (COPD), as well as decreased PO2 in the alveolar air such as at high altitude and during hyperventilation, lead to pulmonary edema and alveolar hypoxia (2, 45). Some of the most significant consequences of alveolar hypoxia are pulmonary vasoconstriction and pulmonary hypertension (46), increased alveolar permeability (38), and impaired alveolar fluid clearance (10), all of which eventually advance pulmonary edema formation. β2-AR stimulation can prevent these adverse effects.

In the lung, stimulation of β2-AR has been shown to cause vasodilatation (13) and endothelial tightening (39) and to increase the rate of alveolar lung fluid removal in various species (for review see Ref. 28). In alveolar epithelium, where β2-AR are highly expressed (6), β2-AR stimulation increases alveolar fluid clearance by increasing activity, expression, and membrane insertion of epithelial Na channels (ENaC) and Na-K-ATPase via cAMP-dependent processes (25, 41). Overexpression of β2-AR in rat alveolar epithelium stimulates fluid clearance by increasing β2-AR signaling and stimulation of ion transport (12). Studies on β2-AR-knockout mice indicate that β2-AR stimulation of reabsorption represents a protective mechanism for removal of excess alveolar fluid in response to lung injury (34).

However, there are indications that hypoxia might impair β2-AR signaling. In rat heart, chronic exposure to hypoxia causes a decrease in β2-AR density (42) and Gα protein activity (40) and increases desensitization (26). In systemic hypoxia effects appear to be due to elevated sympathetic activity. On the other hand, Winter et al. (47) found no change in β2-AR density in spleen and in the left ventricle of rat heart after 28 days of hypoxia. In vitro hypoxia decreases β2-AR density and response to agonist in cultured myocytes (27). Thus changes seem to depend on the tissue as well as the degree and duration of hypoxia.

Evidence for altered β2-AR signaling in the lung is sparse and controversial. Exposure of rats to hypoxia resulted in an increase in the number of β-adrenoceptors in whole lung tissue that seemed mainly due to increased β2-AR but not β1-AR (3, 47). Vivona et al. (44) showed that in rats exposed to hypoxia terbutaline-stimulated fluid reabsorption reached the same level as in normoxic animals, indicating no impairment of
β2-AR function by exposure to hypoxia. In contrast, in a similar experimental setting impaired β2-AR function has been demonstrated by Litvan et al. (24), who found that isoproterenol stimulation of alveolar fluid clearance and Na-K-ATPase was decreased in lungs of hypoxic rats. In the isolated perfused rat lung, acute hypoxia has been found to diminish terbutaline-induced cAMP production and terbutaline does not prevent hypoxia-induced edema formation (10), but the mechanisms are not known. It was therefore the aim of the present study to evaluate the effect of hypoxia on β2-AR function in primary rat alveolar epithelial cells by characterizing ligand binding, receptor density, G protein function, and cAMP production.

MATERIALS AND METHODS

Reagents. Terbutaline hemisulfate, ICI-118,551, pertussis toxin (PTX), cholera toxin (CTX), and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (Taufkirchen, Germany). Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and newborn rat serum (NRS) were from PAA (Coelbe, Germany), forskolin was from Applichem (Darmstadt, Germany), cAMP kits were from Immunotech (Kiel, Germany), (-)-[13]Hiodocyanopindolol (ICYP; 2,000 Ci/mmol) was from GE Healthcare Amersham (Freiburg, Germany), elastase was from WAK-Chemicals (Steinbach, Germany), and rat IgG was from Biomedica (Eching, Germany).

Alveolar epithelial type II cell isolation and culture. Experiments were performed on primary cultures of alveolar type II (ATII) cells isolated from lungs of normoxic male rats (Sprague-Dawley, 150–200 g) as described previously (25). Briefly, lungs from rats anesthetized with pentobarbital sodium (100 mg/kg ip; Trapanal, Byk Gulden, Germany) were perfused with PBS while being ventilated. ATII cells were isolated by elastase digestion, mincing of lung tissue, filtration, and differential adhesion on IgG-coated plates (11). Nonadherent cells were suspended in DMEM supplemented with 10% NCS, glutamine (4 mM), and gentamicin (50 μg/ml) (DMEM-N). Both purity and viability of ATII cells were >85%. For cAMP measurements cells were cultured in 6-well plates, and for RNA preparations cells were cultured in 7.5-cm² petri dishes. Seeding density was 200,000 – 300,000 cells/cm². Confluent cells, usually on day 3 after seeding, were exposed to hypoxia (3% O₂, 5% CO₂, rest N₂) for 24 h in an O₂ and CO₂-controlled tissue culture incubator (Nunc, Germany). Control cells continued to stay in normoxia (room air supplemented with 5% CO₂) for 24 h. On day 4 in culture, cells were used for experiments. In a subset of experiments cells cultured in 24-well plates were reoxygenated for 3 h after hypoxic exposure. Long-term reoxygenation experiments have not been performed because of a possible transdifferentiation from the ATII to the alveolar type I (ATI) cell phenotype (7).

RNA isolation and RT-PCR. After exposure to hypoxia, cells were kept on ice, washed twice with ice-cold PBS and were lysed with Buffer RLT (Qiagen, Hilden, Germany). Total RNA was isolated according to the manufacturer’s instructions. RNA (0.1 μg) was transcribed with Superscript II reverse transcriptase (Life Technologies, Bochum, Germany) with random hexamer primers (Roche, Mannheim, Germany). Real-time quantitative PCR was performed in the Lightcycler with the QuantiTect SYBR Green PCR kit and the primers from Qiagen. To test for the specificity of PCR amplification, products were separated by agarose gel electrophoresis and stained with GelStar (BMA, Rockland, ME). PCR products showed single bands of the predicted size (not shown). Standards for quantification of mRNA expression were prepared from the PCR products after elution from the agarose gel (18). 28S rRNA was used to control for differences in the efficiency of reverse transcription. 28S rRNA did not change during exposure to hypoxia (not shown).

Plasma membrane preparation. Cells were washed two times with ice-cold PBS, harvested by scraping in a buffer composed of 20 mM HEPES (pH 7.4), 1 mM EDTA, 2 μg/ml aprotinin, 0.1 mM PMSF, and 10 μg/ml leupeptin, and passed through a 27-gauge needle. Cell lysates were centrifuged (500 g, 10 min, 4°C) to separate unbroken cells and nuclei. Supernatants were centrifuged (16,000 g, 30 min, 4°C) to pellet plasma membranes that were resuspended in the lysis buffer. Protein was measured with a kit from Bio-Rad (Munich, Germany). Samples were stored at ~80°C until further use.

Western blotting. G proteins were measured by Western blot analysis after electrophoretic separation of membranes (5–20 μg) from cells exposed to normoxia and hypoxia, with antibodies against Gi1/2 (ab-4; Calbiochem, Darmstadt, Germany), Goα, C-18, Goα, C-10, or a common antibody for Gβ subunits (T-20) (Santa Cruz, Heidelberg, Germany). Since the antibody for Goα, protein cross-reacts with Goα2 and to some degree with Goα1 proteins, lysates from T-10 cells (kind gift from H. J. Hippe, Department of Cardiology, University of Heidelberg) that overexpress Goα3 proteins were used as positive controls. To correct the possible differences in protein loading, blots were stripped and reprobed with an antibody against β-actin (Sigma). Bands were detected with secondary antibodies conjugated with horseradish peroxidase (Calbiochem, Sigma) and enhanced chemiluminescence (GE Healthcare Amersham). Band densities were measured with Scion Image software (Scion, Frederick, MD).

Measurement of β2-AR density. To determine the effect of hypoxia on β2-AR density, saturation binding experiments were performed on plasma membranes with the β2-AR antagonist ICYP. Membranes (1 μg) were incubated for 90 min at 37°C in a binding buffer composed of (in mM) 50 Tris-HCl, 100 KCl, and 5 MgCl₂, pH 7.4 in disposable polypolyene tubes containing different concentrations of ICYP (0–150 pM). Nonspecific binding was determined by displacement of ICYP binding with the specific β2-AR antagonist ICI-118,551 (100 μM). Reactions were stopped by dilution with 4 ml of ice-cold 50 mM Tris-HCl, 154 mM NaCl, pH 7.4, followed by rapid filtration through presoaked Whatman GF/C glass fiber filters in a Millipore cell harvester (Millipore, Eschborn, Germany). Filters were washed three times with 4 ml of the same buffer. The radioactivity on the wet filters was counted with a gamma counter (Berthold Technologies, Bad Wildbad, Germany).

Competition binding experiments. Competition binding experiments were performed in cell membranes to assess whether hypoxia affected the ICYP affinity to β2-AR. Plasma membranes were exposed to 30 pM ICYP and increasing concentrations of terbutaline (1 nM to 1 mM), a selective β2-AR agonist. ICYP binding was determined as described above.

Cyclic AMP production. cAMP production was measured in confluent ATII cells cultured in 96-well plates after incubation in DMEM-N containing the phosphodiesterase inhibitor IBMX (1 mM) for 30 min at 37°C. β2-AR-mediated cAMP production was assessed in the presence of terbutaline (1 nM to 1 mM; 10 min). β2-AR-independent activity of AC was measured by incubation with forskolin (1 nM to 0.1 mM). In this case, control cells were treated with DMSO, which did not affect cAMP formation (not shown). Reactions were terminated by aspirating the medium and lysing the cells with ice-cold HCl (0.1 N). cAMP was measured by radioimmunoassay with a kit from Immunotech (Kiel, Germany) according to the manufacturer’s instructions. Values were normalized to total cell protein; the degree of stimulation was shown as percentage of basal values.

To test whether the effect of hypoxia on cAMP production could be reversed by reoxygenation, cells were exposed to normoxia and hypoxia (24 h) and then kept in normoxia for 3 h. At the end of this period, cells were incubated for 30 min with normoxia- or hypoxia-equilibrated DMEM-N containing 1 mM IBMX for 30 min. cAMP production was measured after stimulation of the cells with 1 mM terbutaline for 10 min.

For short-term desensitization experiments, cells were exposed to normoxia or hypoxia (3% O₂) for 22–24 h. Cells then received terbutaline (25 μM) for 0–120 min in the absence of IBMX for desensitization at the respective oxygenation. This concentration of
terbutaline was 10-fold of its potency to stimulate cAMP production as calculated from the terbutaline dose-response curves. Cells were then washed and incubated (30 min) with normoxia- or hypoxia-equilibrated culture medium containing 1 mM of IBMX before restimulation with 1 mM terbutaline for 10 min. cAMP was measured as described above.

For long-term desensitization experiments, cells were incubated with terbutaline (25 μM) for 24 h in hypoxia and normoxia in the absence of IBMX. cAMP production was then measured in the presence of increasing concentrations of terbutaline (1 nM to 1 mM) after incubation of the cells with IBMX (1 mM) for 30 min.

To test the coupling effect of Gαs proteins on cAMP production in hypoxia, we used PTX, which prevents the coupling of Gαs proteins to β2-AR. Cells exposed to normoxia and hypoxia for 22 h were treated with PTX (0.5 μg/ml) for 2 h at the respective oxygenation level. cAMP production was then measured after stimulation with terbutaline (1 nM to 1 mM) as described above.

To determine Gαi protein activity in normoxia and hypoxia, cells were incubated with CTX (3–10 μg/ml for 1 or 2 h). Cells exposed to normoxia and hypoxia for 22 or 23 h were treated with CTX in the presence of IBMX. CTX exposure continued for 1 or 2 h at the respective oxygenation level. cAMP was measured as described above.

Data analysis. Data are reported as means ± SE. Sigmoidal dose-response curves for cAMP production were fitted by nonlinear regression to obtain EC50 values for terbutaline-stimulated cAMP production.

Saturating and terbutaline competition binding experiments were analyzed by nonlinear regression. The maximal number of ICYP binding sites (Bmax) and the equilibrium dissociation constant (Kd) were calculated from saturation binding curves by nonlinear least squares curve fittings for one binding site. Terbutaline competition binding experiments were analyzed by one- or two-site ICYP binding. The goodness of the fit was determined by the F-ratio test. All fits were calculated with Sigma Plot (version 8, Erkrath, Germany).

Student’s unpaired t-test was used to analyze results from Western blots and binding experiments. Multiple comparisons were performed by one-way ANOVA followed by Fisher least significant difference (LSD) post hoc testing. Level of significance was defined by P ≤ 0.05. Statistical analyses were performed with Sigma Stat (version 3).

RESULTS

β2-AR-mediated cAMP production. To examine the effects of hypoxia on β2-AR signaling, dose dependence of terbutaline-stimulated cAMP production was measured. Hypoxia did not change basal cAMP production (normoxia 3.02 ± 0.35, hypoxia 3.74 ± 0.36 pmol/mg; n = 10 each in normoxia and hypoxia, P = 0.192). Terbutaline-stimulated cAMP production was fully inhibited by 100 μM ICI-118,551 (data not shown), indicating that terbutaline acts via β2-AR. As shown in Fig. 1, hypoxia decreased maximum terbutaline-stimulated cAMP production by ~37% (P = 0.007). There was no change in the logEC50 values of terbutaline (Fig. IA, Table 1; P = 0.148).

To test whether the effect of hypoxia on cAMP production can be reversed by reoxygenation, hypoxic cells were exposed to normoxia for 3 h before stimulation with 1 mM terbutaline. Figure 1B shows that 3 h of reoxygenation fully reversed the hypoxia-induced decrease in terbutaline-induced cAMP production (P = 0.001), bringing it back to normoxic values (P = 0.611).

Adenylyl cyclase activity. To test whether the decrease in terbutaline-induced cAMP production in hypoxia was due to a change in AC activity, cells were stimulated by forskolin, a direct stimulator of AC. Forskolin-induced cAMP production did not saturate. Maximal cAMP production at 100 μM forskolin did not change in hypoxia (Fig. 2, Table 1; P = 0.788), indicating that AC activity was not affected by hypoxia.

Hypoxia inhibits mRNA levels of β2-AR and G protein subunits. Effects of hypoxia on the mRNA expression of β2-AR and G proteins were analyzed by PCR. Results summarized in Table 2 show that the main subunits of the Gαi proteins expressed in alveolar epithelial cells are Gαi2 and Gαi3. The expression of Gαi1 protein was very low. Hypoxia significantly decreased β2-AR, Gαi1, Gαi2, and Gαi3 mRNA levels (P = 0.026, P = 0.007, P < 0.001, and P = 0.003, respectively), whereas there was no statistically significant change in Gαs mRNA (P = 0.121).

G protein levels. Western blots were performed to test whether hypoxia affects the protein level of Gαs, Gαi1/2, Gαi3, and Gβ proteins in plasma membranes from normoxia- and hypoxia-exposed cells. Blots from three independent experiments revealed two bands for Gαs, reflecting the long (52 kDa) and
Table 1. Effects of hypoxia on forskolin-, terbutaline-, PTX-, and CTX-induced cAMP production

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Maximal cAMP</th>
<th>Hypoxia</th>
<th>Maximal cAMP</th>
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<tbody>
<tr>
<td><strong>Forskolin</strong></td>
<td></td>
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<tr>
<td>1 µM/ml, 1 h</td>
<td>3.556±0.342</td>
<td></td>
<td>3.329±0.982</td>
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<tr>
<td>CTX</td>
<td></td>
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<tr>
<td>3 µM/ml, 1 h</td>
<td>6.242±1.805</td>
<td></td>
<td>5.173±1.007</td>
<td></td>
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<tr>
<td>5 µM/ml, 2 h</td>
<td>5.901±1.161</td>
<td></td>
<td>5.711±1.053</td>
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<tr>
<td>10 µM/ml, 1 h</td>
<td>5.450±0.914</td>
<td></td>
<td>4.670±0.904</td>
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<tr>
<td><strong>Terbutaline stimulation (10 min)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>−PTX</td>
<td></td>
<td></td>
<td>−5.37±0.14</td>
<td></td>
</tr>
<tr>
<td>+PTX</td>
<td>−5.32±0.08</td>
<td>4.951±0.47</td>
<td>3.092±0.37*</td>
<td></td>
</tr>
<tr>
<td><strong>Terbutaline stimulation (10 min) after incubation with terbutaline (24 h)</strong></td>
<td></td>
<td></td>
<td>−4.78±0.28</td>
<td>4.669±0.67±</td>
</tr>
<tr>
<td>−PTX</td>
<td>−5.21±0.1</td>
<td>1.085±0.179</td>
<td>5.047±1.166*</td>
<td></td>
</tr>
<tr>
<td>+PTX</td>
<td>−5.22±0.14</td>
<td>1.157±0.143</td>
<td>1.164±0.2415%</td>
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</table>

Values are means ± SE of 6–10 independent experiments. Alveolar type II (ATII) cells were exposed to normoxia and hypoxia (3% O2, 24 h). cAMP production is expressed as % of basal cAMP production. cAMP production was stimulated by 10−4 M forskolin; curves are shown in Fig. 2. To determine effect of cholera toxin (CTX) on cAMP production, cells exposed to normoxia and hypoxia (3% O2, 22–23 h) were simulated with CTX for the indicated times and concentrations. To determine terbutaline-stimulated maximal cAMP production and log EC50 values of terbutaline, cells exposed to normoxia and hypoxia (3% O2, 22 h) were incubated with or without terbutaline (25 µM) in the absence or presence of pertussis toxin (PTX; 0.5 µg/ml, 2 h). Total exposure time to hypoxia was 24 h; results are from data shown in Figs. 1, 6, and 7. *P < 0.05; †difference between normoxia and hypoxia; ‡effect of PTX; §effect of 24-h terbutaline-treated cells.

Effects of hypoxia on forskolin-, terbutaline-, PTX-, and CTX-induced cAMP production.

**Hypoxia decreases β2-AR density.** Density of β2-AR in plasma membranes from ATII cells exposed to normoxia or hypoxia was measured by performing saturation binding experiments. Results show that specific ICYP binding was saturable and of high affinity. Dissociation constants of ICYP (Kd) were decreased by ~27% (P = 0.022), but there was no change in Gα2, Gα11/12, and Gβ protein levels in hypoxia compared with normoxia (Fig. 3).

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**Hypothesis.** To test the hypothesis that hypoxia affects binding characteristics of agonist to β2-AR, membranes from normoxia- and hypoxia-exposed cells were used for competition binding experiments by displacement of ICYP binding with terbutaline. Competition binding curves were fitted significantly better with a two-site model than a one-site model (F-test: P < 0.001, Fig. 5) with high (Kh)- and low (Kl)-agonist affinity binding states reflecting G protein-coupled and -uncoupled states of β2-AR, respectively. As summarized in Table 3, hypoxia did not affect the percentage of high (Rh)- and low (Rl)-affinity binding sites of the β2-AR (P = 0.569 and P = 0.564, respectively). Also, terbutaline-mediated displacement of ICYP binding from high (Kh)- and low (Kl)-affinity binding states was not affected by hypoxia (P = 0.597 and P = 0.09, respectively). These data indicate that hypoxia did not change the affinity of terbutaline binding to β2-AR.

**Effects of pertussis toxin on cAMP production.** To test whether decreased β2-AR-mediated cAMP production was due to increased coupling of β2-AR to Gi/o proteins, dose-response curves with terbutaline after pretreatment of cells with PTX were performed. In normoxia, PTX treatment did not affect cAMP production.

Table 2. Effects of hypoxia on mRNA expression of β2-AR and G protein subunits in ATII cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
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<tbody>
<tr>
<td>β2-AR</td>
<td>13.24±0.73</td>
<td>10.43±0.63*</td>
</tr>
<tr>
<td>Gα2</td>
<td>90.35±0.9</td>
<td>70.04±4.41</td>
</tr>
<tr>
<td>Gα11/12</td>
<td>0.036±0.03</td>
<td>0.44±0.02</td>
</tr>
<tr>
<td>Gβ0</td>
<td>17.74±1.4</td>
<td>11.92±0.9*</td>
</tr>
<tr>
<td>Gβ1</td>
<td>200.2±7.25</td>
<td>156.1±2.45*</td>
</tr>
<tr>
<td>Gβ2</td>
<td>85.3±4.34</td>
<td>63.9±1.85*</td>
</tr>
</tbody>
</table>

Values (in mRNA fg/ng 28S rRNA) are means ± SE of 9–12 independent experiments. Cells were exposed to normoxia (room air) and hypoxia (3% O2 for 24 h). Total RNA was extracted for the measurement of mRNA expression by real-time PCR. Amounts of mRNA were corrected for 28S rRNA. *Difference between normoxia and hypoxia, P < 0.05.
maximal terbutaline-stimulated cAMP production ($P = 0.439$; Fig. 6, Table 1). Interestingly, PTX treatment of hypoxia-exposed cells fully prevented the hypoxia-induced decrease in cAMP production ($P = 0.042$), raising cAMP production to normoxic levels. PTX incubation did not change logEC$_{50}$ values of terbutaline ($P = 0.148$) and basal cAMP production in normoxia and hypoxia (normoxia $2.67 \pm 0.23$, hypoxia $3.29 \pm 0.17$ pmol/mg; $P = 0.211$).

Fig. 3. Effects of hypoxia on the expression of G proteins. Plasma membranes from normoxia (N) and hypoxia (H; 3% O$_2$, 24 h) exposed ATII cells were prepared as described in MATERIALS AND METHODS. Amounts of protein loaded were 5 µg (A) and 20 µg (B–D). Western blots were performed with anti-G$_s$ (A), anti-G$_{i1/2}$ (B), anti-G$_{i3}$ (C), and anti-G$_{i12}$ (D) rabbit polyclonal antibodies. Blots were stripped and reprobed for β-actin. G$_s$ and G$_s$, long and short forms of G$_c$, G$_{i3}$/T-10, G$_i$ protein from overexpressing T-10 cells used as positive control for G$_i$ antibody. Western blots from 3 independent samples are shown; bar graphs indicate bands densities ± SE normalized to β-actin. *Significant difference between normoxia and hypoxia ($P < 0.05$).
Nonspecific binding was determined in the presence of ICI-118,551 (100 nM) were incubated with increasing concentrations of ICYP for 90 min at 37°C. Results are summarized in Table 3. Values are representative of 4 independent experiments each performed in duplicate. Activity of Gs protein. To test whether the activity of Gs protein was altered by hypoxia, cells were stimulated with CTX, which irreversibly activates Gs proteins. CTX (3 and 10 μg/ml) incubation of the cells for 1 or 2 h significantly elevated cAMP production at both oxygenation levels. There was no difference between normoxia and hypoxia (P = 0.945; Table 1).

Short- and long-term desensitization. We also tested whether hypoxia affects desensitization of β2-AR in ATII cells. Cells were exposed to terbutaline for different periods of time in the absence of IBMX, washed, and then stimulated again with a saturating concentration of terbutaline (1 mM) in the presence of IBMX. Short-term experiments indicated that desensitization could be detected as early as 10 min after pretreatment, decreasing cAMP production by ~15% (P = 0.002). Pretreatment of the cells with terbutaline for 2 h decreased cAMP production by 65% and 60% in normoxia and hypoxia, respectively (P = 0.001). Kinetics were not different (P = 0.164; Fig. 7A).

For long-term desensitization, cells were stimulated with terbutaline for 24 h. In normoxia, pretreatment of the cells with terbutaline for 24 h decreased maximal terbutaline induced cAMP production by 78% (P = 0.001). Terbutaline pretreatment of hypoxia-exposed cells decreased maximal cAMP production by 83% compared with hypoxia alone (P = 0.001) and by 50% compared with terbutaline-treated normoxic cells (P = 0.016; Fig. 7B). Both in normoxia and in hypoxia 24 h of terbutaline pretreatment did not affect logEC50 values of terbutaline (P = 0.738; Fig. 7B, Table 1) and basal cAMP production (P = 0.489, normoxia-terbutaline preincubation vs. hypoxia).

Table 3. Effects of hypoxia on ICYP binding

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
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<tr>
<td>ICYP saturation binding curves</td>
<td></td>
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<tr>
<td>Bmax, fmol/mg</td>
<td>354.11±62.462</td>
<td>277.27±53.76*</td>
</tr>
<tr>
<td>Kd, nM</td>
<td>10.17±1.93</td>
<td>11.92±2.16</td>
</tr>
<tr>
<td>Terbutaline displacement of ICYP binding</td>
<td></td>
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<tr>
<td>Kb, nM</td>
<td>17.7±0.7</td>
<td>15.7±1.7</td>
</tr>
<tr>
<td>Kd, nM</td>
<td>7.676±2482</td>
<td>12.500±4709</td>
</tr>
<tr>
<td>%Rh</td>
<td>45.19±2.17</td>
<td>42.66±4.8</td>
</tr>
<tr>
<td>%Rl</td>
<td>54.8±2.17</td>
<td>57.3±4.8</td>
</tr>
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</table>

Mean values ± SE of 4 independent experiments for each analysis. After exposure of ATII cells to normoxia and hypoxia (3% O2, 24 h), plasma membranes were prepared. −[125I]iodocyanopindolol (ICYP) binding was performed as described in MATERIALS AND METHODS. ICYP saturation binding curves (examples in Fig. 4) were analyzed by nonlinear regression for best fit to a 1-site binding model. Bmax and Kd represent receptor density and the dissociation constant for specific ICYP binding, respectively. Terbutaline displacement of ICYP binding (shown in Fig. 5) was analyzed by nonlinear regression for the best fit to a 2-site binding model. Kb and Kd indicate dissociation constants for high- and low-affinity terbutaline binding, respectively, and %Rhl and %Rl indicate % of receptors displaying high and low agonist affinity, respectively. *Difference between normoxia and hypoxia, P < 0.05.
hypoxia on β2-AR signaling in lung alveolar epithelium are contradictory. Dehler et al. (10) reported a decrease in terbutaline-induced cAMP production in lung tissue and perfusate of isolated perfused rat lungs and found that terbutaline failed to restore the hypoxia-induced increase in alveolar permeability. Indirect evidence for impaired β2-AR signaling can be derived from results obtained by Litvan et al. (24), who found that isoproterenol restored normoxic fluid clearance rates in hypoxia-exposed rats (8% O2, 24 h). However, in hypoxia isoproterenol-induced maximal reabsorption and Na-K-ATPase activity did not reach values of normoxic rats. In contrast, Vivona et al. (44) showed that in rats exposed to hypoxia (8% O2, 24 h) terbutaline-containing fluid instilled into alveolar space prevented the hypoxia-induced inhibition of reabsorption. Terbutaline-stimulated alveolar fluid clearance in hypoxic rats reached the same rate as in normoxic animals (44), indicating that β2-AR signaling might not have been impaired. The
reasons for the discrepancies are unclear, since similar experimental conditions were used in the studies. Also, the mechanisms require further clarification, since in neither study was cAMP or receptor density measured.

**cAMP production, adenylyl cyclase activity.** Our results indicate that terbutaline-induced cAMP production is decreased when primary rat alveolar epithelial cells are exposed to hypoxia. Thus we performed a series of experiments to determine at which level β2-AR signaling might be impaired. The impairment of cAMP production by hypoxia was not seen when AC was stimulated with forskolin, which predominantly activates the catalytic unit of the enzyme and thus makes it almost independent of receptor-mediated signaling. This is in accordance with data showing unchanged AC activity in the hypoxic lung (31). Therefore, mechanisms impairing cAMP formation on stimulation of β2-AR might affect the receptor itself or the control of AC by G proteins.

**β2-AR density and ligand affinity.** Hypoxia decreased β2-AR density in plasma membranes from ATII cells by 22% (Table 3), as indicated by decreased ICYP binding. Part of this decrease might be due to decreased mRNA expression of β2-AR. Similarly, a decrease in β2-AR density has been reported in rat ventricular myocytes exposed to acute hypoxia, which could be reversed by reoxygenation (27), and in lymphocytes of humans exposed to high altitude (42). Prolonged in vivo hypoxia decreased β2-AR density in left ventricles of Wistar rats with decreased receptor function (20). In contrast, Birnkrant et al. (3) and Winter et al. (47) showed that β2-AR density was increased in lungs of rat exposed to hypoxia in vivo.

Our results indicate that in normoxic cells the number of β2-AR and the binding constants (K_D) for ICYP were in the range reported by others in lung (1, 15). Hypoxia did not affect the potency of terbutaline calculated from the dose dependence of cAMP formation in intact cells. The affinity for ligand binding was evaluated by competition binding of ICYP with terbutaline in plasma membranes prepared from normoxic and hypoxic ATII cells. The results (Fig. 5) indicate that terbutaline binds to high- and low-affinity sites of β2-AR, which represent the G protein-coupled and -uncoupled forms of the β2-AR, respectively (9). The potency of terbutaline to stimulate cAMP production in intact ATII cells was found to be in the range of the low-affinity binding constant. Neither of the two binding constants was altered by hypoxia. Together these results indicate that altered agonist binding affinity does not explain the decreased terbutaline-induced cAMP production in hypoxia-exposed ATII cells.

**Role of G proteins.** β2-AR activates Gs and Gi proteins to regulate AC activity (for review see Ref. 17). It was therefore important to test whether altered G protein expression and function caused the decrease in terbutaline-stimulated cAMP production by hypoxia. Western blot analysis of plasma membranes of hypoxia-exposed ATII cells revealed that hypoxia decreased Goα, protein levels by 27%, whereas there was no change in mRNA expression. In contrast, levels of the inhibitory Goα (1,2) and Goα (3) proteins and the Gβ proteins did not change despite decreased mRNA. Thus the relative ratios between β2-AR and stimulatory and inhibitory G proteins might have changed in hypoxia, causing β2-AR to couple to Gi proteins, which could inhibit cAMP formation (48).

To test whether the hypoxia-induced decrease in terbutaline-mediated cAMP production was in fact due to increased coupling of Gs proteins to β2-AR, cells were incubated with PTX, which prevents Goα, protein interaction with β2-AR. PTX did not affect cAMP formation in normoxic cells, indicating a predominant effect of Goα. However, PTX fully prevented the hypoxia-induced decrease in maximal cAMP production. This result might indicate that in hypoxia the inhibitory activity of Gi proteins contributes to decreased cAMP formation. It also implies that despite the decrease in Gi proteins found in Western blots there is sufficient activity of Gi proteins present to stimulate cAMP formation even in hypoxic ATII cells. Results from experiments with CTX, a potent stimulator of Gi proteins, support this notion, since CTX stimulates cAMP formation in normoxic and hypoxic ATII cells, reaching the same levels in both oxygenation states. Therefore, the results on PTX and CTX indicate that despite the decrease in Goα measured by Western blot in plasma membranes functional Goα is not impaired, and that there seems to be an increased Gi protein activity relative to Gi proteins in hypoxic ATII cells, which inhibits cAMP production on stimulation of β2-AR.

The mechanisms causing the relative increase in Gi activity in hypoxia are not clear. Distinct Gβ and Gγ protein combinations can assemble with different Goα proteins, modify signaling (23), and stabilize Goα proteins in the plasma membrane (21). Western blots using a common antibody show identical amounts of Gβ in plasma membranes of hypoxia- and normoxia-exposed ATII cells. We failed to detect Gγ subunits. We do not know whether individual isoforms of Gβ and Gγ proteins are changed by hypoxia. However, our results showing that after PTX treatment of hypoxic cells β2-AR-Gα coupling still effectively produces cAMP might rule out the possibility of a loss of association of Gi proteins with Gβγ complexes.

Altered interactions with the cytoskeleton might be another potential mechanism explaining altered G protein interaction with β2-AR (for review see Ref. 37). In vitro exposure of ATII cells to hypoxia causes disruption of the cytoskeleton (5), which might cause the internalization of membrane standing proteins (41) and thus might affect the stability and mobility of proteins. The fact that brief reoxygenation was sufficient to reverse hypoxia-induced inhibition of terbutaline-stimulated cAMP production might support this notion and most likely excludes the involvement of altered expression and synthesis of these proteins.

One striking result of our study is that hypoxia also increased the sensitivity of β2-AR to desensitization on long-term agonist stimulation, whereas short-term desensitization was not affected. As in untreated cells, reversal with PTX indicates an involvement of Gi protein. Thus it is possible that hypoxia affects processes involved in β2-AR desensitization such as phosphodiesterases, possibly PDE4, receptor phosphorylation by G protein-coupled receptor kinases (GRKs), recruitment of β-arrestin, and activation of Src tyrosine kinases (for review see Ref. 22). Further research is warranted to clarify their involvement.

Another possible mechanism causing inactivation of β2-AR signaling might be heterologous desensitization by activation of other receptors (16). Likely candidate stimuli might be hypoxia-induced proinflammatory mediators (43), which have
been observed to cause β2-AR desensitization in the lung (14, 19), e.g., by a hypoxia-induced increase in PGE2 (29), endothelin-1 (36), or adenosine (30). However, this subject requires further exploration.

Exposure of rat lungs and ATII cells to hypoxia has been used as a model to study various lung diseases that lead to pulmonary edema such as pulmonary hypertension, permeability edema by inflammatory processes, and alveolar edema on inhibition of alveolar reabsorption (10, 25, 46). On the basis of observations indicating that long-term treatment with β2-AR agonists improved alveolar fluid clearance, β2-AR agonist therapy has been suggested as a treatment for pulmonary edema (35). However, our data presented here and results from hypoxia-exposed rat lungs and lung injury models indicate potential limitations of this approach. In ALI, β2-AR function appears diminished (32). Prolonged in vivo administration of β2-AR agonists to normoxic rats impairs the stimulation of alveolar fluid clearance by terbutaline and decreases β2-AR density (33). These effects have been explained by desensitization of β2-AR due to elevated catecholamine levels. A similar mechanism might be effective in hypoxia (42) and possibly explains the lack of full stimulation of alveolar fluid clearance and Na-K-ATPase activity with isoproterenol in hypoxic rats (24). We show here that hypoxia of ATII cells directly impairs β2-AR signaling, probably by a G protein-mediated process. It therefore appears likely that dysfunction of β2-AR signaling in hypoxia might decrease the response to endogenous and exogenous β2-AR agonists. Whether this applies only to alveolar epithelium or also to the pulmonary vasculature, where it might impair β2-AR agonist-induced vasodilatation, or to the endothelium, where barrier permeability might be impaired, requires further studies.

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REFERENCES


