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Quantitation of translatable (Cu^{2+},Zn^{2+})superoxide dismutase messenger-RNA in lungs of endotoxin-treated O_2-exposed rats

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The lung injury characteristically resulting from prolonged exposure of adult rats to hyperoxia is largely prevented by treatment with low doses of bacterial endotoxin [1]. Nearly all untreated rats die within 96 hr of >95% O_2 exposure, whereas over 95% of endotoxin-treated rats survive [2]. The increased tolerance to hyperoxia produced by endotoxin treatment is associated with elevated levels of lung antioxidant enzymes, including superoxide dismutase (SOD*) [3]. The heightened SOD activity in lungs of endotoxin-treated O_2-exposed rats is due to a greater number of (Cu^{2+},Zn^{2+})SOD molecules, which is in turn caused, at least in part, by a 50% elevation in the rate of (Cu^{2+},Zn^{2+})SOD synthesis [4]. The rate of total protein synthesis in the lung under these same conditions is increased 100%. The present study was undertaken to determine whether the molecular mechanism by which endotoxin stimulates the synthesis of (Cu^{2+},Zn^{2+})SOD and total protein leads to increased concentrations of the respective messenger-RNA species.

Materials and methods

Animals. Male Long–Evans rats weighing approximately 250 g were obtained from Charles River Breeding Laboratories and were maintained in the University of Miami Animal Care Facility, where they were allowed food and water *ad lib*.

Materials. The rabbit reticulocyte lysate translation system and oligo(dT) cellulose were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Aquasol and [3,4,5-^{3}H]leucine were obtained from New England Nuclear (Boston, MA). Endotoxin (*Salmonella typhimurium* lipopolysaccharide, phenol-water extraction) and all biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Soluene-350 was obtained from Packard Instruments (Downers Grove, IL).

Exposure conditions. Rats were exposed to hyperoxia in a 3.4 cu. ft. exposure chamber in which O_2 (>95%), CO_2 (<0.5%), temperature (22–25°) and humidity (60–80%) were monitored closely. Endotoxin was prepared in phosphate-buffered saline (pH 7.8) and injected intraperitoneally (500 μg/kg) at the beginning of the exposure period and again 24 hr later (250 μg/kg). Control rats were injected with an equal volume of phosphate-buffered saline.

Extraction of mRNA. Rats treated with saline or endotoxin, and exposed to either air or >95% O_2 for 72 hr, were killed by intraperitoneal injection of sodium pentobarbital (50 mg/kg), followed by exsanguination. The lungs were excised and perfused with phosphate-buffered saline to remove blood from the microvasculature. One lobe was saved for DNA assay [5]. The remainder of the lung was homogenized with a Brinkmann Instruments Polytron for 30 sec in a 4 M solution of guanidinium thiocyanate, and total RNA was extracted [6]. PolyA-mRNA was isolated by chromatography on oligo(dT) cellulose, precipitated with ethanol (−20°), and dissolved in sterile H_2O [7].

PolyA-mRNA was translated *in vitro* using a rabbit reticulocyte lysate containing [^{3}H]leucine for 60 min at 30°. To determine incorporation of [^{3}H]leucine into total protein, 200 μg of bovine serum albumin was added and protein was precipitated with 25% trichloroacetic acid (0°). The washed pellet was dissolved in 2 N sodium hydroxide, and a portion was counted in Aquasol. To determine incorporation into ($Cu,^{2+}$,Zn^{2+})SOD, 5 μg of pure rat liver (Cu^{2+},Zn^{2+})SOD was added to the translation mixture, and the enzyme was immunoprecipitated with monospecific goat anti-rat (Cu^{2+},Zn^{2+})SOD. The immunoprecipitate was denatured, subjected to electrophoresis on SDS-polyacrylamide gels, and radioactivity in (Cu^{2+},Zn^{2+})SOD was extracted from the gel [4].

Results and discussion

The reticulocyte-lysate incorporation of [^{3}H]leucine into protein was linear in the range 0.04 to 0.20 μg lung polyA-mRNA, and subsequent experiments utilized 0.15 μg polyA-mRNA. Optimal incorporation was found to occur at 1.2 mM Mg^{2+}, which was the Mg^{2+} concentration of the reticulocyte lysate, and at 0.2 M K^+.

Treatment of >95% O_2-exposed rats with endotoxin results in a (Cu^{2+},Zn^{2+})SOD synthesis rate 50% higher than that of saline-treated controls breathing air or >95% O_2 [4]. The level of lung polyA-mRNA encoding for (Cu^{2+},Zn^{2+})SOD, however, was not elevated significantly in these rats (Table 1), denying the hypothesis that the increased rate of (Cu^{2+},Zn^{2+})SOD synthesis is the result of enzyme induction. Similarly, the absence of an increase in total lung polyA-mRNA concentration (Table 2) when total protein synthesis was elevated 100% indicates that another molecular mechanism was responsible for the elevation in protein synthesis.

The proportion of (Cu^{2+},Zn^{2+})SOD polyA-mRNA in the lungs of the four experimental groups remained constant at 0.2% of the polyA-mRNA encoding for total lung protein. Translation of polyA-mRNA yielded (Cu^{2+},Zn^{2+})SOD of a subunit molecular weight which did not differ from that of the enzyme as isolated from rat lung (16,000), indicating that subsequent processing of the nascent enzyme by the adult lung likely did not occur (data not shown).

In endotoxin-treated O_2-exposed rats, lung RNA and DNA are increased compared to saline-treated controls,

Table 1. Levels of translatable lung (Cu^{2+},Zn^{2+})superoxide dismutase polyA-mRNA

Exposure (72 hr)	(Cu^{2+},Zn^{2+})superoxide dismutase mRNA	
	dpm × 10^{-3}/lung	dpm/μg DNA
Air–saline	187 ± 15 (3)	5.49 ± 1.35 (3)
Air–endotoxin	239 ± 39 (2)	7.00 ± 1.93 (2)
O_2–saline	153 ± 45 (3)	5.28 ± 1.54 (3)
O_2–endotoxin	178 ± 46 (3)	6.36 ± 1.16 (3)

Values represent dpm [^{3}H]leucine incorporated into (Cu^{2+},Zn^{2+})superoxide dismutase by rabbit reticulosyte lysate upon addition of the respective polyA-mRNA fraction isolated from the lungs of rats exposed to air or >95% O_2. Mean values ± SEM are shown, with the number of experiments in parentheses.

* Abbreviations: SOD, superoxide dismutase; SDS, sodium dodecyl sulfate

Table 2. Levels of translatable lung total protein polyA-mRNA

Exposure (72 hr)	Total protein mRNA	
	dpm × 10^{-6}/lung	dpm × 10^{-3}/μg DNA
Air–saline	74.7 ± 22.0 (3)	2.48 ± 0.46 (3)
Air–endotoxin	85.3 ± 3.5 (2)	2.88 ± 0.51 (2)
O_2–saline	77.2 ± 22.0 (3)	2.59 ± 0.56 (3)
O_2–endotoxin	77.2 ± 7.5 (3)	2.48 ± 0.27 (3)

Values represent dpm [^{3}H]leucine incorporated into total protein by rabbit reticulocyte lysate upon addition of the respective polyA-mRNA fraction isolated from the lungs of rats exposed to air or >95% O_2. Mean values ± SEM are shown, with the number of experiments in parentheses.

with a resulting increase in the RNA/DNA ratio [8]. The majority of cellular RNA was ribosomal RNA, and endotoxin-treatment prevented the O_2-induced dissociation of lung polyribosomes to their various subunits.* Endotoxin treatment may therefore lead to an increased rate of protein synthesis by increasing total lung RNA and hence total lung polyribosome concentration, thereby increasing the capacity of the lung for protein synthesis.

It remains possible that lung transcriptional rates are elevated shortly after the exposure to O_2 and endotoxin begins, but that low mRNA stability results in no discernable elevation after 72 hr of exposure. This possibility is considered unlikely, however, because lung protein synthesis was elevated only slightly within 24 hr of exposure to O_2 and endotoxin.†

In summary, the present experiments indicate that, despite increased rates of (Cu^{2+},Zn^{2+})SOD and total protein synthesis in lungs of endotoxin-treated O_2-exposed rats, there was no alteration in polyA-mRNA level for either (Cu^{2+},Zn^{2+})SOD or total protein. These observations suggest that specific induction of lung protein synthesis via increased transcriptional rates does not play an important role in this phenomenon, but rather suggests that endotoxin treatment increases lung protein synthetic capacity by a post-transcriptional mechanism, perhaps via elevated lung polyribosome concentration.

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* D. Fan and D. Massaro, unpublished observations.

† M. Hass and D, Massaro, submitted for publication.

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