Effects of lisofylline on hyperoxia-induced lung injury

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George, Caroline L. S., Giamila Fantuzzi, Stuart Burstsen, Laura Leer, and Edward Abraham. Effects of lisofylline on hyperoxia-induced lung injury. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L776–L785, 1999.—Lisofylline [1-(5-hydroxyhexyl)-3,7-dimethylxanthine] decreases lipid peroxidation in vitro and in vivo suppresses proinflammatory cytokine expression in models of lung injury due to sepsis, blood loss, and oxidative damage. In the present experiments, we used a murine hyperoxia model to examine the effects of lisofylline on the activation of nuclear transcriptional regulatory factors [nuclear factor-κB (NF-κB) and cAMP response element binding protein (CREB)], the expression of proinflammatory cytokines in the lungs, and circulating levels of oxidized free fatty acids as well as on hyperoxia-induced lung injury and mortality. Treatment with lisofylline inhibited hyperoxia-associated increases in tumor necrosis factor-α, interleukin-1β, and interleukin-6 in the lungs as well as decreased the levels of hyperoxia-induced serum oxidized free fatty acids. Although hyperoxic exposure produced activation of both nuclear factor-κB and CREB in lung cell populations, only CREB activation was reduced in the mice treated with lisofylline. Lisofylline diminished hyperoxia-associated increases in lung wet-to-dry weight ratios and improved survival in animals exposed to hyperoxia. These results suggest that lisofylline ameliorates hyperoxia-induced lung injury and mortality through inhibiting CREB activation, membrane oxidation, and proinflammatory cytokine expression in the lungs.

Cytokine expression; nuclear factor-κB; adenosine 3′,5′-cyclic monophosphate response element binding protein; lipid oxidation

HYPEROXIA-INDUCED LUNG INJURY is characterized by an intense inflammatory response initiated and exacerbated by the generation of reactive oxygen species (ROS) (14, 21). Histological changes include alveolar and interlobular septal edema, neutrophil and macrophage infiltration, type II cell hyperplasia, and fibroblastic proliferation (12, 25). Increased expression of proinflammatory cytokines accompanies hyperoxia and appears to contribute to the development of lung injury in this setting. Tumor necrosis factor (TNF-α) is present in the lung in increased amounts early in the course of hyperoxia, even before histological changes are seen (38, 43). Survival during hyperoxia was improved when an anti-TNF-α antibody was given during hyperoxic exposure (22, 43). Interleukin (IL)-1β and IL-6 levels are also elevated in the lung during exposure to high concentrations of oxygen (23, 28).

Intracellular signaling pathways leading to the activation of transcriptional regulatory factors such as nuclear factor-κB (NF-κB) and cAMP response element binding protein (CREB) can be affected by ROS (36, 39). The activity of ROS to modulate the activity of NF-κB or CREB may be particularly important in the setting of hyperoxia where increased amounts of ROS are generated in the lungs (21). NF-κB is activated in lung cells after as short a period of hyperoxic exposure as 24 h (38). Binding sites for NF-κB and CREB are present in the promoter regions of proinflammatory cytokine genes, including TNF-α, IL-1β, and IL-6 (27, 31, 39). Therefore, increased proinflammatory cytokine expression as a result of ROS-induced activation of transcriptional factors may play an important role in initiating lung injury during hyperoxia.

Lisofylline [LSF; 1-(5-hydroxyhexyl)-3,7-dimethylxanthine] reduces lung free fatty acid ratios in vitro models of IL-8-stimulated neutrophils and lung injury (16, 17). Patients with acute respiratory distress syndrome or those who are at risk for developing acute lung injury have increased serum levels of free fatty acids, which are reduced by treatment with LSF (6).

The addition of high concentrations (100 µM) of LSF to peripheral blood mononuclear cells stimulated with lipopolysaccharide decreased the release of TNF-α, IL-1β, and IL-6 (33). Nevertheless, LSF improves survival from endotoxemia at doses that produce concentrations substantially below 100 µM even when administered 4 h after the endotoxin challenge (32). In murine and porcine models of acute lung injury induced by ischemia-reperfusion or sepsis, LSF treatment decreased proinflammatory cytokine levels in the lungs and diminished the severity of inflammatory lung injury (1, 17).

Because LSF decreases membrane peroxidation, inhibits proinflammatory intracellular signaling, and diminishes proinflammatory cytokine expression, we hypothesized that it may have protective effects on hyperoxia-induced lung injury. To evaluate this question, we examined the effects of LSF on the transcriptional regulation of proinflammatory cytokine genes in the lungs and the oxidation of membrane lipids as well as on the severity of lung injury and mortality during hyperoxic exposure.

METHODS

Materials. LSF was provided by Cel Therapeutics (Seattle, WA). Methoxyflurane was obtained from Pittman-Moore (Mundelein, IL). Percoll and poly(dI-dC)-poly(dI-dC) were obtained from Pharmacia (Uppsala, Sweden). The colorimet-
ric protein assay kit, bovine serum albumin standard, N,N,N',N'-tetramethylethylene diamine (TEMD), and ammonium persulfate were purchased from Bio-Rad Laboratories (Hercules, CA). The conserved CRE and xB oligonucleotides were synthesized by Operon (Alameda, CA) with previously published sequences (Roesler/Zabel). All murine PCR primers (TNF-α, IL-1β, IL-6, and hypoxanthine phosphoribosyltransferase (HPRT)) were obtained from Clontech (Palo Alto, CA). Moloney murine leukemia virus (MMLV) reverse transcriptase, 0.1 M dithiothreitol, and 5× first-strand buffer were all purchased from Life Technologies (Grand Island, NY). Recombinant RNA and random hexamers were obtained from Promega (Madison, WI). AmpliTaq DNA polymerase and 10× PCR buffer were purchased from Perkin-Elmer (Branchburg, NJ). [α-32P]dATP was obtained from DuPont-NEN Life Science Products (Boston, MA). Sequenase version 2.0 T7 DNA polymerase was obtained from United States Biochemical (Cleveland, OH). Antibodies to the p50 and p65 subunits of NF-κB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to phosphorylated (phospho)-CREB was obtained from Upstate Biotechnology (Lake Placid, NY). NucTrap purification columns were obtained from Stratagene (La Jolla, CA). Rabbit anti-murine polyclonal antibody, recombinant TNF-α standard, and the ELISA kit for mouse IL-6 were purchased from Endogen (Woburn, MA). The hamster anti-murine TNF-α monodonal antibody was obtained from Genzyme Diagnostics (Cambridge, MA). Biotin and ruthenium (II) trisbipyridine were obtained from Igen (Gaithersburg, MD). The streptavidin-coated paramagnetic beads were obtained from Dynal (Lake Success, NY). Biscyclamide (2% solution) was purchased from Fisher (Pittsburgh, PA). Biomax MS film was obtained from Eastman Kodak (Rochester, NY). All HPLC grade reagents were purchased from Burdick-jackson (Seattle, WA). Standards for HPLC, 9-(S)- and 13-(S)-hydroxyoctadecadienoic acid (HODE), 9-(S)- and 13-(S)- hydroperoxyoctadecadienoic acid (HPODE), and 5-hydroxyicosatetraenoic acid lactone were obtained from Cayman Chemical (Ann Arbor, MI). RPMI 1640 medium, antibiotics, 25 mM HEPES, 1-glutamine, penicillin, streptomycin, fetal calf serum, collagenase, deoxyribonuclease, and all other chemicals were all obtained from Sigma (St. Louis, MO). 

Animals. The study protocol was approved by the University of Colorado (Denver, CO) Health Sciences Center Animal Subject Protection Committee, and all protocols followed National Institutes of Health guidelines for the use of laboratory animals. Male BALB/c mice, 4 wk of age, were obtained from The Jackson Laboratories (Bar Harbor, ME) and then allowed to acclimate to Denver’s altitude for at least 2 wk before use. All mice were 6–7 wk old when used for experiments. The mice were kept on a 12:12-h light-dark cycle and provided mice chow (Agway Prolab 3000, Syracuse, NY) and water ad libitum. BALB/c mice were used in these experiments because of their intermediate sensitivity to hyperoxic injury compared with that of other mouse strains (20).

Interventions. Study groups of mice were placed in hyperbaric chambers lined with lime soda. The chambers were pressurized to 760 mmHg and, when needed, depressurized over 30 min. Timed hyperoxic exposures (inspired O2 fraction 1.0, 760 mmHg) lasted 24–72 h. Test groups of animals (n = 5–8) were treated intraperitoneally with either LSF (100 mg·kg−1·dose−1) or PBS (0.75 ml), or PBS (n = 20) or LSF (n = 20) for the first 72 h of hyperoxia, then were observed under hyperoxic conditions until all animals had expired. The 72-h period of treatment was used because after 72 h, depressurizing the chambers appeared to affect mouse mortality, with increased animal deaths occurring during the depressurization period.

Bronchoalveolar lavage. The animals were first anesthetized with methoxyflurane, then killed by cervical dislocation. The thoracic skin and fascia were opened by a midline incision. The trachea was exposed by blunt dissection. Bronchoalveolar lavage (BAL) fluid was obtained as previously described (34). In brief, the lungs were lavaged three times with the same 1-ml aliquot of cold, sterile PBS. The final returned volume was consistently >0.75 ml. The fluid was immediately centrifuged at 2,500 rpm for 30 s. The supernatant was removed and stored at −70°C until used for cytokine measurements.

Cytokine protein measurements. TNF-α protein was measured with an electrochemiluminescence method (11). Briefly, a pre-purified immunoglobulin G preparation from a rabbit anti-murine polyclonal antibody was labeled with ruthenium (II) trisbipyridine chelate as per the manufacturer’s instructions (Igen). Twenty-five microliters of ruthenylated antibody (2 μg/ml) were combined with 25 μl of a biotinylated hamster anti-murine TNF-α monoclonal antibody (1 μg/ml) and diluted in an electrochemiluminescence buffer (PBS, pH 7.4, with 0.25% bovine serum albumin, 0.5% Tween 20, and 0.01% sodium azide). The antibody solution was combined with 25 μl of a recombinant TNF-α standard or 25 μl of a BAL fluid sample in a 6-ml polypropylene tube for incubation at room temperature overnight. Twenty-five microliters of a 1 mg/ml solution of streptavidin-coated paramagnetic beads were added to each tube, and the tubes were agitated for 15 min at room temperature. The reaction was quenched by adding 200 μl/tube of PBS, pH 7.4. The sample and standards were quantitated with an Origen 1.5 analyzer (Igen). The sensitivity of the assay was 50 pg/ml.

Semiquantitative PCR from whole lung homogenates. Groups of five mice, with results obtained from individual mice, were used for each experimental condition. Semiquantitative PCR was used in these studies because the amount of RNA obtained from each mouse was insufficient to prepare Northern blots for some cytokines. The animals were anesthetized and prepared for dissection as described in Bronchoalveolar lavage. The thorax was opened with two lateral incisions along the rib cage. The right heart was injected with cold, sterile PBS (1–2 ml) until the lungs had been thoroughly flushed. The lungs were excised with care to avoid the peritracheal lymph nodes and rinsed in PBS. The lungs were briefly blotted, then snap-frozen in liquid nitrogen. The lungs were homogenized for 30 s on ice in a denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol as per Chomczynski and Sacchi (9); then mRNA was phenol extracted. cDNA was synthesized from 1 μg of mRNA with MMLV reverse transcriptase and random hexamer oligonucleotide primers as described by Kawasaki (24). Semiquantitative PCR was performed with primers specific for murine TNF-α, IL-1β, and IL-6. A single PCR...
master mix was prepared. Aliquots used for each sample contained 1× PCR buffer, 0.188 mM each deoxyribonucleotide triphosphate, 0.4 µM each single-strand DNA primer, 0.04 U of AmpliTaq DNA polymerase, and cDNA from 0.25 µg of mRNA, and the final volume was adjusted to 50 µl with sterile deionized water. After an initial 2-min denaturation step at 95°C, between 26 and 38 cycles of PCR were performed as follows: 1 min, 95°C denaturation; 1 min, 60°C anneal; and 1 min, 72°C extension. Complification of the housekeeping gene HPRT was used to standardize the PCR products. PCR products were electrophoresed on a 1.6% agarose gel and stained with ethidium bromide. The number of PCR cycles was selected so that the ethidium-stained amplified DNA products were below the level of saturation. Analysis of the gel was performed with a gel-documentation system (ImageStore 5000 with GelBase Windows Software, Ultraviolet Products, San Gabriel, CA). Absorbance for each cytokine product was normalized to the respective HPRT absorbance.

Preparation of nuclear extracts. Isolation of intrapulmonary monocytes and neutrophils has previously been described by our laboratory (2). The mean number of cells isolated per mouse after 24 h of hyperoxic exposure was 9.26 ± 1.22 × 10^8 in PBS-treated animals and 7.76 ± 2.56 × 10^8 in LSF-treated animals. After isolation, the intrapulmonary monocytes and neutrophils pooled from 10 mice were washed with PBS, and the nuclear protein was isolated as described by Hillman et al. (19). Briefly, 2–3 × 10^6 cells were resuspended in 250 µl of buffer A (13), incubated on ice for 15 min, and homogenized by 15 passages through a 25-gauge needle. After centrifugation for 6 min at 600 g at 4°C, the nuclear pellet was resuspended in 50 µl of buffer C (13) and incubated on ice for 15 min. The nuclear extracts were centrifuged for 10 min at 12,000 g at 4°C. The supernatant was collected and stored in aliquots at −70°C. Protein concentrations were determined with a colorimetric assay (Bio-Rad protein assay, Bio-Rad Laboratories) and standardized with bovine serum albumin.

Electrophoretic mobility shift assay. This procedure has been previously described by our laboratory (39). The k-b site of the immunoglobulin gene (44) and the CRE conserved site (35) were used for identifying DNA-protein complexes. Synthetic double-stranded oligonucleotides of the following sequences (enhancer motif underlined) were fill-in labeled with [α-32P]dATP with T7 DNA polymerase: k-b, 5′-TTTTCCGAC TCGGGAGCTTTCGAGC-3′ and 3′-GCTGAGGCTCCTGAAA GGCTCTTTTT-5′ and CRE, 5′-TTTTCCGAGCTCTGAGCT CAGGAC-3′ and 3′-GCTGAGACTCGAGCTCCTTGGG. The DNA binding reaction was performed at room temperature for 20 min with a total volume of 20 µl. The reaction mixture contained 2.5 µg of nuclear extract, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM diithiothreitol, 1 mM MgCl₂, 4% glycerol, 0.08 µg poly(dI-dC)·poly(dI-dC)/µg nuclear extract, and 32P-labeled double-stranded oligonucleotides at 0.7 fmol/µg nuclear extract. For supershift reactions, antibodies to the p50 (5 µl) or p65 (1 µl) subunits of NF-kB or phospho-CREB (1 µl) were added to the reaction mixture just before the 20-min incubation period. After incubation, 2 µl of 10× gel loading buffer (250 mM Tris-Cl, pH 7.5, 0.2% bromphenol blue, 0.2% xylene cyanol, and 40% glycerol) were added to each sample reaction; then each sample was loaded onto a 4% polyacrylamide gel (acrylamide-bis-acrylamide 80:1, 2.5% glycerol in 0.5× Tris-borate-EDTA, TEMED, and ammonium persulfate) and electrophoresed at 10 V/cm. The gel was then dried and analyzed by autoradiography.

Determination of lipid oxidation from serum by reverse-phase HPLC. Serum was obtained from control unmanipulated mice and from mice exposed to hyperoxia for 48 h and treated with either PBS or LSF. The serum was transferred to silanized (100 µl of methanol and 0.0375% butylated hydroxytoluene) glass vials and immediately placed at −70°C before lipid analysis was performed. For analysis of 9- and 13-HODEs and 9- and 13-HPODEs, serum was transferred to a pre-argoned tube, and 10,000 counts/min of 13-[14C]HODE and 13-[14C]HPODE in 50 µl of absolute ethanol were added to each sample. Chloroform-methanol (2:1) followed by PBS (pH 6.5) was added to each sample, and the partitioned phases were separated by centrifugation. The lower (organic) phase was collected and dried completely under nitrogen. Mobile-phase solvent [400 µl of 10 parts 0.15% acetic acid, 7 parts acetonitrile, and 5 parts tetrahydrofuran (vol/vol)] was added, and the tubes were vortexed and sonicated. The suspended lipid residue was collected and separated on a C18 reverse-phase HPLC Jones Genesis column (4 µm, 25 cm × 4.6 mm) with a flow rate of 1 ml/min, then analyzed by the integrated absorbance signal between 195 and 300 nm monitored via a photodiode array (Shimadzu, Columbia, MD). Standards for each chromatogram included 9-(S)HODE, 9-(S)HPODE, 13-(S)HODE, and 13-(S)HPODE. Quantitation was accomplished by interpolating the peak area response of a sample within the peak area dose-response curve for the standards.

Wet-to-dry lung weight ratios. All mice used for lung wet-to-dry weight ratios were of identical ages. The lungs were excised, rinsed briefly in PBS, blotted, then snap-frozen in liquid nitrogen to obtain the “wet” weight. The lungs were then dried in an oven at 105°C for 24 h to obtain the “dry” weight.

Statistical analysis. Due to the inherent variability between groups of mice, for each experiment the entire group of animals had the same birth date, were exposed to either hyperoxia or room air in the identical chambers, and were prepared and studied at the same time. Data from semiquantitative PCR were obtained from individual mice and analyzed individually before group means and SE were calculated. Data presented are means ± SE and were analyzed by one-way analysis of variance with a Student-Newman-Keuls test of multiple comparisons. Survival curve data were analyzed by log-rank analysis. A P value < 0.05 was considered significant.

RESULTS

Effects of LSF on hyperoxia-induced proinflammatory cytokine expression in the lung. Representative semiquantitative PCR results are shown in Fig. 1. Levels of TNF-α mRNA in the lung were significantly increased after 24 h of hyperoxic exposure and continued to be elevated at 48 h (P < 0.01; Fig. 2). IL-1β and IL-6 mRNA levels were not increased in the lung at 24 h of hyperoxia but were significantly increased after 48 h of exposure (P < 0.01; Figs. 3 and 4).

TNF-α protein in BAL fluid was significantly elevated versus the control levels after 48 h of hyperoxia (P < 0.001; Fig. 5). No IL-1β protein was found in BAL fluid from control or hyperoxia-exposed mice. IL-6 protein levels were increased in BAL fluid after 48 h of hyperoxia (P < 0.05; Fig. 6).

Therapy with LSF completely prevented hyperoxia-induced increases in lung TNF-α and IL-1β mRNA levels and suppressed the hyperoxia-induced increase in IL-6 mRNA levels by 38% (Figs. 2–4). Treatment with LSF also reduced TNF-α and IL-6 protein levels in BAL fluid by 20 and 38%, respectively, after 48 h of exposure to hyperoxia compared with treatment with PBS (Figs. 5 and 6).
Effects of LSF on hyperoxia-induced activation of nuclear transcription factors NF-κB and CREB in the lung. To explore the effects of LSF on transcriptional regulatory events that precede hyperoxia-induced increases in lung cytokine protein, we examined the activation of the transcriptional factors NF-κB and CREB in lung cells of mice exposed to hyperoxia for 24 h and treated with either LSF or PBS during that period. In previous experiments, Shea et al. (38) demonstrated that 24 h of hyperoxia results in increased activation of the transcriptional factor NF-κB in lung cell populations. Such activation of NF-κB occurs before increased levels of TNF-α protein are detectable in the lungs, and activated NF-κB continues to be present in lung cells for periods of hyperoxic exposure > 24 h in length.

NF-κB was constitutively expressed in lung cells from control animals (Fig. 7A). Increased activation of NF-κB was present in mice exposed to hyperoxia for 24 h (Fig. 7A, 24P). Specificity for the observed DNA-protein complex was confirmed by the complete ablation of the NF-κB band when a 500-fold excess of unlabeled κB DNA oligonucleotide was added to the reaction (Fig. 7A, 24P + cold κB). The hyperoxia-induced NF-κB complex contained both the p65 and

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**Fig. 1.** Effect of hyperoxia and lisofylline (LSF) on tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 mRNA levels in lungs. Representative experiments show amplified cytokine product from control animals, animals exposed to hyperoxia for 24 h and treated with PBS (24P) or LSF (24L), and animals exposed to hyperoxia for 48 h and treated with PBS (48P) or LSF (48L). Housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) from each individual animal was included in all experiments.

**Fig. 2.** Effect of hyperoxia on TNF-α mRNA levels in lungs of control (C), 24P, 24L, 48P, and 48L animals (n = 5/group). TNF-α mRNA was significantly increased in animals that received PBS (**P < 0.01) but not in those given LSF during hyperoxia compared with that in C group.

**Fig. 3.** Effect of hyperoxia on IL-1β mRNA levels in lungs of C, 24P, 24L, 48P, and 48L animals (n = 5/group). IL-1β mRNA level was significantly increased in 48P animals (***P < 0.01) but not in those given LSF compared with that in C animals.

**Fig. 4.** Effect of hyperoxia on IL-6 mRNA levels in lungs of C, 24P, 24L, 48P, and 48L animals (n = 5/group). IL-6 mRNA level was significantly increased in 48P animals (***P < 0.01) but not in those given LSF compared with that in C animals.
p50 subunits as shown by the supershifts when antibodies to p65 or p50 were added to the reaction mixture (Fig. 7A, 24P + anti-p65 and 24P + anti-p50). Treatment with LSF during exposure to hyperoxia for 24 h did not affect NF-κB activation (Fig. 7A, 24P and 24L) as confirmed by densitometric analysis (Fig. 7B).

CREB was constitutively expressed in lung cells from control animals in both the phosphorylated and unphosphorylated forms (Fig. 8A). Specificity for the observed DNA-protein complexes was confirmed by complete ablation of both CREB bands when a 500-fold excess of unlabeled CREB-specific DNA oligonucleotide was added to the reaction (Fig. 8A, 24P + cold CREB). Increased phospho-CREB was present in mice exposed to 24 h of hyperoxia (Fig. 8A, 24P). Treatment with LSF consistently reduced the hyperoxia-associated increase in phospho-CREB by 45–50% (Fig. 8A, 24L) as confirmed by densitometric analysis (Fig. 8B).

Effects of LSF on hyperoxia-induced generation of oxidized free fatty acids. A representative HPLC trac-
of the oxidized products of linoleic acid (HODE and HPODE) as well as the total amount of oxidized linoleic acid and arachidonic acid products (total diene chromophores) compared with room air control mice (P, 0.05, P, 0.001, and P, 0.001, respectively; Figs. 10–12). No alterations in serum levels of these oxidized lipids was found after 24 h of hyperoxia.

Treatment with LSF prevented hyperoxia-associated increases in HODE and total diene chromophore levels (Figs. 10 and 12). Although HPODE levels were significantly increased in LSF-treated animals after 48 h of hyperoxia, treatment with LSF prevented hyperoxia-associated increases in HODE and total diene chromophore levels (Figs. 10 and 12). Although HPODE levels were significantly increased in LSF-treated animals after 48 h of hyperoxia, no alterations in serum levels of these oxidized lipids was found after 24 h of hyperoxia.

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one representative set of experiments gave similar results.

Fig. 8. Effects of hyperoxia and LSF on CREB activation. A: CREB binding in intrapulmonary monocyte and neutrophil nuclear extracts (2.5 µg) from pooled lung cells from C, 24P, and 24L + animals (n = 10/group) as shown by EMSA. Supershift results with anti-phosphorylated (phospho)-CREB antibody demonstrate increased CREB activation (i.e., phospho-CREB levels). B: densitometry results show that treatment with LSF reduces by 45–50% the amount of phospho-CREB induced by hyperoxia compared with PBS treatment. One representative experiment is shown. An additional experiment with a separate set of animals gave similar results.

Fig. 9. Representative HPLC tracings demonstrate 9-(S)- and 13-(S)-hydroxyoctadecadienoic acid (9- and 13-HODE, respectively) and 9-(S)-hydroperoxyoctadecadienoic acid (9-HPODE) peaks as well as other oxidized lipids in serum after 48 h of hyperoxia and in room air control serum. Internal standard shown is 5-hydroxyeicosatetraenoic acid lactone. Samples from 4–6 other mice in each experimental group gave similar and reproducible results.

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hyperoxia compared with that in room air control animals, these levels were significantly less than those in animals treated with PBS and exposed to hyperoxia for 48 h (P < 0.05; Fig. 11).

Effect of LSF on lung injury and survival during hyperoxic exposure. Lung wet-to-dry weight ratios were significantly increased in animals exposed to 72 h of hyperoxia compared with control, unmanipulated animals (P < 0.05; Fig. 13). Treatment with LSF prevented the hyperoxia-induced increase in lung edema. Wet-to-dry lung weight ratios in mice that received LSF did not differ significantly from that in unmanipulated control animals not exposed to hyperoxia (Fig. 13). The effects of LSF on hyperoxia-associated mortality was examined by treating mice with either LSF or PBS during the first 72 h of hyperoxic exposure (Fig. 14). LSF treatment significantly prolonged survival (P < 0.02 by log-rank analysis).

Fig. 11. Effect of hyperoxia and LSF on serum levels of linoleic acid peroxide product HPODE in C, 48P, and 48L animals (n = 5–7/group). Both 48P and 48L groups had increased levels of HPODE compared with that in C animals (**P < 0.001 and *P < 0.05, respectively). 48L animals demonstrated significantly less serum HPODE compared with that in 48P animals (#P < 0.05).

Fig. 12. Effect of hyperoxia and LSF on serum levels of total diene chromophores in C, 48P, and 48L animals (n = 5–7/group). 48P animals had significantly higher serum total diene chromophores than C animals (**P < 0.001). Total serum diene chromophore level from 48L mice did not differ significantly from level present in C animals.

Fig. 13. Effect of hyperoxia on wet-to-dry lung weight ratios from C animals and animals exposed to hyperoxia for 72 h and treated with PBS (72P) or LSF (72L) (n = 6/group). 72P animals had significantly greater lung edema compared with that in C animals (***P < 0.005). Level in 72L mice did not differ significantly from that in C animals.

DISCUSSION

In the present experiments, treatment with LSF diminished the severity of hyperoxia-induced lung injury and improved survival. Although hyperoxia resulted in activation of the transcriptional regulatory factors NF-κB and CREB in lung cells, only CREB activity was decreased by LSF, suggesting that LSF may affect intracellular signaling pathways leading to CREB phosphorylation. Functional CRE sites are present in the promoter regions of proinflammatory cytokine genes, including TNF-α, IL-1β, and IL-6, and can modulate the transcription and expression of these cytokines. The ability of LSF to inhibit CREB activation may therefore be a mechanism by which this agent prevents proinflammatory cytokine expression and the development of lung injury during hyperoxic exposure.

ROS can enhance the activation of transcriptional factors such as NF-κB and CREB (4, 36, 39). This interaction between ROS and transcriptional factors may be due, in part, to the stimulatory effects of ROS on intracellular kinases or phosphatases that regulate
phosphorylation and downstream activation of transcription factors. In particular, ROS appear to contribute to the activation of NF-κB (4) through enhancing phosphorylation of serine residues Ser$^{32}$ and Ser$^{36}$ of the inhibitory peptide IκB-α via a specific IκB ubiquitination-dependent protein kinase (8). After phosphorylation, IκB-α is degraded and the transcriptionally active NF-κB heterodimer translocates to the nucleus (8). Kinases known to be affected by ROS and involved in the activation of CREB include p38 mitogen-activated protein kinase (41) and the extracellular signal-regulated kinases (ERK1 and ERK2) (30).

ERK1 and ERK2 have been shown to regulate early-response genes via signal transduction and activator of transcription (STAT) proteins (10) and to induce transcription by activating CREB through the phosphorylation of Ser$^{133}$ (30). LSF inhibits IL-12-driven signaling, transcription by activating CREB through the phosphorylation (STAT) proteins (10) and to induce transcription factors via signal transduction and activator of regulated kinases (ERK1 and ERK2) (30).

In a previous report (7), LSF did not affect TNF-α-induced activation of NF-κB in the macrophage cell line RAW 264.7. The present results are consistent with a lack of effect of LSF on NF-κB activation. Even though our experiments demonstrated that hyperoxia produced activation of NF-κB in lung cells, treatment with LSF did not modify this effect.

Exposure to hyperoxia and ROS produces lipid peroxidation (29) as demonstrated in the present study by increased circulating levels of HODE, HPODEs, and total diene chromophores after 48 h of hyperoxia. Oxidized linoleic acid derivatives are biologically active, inducing IL-1β release in human monocytes (26, 42), and serve as modulators in the mitogenic response induced by epidermal growth factor (15). In a previous study (40) that investigated patients undergoing allogenic bone marrow transplantation, LSF suppressed radiotherapy-induced increases in serum HODE and HPODE. The present results show similar inhibitory effects of LSF in preventing hyperoxia-associated generation of oxidized linoleic acid products, including HODE, HPODE, and total diene chromophores.

In vitro studies (3, 18) of endothelial cells demonstrated that increased lipid peroxidation induced by ROS or the addition of linoleic acid to cell cultures results in the activation of NF-κB. It is unknown at present whether oxidized lipids also induce activation of CREB, but if so, then the prevention of lipid oxidation by LSF could provide another mechanism for the LSF-associated inhibition of CREB phosphorylation found in the present experiments. However, the effects of oxidized lipids on CREB activation would have to be relatively greater than those affecting NF-κB to explain the observed ability of LSF to inhibit hyperoxia-induced activation of CREB, but not of NF-κB, in the lungs.

In the present experiments, LSF significantly decreased TNF-α, IL-1β, and IL-6 expression in the lungs over the first 48 h of hyperoxic exposure. However, even though the amounts of TNF-α and IL-6 protein from mice exposed to hyperoxia and treated with LSF were reduced compared with that in hyperoxia-exposed animals not treated with LSF, cytokine levels were still increased in the LSF-treated mice compared with those in the unmanipulated room air control animals. These results indicate that hyperoxia produces activation of signaling pathways that induce expression of proinflammatory cytokines that are not affected by the administered dose of LSF. It is also possible that hyperoxia activates other cytokine-inducing pathways that are not as responsive to LSF. Transcriptional mechanisms associated with NF-κB are possible candidates for this effect because NF-κB is important in inducing the transcription of multiple proinflammatory mediators, including TNF-α and IL-6, and remains activated in the lung during hyperoxia despite LSF treatment. Other transcriptional factors involved in proinflammatory cytokine regulation, including activator protein-1, are oxidant sensitive (37). The ability of LSF to modulate the activation state of transcriptional factors other than NF-κB and CREB after hyperoxic exposure is unknown. Cytokine protein production may also be affected by posttranscriptional events, and the effects of LSF on posttranscriptional regulation have not been examined.

Although LSF did significantly improve survival after hyperoxic exposure, all mice that received LSF during the first 72 h of hyperoxia still died by 140 h of exposure, demonstrating that the protection provided by LSF was incomplete. These survival results are consistent with the incomplete suppression of proinflammatory cytokine expression in the lungs achieved with LSF therapy with the present dose and schedule. Therefore, the present results, although encouraging in suggesting that LSF may have a therapeutic role in treating patients exposed to high concentrations of oxygen, do not indicate that LSF therapy alone will be entirely protective against the deleterious effects of hyperoxia. Combining LSF with other therapies able to protect cells from oxidant injury through mechanisms different from those of LSF or using LSF on a more intensive schedule might be expected to provide additional benefit.

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