Abstract

We previously reported that exposure of mice to hyperoxia is characterized by extensive lung cell necrosis and apoptosis, mild inflammatory response, and elevated circulating levels of corticosterone. Administration of hydroxycortisone acetate during hyperoxia aggravated lung injury. Using adrenalectomized (ADX) and sham-operated (sham) mice, we studied the role of the glucocorticoids in hyperoxia-induced lung injury. Lung damage was attenuated in ADX mice as measured by lung weight and protein and cell content in bronchoalveolar lavage and as seen by light microscopy. Mortality was delayed by 10 h. Nuclear factor-kappaB (NF-kappaB) activity was significantly decreased in lungs of sham mice exposed to hyperoxia but was preserved in ADX mice. There was a correlation between NF-kappaB activity in ADX mice and decreased levels of IkappaBalpha. In contrast, activator protein-1 activity increased similarly in both groups of mice. Levels of interleukin-6 (IL-6), a transcriptional target of NF-kappaB, were higher in bronchoalveolar lavage and serum of ADX than sham mice. However, the protective effect of ADX was not mediated by [...]
Glucocorticoids aggravate hypoxia-induced lung injury through decreased nuclear factor-κB activity

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Glucocorticoids aggravate hypoxia-induced lung injury through decreased nuclear factor-κB activity. Am J Physiol Lung Cell Mol Physiol 284: L197–L204, 2003. First published August 23, 2002; 10.1152/ajplung.00239.2002.—We previously reported that exposure of mice to hypoxia is characterized by extensive lung cell necrosis and apoptosis, mild inflammatory response, and elevated circulating levels of corticosterone. Administration of hydroxyxortisone acetate during hypoxia aggravated lung injury. Using adrenalectomized (ADX) and sham-operated (sham) mice, we studied the role of the glucocorticoids in hypoxia-induced lung injury. Lung damage was attenuated in ADX mice as measured by lung weight and protein and cell content in bronchoalveolar lavage and as seen by light microscopy. Mortality was delayed by 10 h. Nuclear factor-κB (NF-κB) activity was significantly decreased in lungs of sham mice exposed to hypoxia but was preserved in ADX mice. There was a correlation between NF-κB activity in ADX mice and decreased levels of ICs. In contrast, activator protein-1 activity increased similarly in both groups of mice. Levels of interleukin-6 (IL-6), a transcriptional target of NF-κB, were higher in bronchoalveolar lavage and serum of ADX than sham mice. However, the protective effect of ADX was not mediated by IL-6, because administration of recombinant human IL-6 to sham mice did not prevent lung damage. These results demonstrate that the adrenal response aggravates alveolar injury and is likely to be mediated by the decrease of NF-κB function involved in cell survival.

Keywords: NF-κB; apoptosis; steroid

HIGH-OXYGEN EXPOSURE has been used as a valuable model of idiopathic respiratory distress syndrome or acute respiratory distress syndrome in rodents and is characterized by extensive alveolar cell death, leading to disruption of the alveolocapillary barrier and plasma leakage into the alveoli. Many factors can contribute to alveolar cell death, including the increased presence of reactive oxygen species, the modulation of the cell signaling response, and the inflammatory and stress response of the host. Modulation of the cell response by different strategies, such as administration of keratinocyte growth factor, overexpression of IL-6 in Clara cells, or transfection of Akt into alveolar cells, can prevent lung damage during hypoxia (5, 24, 43). The lung inflammatory response during hypoxia varies with species, strain, and age (15, 30). We have shown that adult C57BL/6 mice display only mild inflammatory cell recruitment into the lungs (6) and that endogenous corticosterone increased significantly during hypoxia in mice. Administration of hydroxyxortisone acetate at high doses aggravated lung injury (8).

Glucocorticoid (GC) effects have been largely studied with respect to lung development, because they impaired alveolar septation (10). However, their direct effects on lung epithelial cells of adult animals are less evident. GC decreased lung epithelial cell proliferation in rats exposed to ozone (35) and facilitated apoptosis in cultured airway epithelial cells (13). It has been established for 20 years that GC are potent inducers of apoptosis in lymphocytes (45), whereas they protect neuronal cells and hepatoma cell lines from cell death (14, 19).

GC bound to their receptors target the nuclear DNA and are able to transactivate several genes by binding the GC-responsive element but are also able to repress the DNA binding of transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) (29).

NF-κB is a redox-sensitive transcription factor, because antioxidant equilibrium can modulate its activation in vitro (31) and it is recognized to act mostly as a cell survival and a proinflammatory factor. NF-κB activity has not been reported during hypoxia-induced lung injury in vivo, nor have its regulatory effects on lung cell death been reported. Therefore, modulation of NF-κB activity and its relationship to GC by different approaches are important in the understanding of lung cell death during oxygen toxicity.

We have explored the effects and the possible roles of stress response in lung damage. We demonstrate that the adrenal response aggravates hypoxia-induced lung injury. The deleterious effect of the stress response was mainly GC dependent, as demonstrated by

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administration of GC and catecholamine antagonists. Lung NF-κB activity decreased significantly during hyperoxia, while, in the absence of the adrenal gland, NF-κB activity was conserved. The preserved NF-κB activity in adrenalectomized (ADX) mice correlated with decreased levels of IkBα. Higher NF-κB activity in ADX mice exposed to oxygen was also supported by significantly higher levels of IL-6 (transcriptionally regulated by NF-κB) in their serum and bronchoalveolar lavage (BAL). Administration of pyrrolidine dithiocarbamate (PDTC), which has been shown to inhibit NF-κB activity in several experimental inflammatory diseases (12, 23, 28), blocked IkBα degradation but did not modify lung NF-κB activity. In conclusion, our study shows that the adrenal response aggravates hyperoxia-induced lung injury, possibly by decreasing NF-κB activity and its cell survival property.

METHODS

Mice. ADX and sham C57BL/6 mice were purchased from Iffa Credo (Labsrele, France) or adrenalectomized at 6 wk of age by us according the same protocol. Mice were kept in our animal facilities for 2 wk before use. The water of ADX mice was supplemented with 0.9% NaCl and 1% glucose. Experiments were performed with 2- to 3-mo-old mice. Corticosterone plasma levels were checked in all mice before the experimental procedures and determined by RIA using 125I-labeled corticosterone (Diagnostic Systems Laboratories, Webster, TX), as described elsewhere (8, 42).

Hyperoxia exposure and in vivo treatment. Mice were placed in a sealed Plexiglas chamber and exposed to 100% oxygen or room air in the same conditions described previously (6). Food and water were available ad libitum. Mice were killed after 72 h of hyperoxia or, for mortality experiments, when the temperature, measured rectally with a clinical thermometer (type HP5310, Philips), dropped below 32°C, an event followed by death within 2 h. All animals (control or oxygen-exposed) were anesthetized with an injection of pentobarbital sodium (50 mg/kg ip) and then bled from the heart. Lung NF-κB and the 21-mer sequence (5'-CGCTTGATGAGT-3') for NF-κB and AP-1 consensus NF-κB and AP-1 activities. Nuclear proteins were extracted according a modification of a previously described protocol (40). Fresh lung tissue (10 mg) was homogenized with a Dounce homogenizer in 0.5 ml of buffer A [10 mM KCl, 1.5 mM MgCl2, 10 mM HEPES, pH 7.9, 1 mM dithiothreitol (DTT), 1 mM NaVO4, 5 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. This procedure was repeated after centrifugation at 2,000 g for 10 min at 4°C. The nuclei-containing pellet was carefully resuspended in 2 vol of buffer B [420 mM NaCl, 10 mM KCl, 20 mM HEPES, pH 7.9, 20% glycerol, 1 mM DTT, 1 mM NaVO4, 5 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride] and subjected to vigorous agitation for 30 min at 4°C. After centrifugation at 13,000 g for 20 min at 4°C, the supernatant was collected and stored at −80°C. NF-κB and AP-1 activity in nuclear proteins was evaluated using the electrophoretic mobility shift assay, which measures the abundance of protein species with the ability to bind a specific nucleotide sequence. The oligonucleotide probes were the specific consensus-bind site NF-κBcons2 (5'-ATGTGAAGGAGCTTATC-3') for NF-κB and the 21-mer sequence (5'-GGCAGTATGAGTTTACA-3') for AP-1. Probes were labeled with [γ-32P]dCTP using the Klenow method. Binding reactions were performed in DNA binding buffer containing poly(dI-dC) (0.5 μg/μg protein), 1 mM EDTA, 1 mM DTT, 4% Ficoll-400, 4 mg/ml BSA (Boehringer, fraction V), the nuclear extracts (2 μg), and 30,000 counts/min (ideally 30,000 counts/min/34 fmol in 1 μl) of radiolabeled oligonucleotide probe. Specificity of the bands in the retardation gels was ascertained by supershift (p65 of NF-κB, using anti NF-κB p65, TransCruz gel supershift antibody, sc-109 X) and/or by competition with cold probes. Briefly, competition was performed by preincubating the samples with the specific 10-nucleotide sequence 5'-GGGTATTTCC-3' (core sequence for NF-κB) or the 11-nucleotide sequence 5'-GGGTTATTTCC-3' (containing the core sequence for AP-1) before incubation with the respective radiolabeled probes at molar ratios of 1:1, 1:10, 1:100, and 1:1,000.
Western blots. Lungs were homogenized as described previously (5), and 40 μg of protein were loaded per lane onto 10% polyacrylamide gels and electrophoresed. Proteins were transferred to nitrocellulose membranes, and nonspecific binding was blocked with 5% nonfat milk in Tris-buffered saline + 0.1% Tween 20 (TBST) overnight at 4°C. Membranes were blotted with the polyclonal antibody anti-IkBα (catalog no. 554135, PharMingen, 1:1,000 dilution) in TBST and 5% nonfat milk. A goat anti-rabbit IgG-horseradish peroxidase (catalog no. 170-6515, Bio-Rad) diluted 1:3,000 in TBST and 5% nonfat milk was used as secondary antibody. Membranes were washed again with TBST and revealed with an enhanced chemiluminescence detection reagent kit (Amersham International, Amersham, UK) at room temperature before being exposed to Biomax MR film (Eastman Kodak, Rochester, NY). Results were quantified using sub-saturated emulsions on X-ray film, as described elsewhere (5), and normalized with the actin signal detected with a polyclonal antiactin (kind gift of G. Gabbiani, Dept. of Pathology, University of Geneva).

Statistical analysis. For each parameter measured or calculated, the values for all animals in an experimental group were averaged, and the standard deviation of the mean was calculated. The significance of differences between the values of an experimental group and those of the control group was determined with the unpaired Student’s t-test. Where appropriate, two-way ANOVA with multiple comparisons followed by an unpaired t-test was used. Significance levels were set at \( P < 0.05 \).

RESULTS

Adrenalectomy protects mice from alveolar edema. To evaluate lung injury and alveolar damage, we measured lung injury markers at 72 h of oxygen exposure. ADX mice showed a significant decrease in lung weight and BAL protein content compared with sham mice (Table 1). When corticosterone was reinjected into ADX mice, the susceptibility to hyperoxia was restored. Although BAL cell count increased during hyperoxia in sham mice, it was not significantly different from that in air-breathing sham mice. Lung histology of sham mice showed interstitial and alveolar edema, septal breakdowns, and focal alveolar hemorrhages, which were attenuated in ADX mice (Fig. 1). Mortality in ADX mice was delayed by 6–10 h (data not shown). To determine whether the adrenal medulla or cortex was implicated in lung damage, we subcutaneously inserted selective inhibitors of GC receptors (RU-486) or β-blockers (8, 17). Only RU-486 treatment significantly decreased alveolar damage (Fig. 2). These experiments demonstrate that GC aggravate the alveolar damage induced by hyperoxia.

Hyperoxia induces thymocyte apoptosis via endogenous increase of corticosterone. Steroids are potent inducers of thymocyte apoptosis; therefore, we explored the role of endogenous GC elevation in thymic atrophy induced by hyperoxia. Thymus weight was significantly reduced by hyperoxia (84 h) in sham, but not ADX, mice (Fig. 3A). Adrenalectomy in air-breathing mice increased thymus weight, which did not change during hyperoxia (Fig. 3A).

Activated caspases were detected in isolated thymocytes using a specific probe (CaspTag). The percentage of positive cells was increased in thymocytes isolated from hyperoxic sham mice compared with air-breathing mice (42% vs. 8%) and was not affected by hyperoxia in ADX mice (7%). Moreover, daily injection of hydroxycorticosterone into ADX mice reversed this effect (29% of positive cells). These results demonstrate that thymocyte apoptosis during hyperoxia depends on increased circulating levels of endogenous corticosterone (Fig. 3B).

Modulation of NF-κB and AP-1 activities by hyperoxia. We examined DNA binding of NF-κB and AP-1 in lung extracts during hyperoxia in sham and ADX mice by gel-shift assay (Fig. 4A). Two bands (p55 and p65) were detected in lung nuclear extracts (37). These bands were specific for NF-κB and AP-1: they could be abolished by competition with the oligonucleotide specific for the core sequence and not for the mutated sequence (PU.1; Fig. 4, B and D), and the p65 protein was displaced by supershift (data not shown). NF-κB activity decreased significantly during hyperoxia in sham mice (\( P < 0.01 \)), whereas it was conserved in ADX mice (Fig. 4A). We also analyzed AP-1 activity, which increased in sham and ADX mice during hyperoxia (Fig. 4C). Although AP-1 activity was slightly more elevated in ADX than in sham mice exposed to oxygen, the difference was not significant (Fig. 4C).

Inasmuch as oxidative stress modulates NF-κB activity by degrading IκBα, a protein that is bound to NF-κB within the cytosol and, thereby, prevents translocation of NF-κB into the nucleus, we examined IκBα

Table 1. Lung injury score in sham and ADX mice

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>Hyperoxia</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>ADX</td>
</tr>
<tr>
<td>Right lung wt, g</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Protein, mg/ml</td>
<td>0.09 ± 0.05</td>
<td>0.13 ± 0.09</td>
</tr>
<tr>
<td>Cell count, × 10(^3)</td>
<td>26.7 ± 13.6</td>
<td>27.3 ± 12.3</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Serum IL-6, pg/ml</td>
<td>4.3 ± 2.4</td>
<td>5.2 ± 0.6</td>
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Values are means ± SD of 5–10 animals in each group. Sham-operated (sham) mice, adrenalectomized (ADX) mice, and ADX mice treated with hydrocortisone acetate (HCS, 140 μg/day) were used. Lungs were harvested at 72 h, and bronchoalveolar lavage was performed by intratracheal instillation of 2 ml of PBS, with hydrostatic pressure of 20 cmH\(_2\)O. Cells were counted, and proteins and interleukin-6 (IL-6) were measured in supernatant and serum. Significantly different from air: \(^aP < 0.05\) and \(^bP < 0.001\). Significantly different from sham: \(^cP < 0.01\) and \(^dP < 0.001\). Significantly different from ADX: \(^eP < 0.05\) and \(^fP < 0.001\). ND, not determined.
expression by Western blot (Fig. 5). Hyperoxia did not change IκB expression in sham mice. However, in ADX mice, where NF-κB activity was conserved, IκB expression vanished (Fig. 5). These results suggest that GC could act not only directly by repressing NF-κB but also by decreasing IκB.

**PDTC prevents IκB degradation.** We administered PDTC, a drug that can block NF-κB by preventing IκB degradation (23), to sham and ADX mice (Fig. 5). Lung NF-κB activity was not affected by daily administration of PDTC in sham or ADX mice, and lung weight was not modified by PDTC treatment during hyperoxia (data not shown). IκB expression, analyzed by Western blot, was higher in PDTC-treated mice than in untreated mice (Fig. 5). These data suggest that PDTC does not modify total lung NF-κB activity in hyperoxia-induced injury in sham or ADX mice, although it prevents IκB degradation.

**Effect of IL-6 on hyperoxia-induced lung damage.** IL-6 is a cytokine that is transcriptionally regulated by NF-κB (41). Inasmuch as mice overexpressing IL-6 in Clara cells were clearly protected in hyperoxia (43), we first examined IL-6 levels in serum and BAL of ADX and sham mice (Table 1). During hyperoxia exposure, the level of IL-6 was 10-fold higher in ADX than in sham mice (Table 1). Interestingly, the number of cells counted in BAL was similar in both groups of mice exposed to hyperoxia. These data correlate with an enhanced NF-κB activity in ADX mice compared with sham mice during hyperoxia.

To investigate whether the protective effect exerted by the absence of the adrenal gland could be mediated by IL-6 elevation, we administered rhIL-6 to wild-type mice by continuous infusion at a dose previously shown to be efficient (39). rhIL-6 levels reached 0.8–1.7 ng/ml and were even higher than those obtained during hyperoxia in ADX mice. This treatment conferred no protective effect on hyperoxia-induced alveolar edema (Fig. 6). These results indicate that IL-6 does not play a major role in the prevention of lung damage in ADX mice.

**DISCUSSION**

We have demonstrated that lung damage is aggravated by the GC produced in response to hyperoxia. Lung weight and protein content, reflecting alveolar damage, were significantly reduced in ADX mice, and when hydroxy cortisone acetate was administered to ADX mice, the susceptibility to hyperoxia was restored. If at 72 h of oxygen exposure the attenuation of lung injury was evident, ADX mice showed only a delayed mortality of several hours, suggesting that the adrenal response contributes to lung damage. We previously reported that hyperoxia...
increased circulating levels of endogenous corticosterone in mice and that administration of hydroxycorticosterone at high doses worsened lung injury (8). In humans, cortisol elevation has been reported in acute respiratory distress syndrome and is part of the normal stress response (27). Furthermore, low levels of cortisol have been associated with a poor outcome in septic patients (9).

**Fig. 3.** A: thymus weight of air-breathing and hyperoxia (Hox)-exposed sham and ADX mice. Thymus weight of sham mice exposed to hyperoxia was significantly reduced compared with air-breathing mice ($P < 0.01$). Adrenalectomy significantly increased thymus weight in air-breathing animals ($P < 0.01$) and protected thymus from atrophy during hyperoxia. Values are means ± SD ($n = 8$). B: detection of activated caspase on isolated thymocytes. Thymocytes of air-breathing and hyperoxia-exposed mice were isolated and labeled with the FITC-labeled CaspaTag fluorescein (VAD) probe and examined by flow cytometry. Results are from a representative experiment. HCS, hydroxycorticosterone acetate.

**Fig. 4.** A and C: detection of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activity in lung nuclear extracts. Two micrograms of lung nuclear protein extract were loaded in each lane, and gels were incubated with $^{32}$P-labeled oligonucleotide probes for NF-κB (A) or AP-1 (C) and subjected to EMSA. A: NF-κB activity decreased during hyperoxia in sham mice compared with air-breathing mice: $^*P < 0.05$. NF-κB activity was preserved during hyperoxia in ADX mice compared with sham mice: $^#P < 0.01$. C: AP-1 activity increased during hyperoxia in sham and ADX mice: $^*P < 0.05$. Values are means ± SD of 3–5 different samples for each condition. A representative autoradiogram is shown at top. AU, arbitrary units. B and D: competition experiments performed by preincubating lung nuclear extracts with cold specific DNA 10-nucleotide or PU.1-specific DNA 11-nucleotide sequence at molar ratios of 1:1, 1:10, 1:100, and 1:1,000.
In the majority of lung illness, there is a component of alveolar damage and a component of inflammatory response. GC effects are diverse and often opposite, depending on the type of cell. Several studies have demonstrated negative effects of steroids on lung development when given to pregnant rats or newborn pups (see Ref. 18 for review). Luyet et al. (25) reported recently that early treatment of newborn rats with dexamethasone decreased cell proliferation and increased the number of TdT-mediated dUTP nick end label-positive cells. In adult rats, absence of the adrenal gland resulted in a higher compensatory growth of the contralateral lung after pneumonectomy (18); however, this increased growth phenomenon was not detected in ADX rats without pneumonectomy. We were unable to detect any DNA ladder in lungs of control mice treated with dexamethasone in vivo (unpublished observations), suggesting that GC alone do not induce significant alveolar cell apoptosis in adult mice but are likely to synergize with the effects of oxygen on alveolar damage.

Corticosterone elevation is part of the normal stress response to injuries, and in this context it is still debated whether this normal response to stress can overwhelm the normal defense mechanisms and deleterious for the organism (27). Among its effects, the stress response has been known for a very long time to result in thymus atrophy (38). Thymus atrophy was later shown to be due to an apoptosis-dependent mechanism, which was induced by GC (45). Indeed, thymocyte apoptosis, evaluated by CaspaTag labeling, was completely abrogated in ADX mice exposed to hyperoxia and reversed by hydrocortisone administration. These data demonstrate that the elevation of GC is clinically relevant since it induces thymus atrophy and that GC are directly responsible for thymus apoptosis during hyperoxia-induced lung injury.

Taking into account that among the major effects of GC is repression of the activity of NF-κB and, to a lesser extent, AP-1 (29), we examined NF-κB and AP-1 activities in vivo during hyperoxia. Our results showed that lung NF-κB activity decreased significantly during hyperoxia in sham mice, and, in contrast, AP-1 activity increased. Although the pattern of AP-1 activity was similar in sham and ADX mice, this was not the case for NF-κB. Indeed, NF-κB activity did not decrease but was conserved in ADX mice during hyperoxia. These data suggest that endogenous corticoste-
erase under the control of NF-κB promoter (34). Dexamethasone did not decrease luciferase expression after LPS administration in the lungs, whereas it decreased NF-κB activity in peritoneal macrophages (34). It is likely that different cell types did not respond similarly in terms of NF-κB activation and might be more or less sensitive to PDTC treatment.

Recently, Ward and co-workers (43) reported that mice overexpressing IL-6 in Clara cells removed were protected from hyperoxia, and they explained lung cell resistance through increased basal levels of Bcl-2 in their transgenic mice. IL-6 is a well-known proinflammatory cytokine synthesized within the lungs mainly by alveolar macrophages. Inasmuch as NF-κB is almost the exclusive transcription factor for IL-6 (41), we measured IL-6 levels in response to hyperoxia. ADX mice display much higher levels of IL-6 in serum and BAL than sham mice in response to hyperoxia. IL-6 levels were completely dependent on the presence of GC, since administration of hydrocortisone to ADX mice restored the sham phenotype. These results confirm the belief that adrenal hormones and, most likely, GC act on IL-6 production via increased NF-κB activity. To further test the hypothesis that higher IL-6 levels in ADX mice might prevent oxygen-induced lung damage, we performed continuous infusion of rhIL-6. Although we reached IL-6 levels in serum that were similar to those measured in ADX mice exposed to oxygen reported by Ward et al. (0.5–1.5 ng/ml; Ref. 43), we did not observe any protective effect with rhIL-6 administration, suggesting that the protective mechanism of ADX mice might not be related to the IL-6 response. However, it cannot be excluded that subcutaneous administration did not reach the epithelial side of the alveoli in sufficient quantity.

Taken together, our data show that the adrenal response and, in particular, GC aggravates alveolar damage, which is correlated with the extinction of NF-κB activity. This work provides an explanation for the failure of the steroid to markedly improve several acute or chronic inflammatory lung disorders, since its effects on inflammatory cells and/or epithelial and endothelial cells might be opposed (3, 11, 26, 27). The concomitant ongoing destructive phase, where steroid treatment might accelerate cell death, and proliferative inflammatory phase, where steroids might stop cell proliferation and prevent proinflammatory cytokine transcription, could explain the conflicting results in assessing the efficacy of steroid treatment.

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