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RAPID COMMUNICATION

PROTECTION AGAINST HYPEROXIA BY SERUM FROM ENDOTOXIN TREATED RATS: ABSENCE OF SUPEROXIDE DISMUTASE INDUCTION

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ABSTRACT. Endotoxin greatly reduces lung injury and pleural effusions in adult rats exposed to normobaric hyperoxia (> 98% oxygen for 60 hours). This study reports that serum from endotoxin treated donor rats protects serum recipients against hyperoxic lung injury without altering lung superoxide dismutase (SOD) activity. Rats pretreated with endotoxin alone were protected and exhibited an increase in lung SOD activity as previously reported by others. Protection by serum was not due to the transfer of residual endotoxin or SOD. These results show that protection from oxygen toxicity can occur in rats without an increase in lung SOD and suggest that a serum factor may be involved.

INTRODUCTION. Rats develop massive bilateral pleural effusions during the third day of exposure to normobaric hyperoxia (> 98% oxygen at 1 ATA). Their lungs are hemorrhagic and death typically occurs at 60 hours. In contrast, rats pretreated with endotoxin (1 mg/kg body weight, i.p.) exhibit only slight pleural effusions during the third day of hyperoxia. Their lungs appear normal and survival extends well beyond 60 hours (1-4).

The ability of endotoxin to protect rats from hyperoxia was first reported by Frank, Yam and Roberts (4). These investigators observed increased antioxidant enzyme activity (e.g., superoxide dismutase, SOD) in the lungs of endotoxin protected rats and proposed that the mechanism of protection involved antioxidant enzyme induction. According to this hypothesis potentially damaging free radicals are quenched by the increased antioxidant enzymes during hyperoxia and lung injury is prevented.

Interestingly, endotoxin alone, or sera from endotoxin treated mice, also prevents free radical injury during whole body irradiation (5,6). The similarity between radiation and oxygen toxicity (7) prompted us to examine the possibility that sera from endotoxin treated rats may protect recipients from oxygen toxicity.

MATERIALS and METHODS. Animals: Male Sprague-Dawley rats (200-350 g) were obtained from Simonsen (Gilroy, CA.) and maintained on standard laboratory chow and water ad libitum. Rats were acclimatized to the laboratory for at least 10 days prior to use.

Serum collection: Colonic temperatures and body weights were recorded from serum donor rats immediately prior to vehicle (1 ml sterile 0.9% NaCl, i.p.) or endotoxin administration (E. coli 055:B5, Boivin extraction, Difco, 1 mg/kg body weight, i.p.). Donor rats were sedated lightly with Fluothane (Fort Dodge Laboratories, Fort Dodge,

Iowa) at the time of saline or endotoxin administration and three hours later when sodium pentobarbitol (Veterinary Laboratories, Lenexa, Kansas) was used as a general anesthetic (45 mg/kg body weight, i.p.) during blood collection. Unheparinized blood was collected from the abdominal aorta and clotted on ice, with occasional stirring, for 75 minutes. Serum was harvested by centrifugation (4,ooo g's for 10 minutes), pooled, and immediately injected via tail vein into recipients. Selected samples of pooled serum were frozen at -20 ^{o}C for later measurement of residual endotoxin.

Serum administration and oxygen exposure: Serum recipients received fresh serum (15 ml/kg body weight, i.v.) via a lateral tail vein immediately prior to oxygen exposure. Other rats received either sterile saline (1 ml, i.p.), endotoxin dissolved in saline (1 mg/kg body weight, i.p.), or low-dose (residual) endotoxin dissolved in saline (15 ml saline/kg body weight at a concentration of 2, 4, 6, or 8 µgrams endotoxin/ml). Rats were housed individually in a plexiglass chamber during exposure. Oxygen was maintained at a concentration > 98% at 1 ATA (sea level) and CO_2 levels were below 0.25% as measured by mass spectrometry (Perkin-Elmer, Model 1100). All rats were fasted during the exposure period but each had access to water. Pretreatment and exposure of air/saline control rats was the same as for the oxygen exposed groups except that compressed air replaced pure oxygen.

Determination of total lung SOD activity: Rats were removed from the oxygen chamber following the 60 hour exposure period, anesthesized as above and exsanguinated. Their lungs were removed, rinsed, trimmed and blotted. The right lobe was weighed and homogenized in iced 0.017 M phosphate buffered saline (pH = 7.8, 10:1 V:W) using a polytron homogenizer (Polyscience Corp., Niles, Il.). The homogenate was then frozen (-20 ^{o}C) and stored overnight prior to further treatment. Lung homogenates were freeze/thawed twice in a dry ice/acetone bath, centrifuged at 4,000 g's for 10 minutes, and the supernatant assayed immediately for total SOD activity using the standard ferricytochrome c assay (8). The reaction mixture for this assay consisted of 100 µM EDTA, 10 µM cytochrome c, and 50 µM xanthine dissolved in 0.05 M phosphate buffered saline (pH = 7.8). At these concentrations the addition of 0.01 units xanthine oxidase to 3 ml reaction mixture produced a rate of change at 550 nanometers equal to 0.025 absorbance units/minute (Beckman spectrophotometer, Model 35). SOD activity in the sample was determined by finding the volume of homogenate (usually 7-20 µL) which produced a 50% reduction in reaction rate (calculated as the slope of the line of best fit) from 1 1/2 to 4 minutes following the addition of xanthine oxidase; this volume contained one unit of SOD by definition (8). The 1 1/2 to 4 minute time period was chosen because of the presence of oxidase activity during the first 20 seconds following the addition of lung homogenates to the reaction mixture. These changes were slight (about 10% of the absorbance change produced by xanthine oxidase alone over 4 minutes) and were of similar magnitude in samples from the five different groups. Because of these similarities, and the linearity of the absorbance change during the 1 1/2 to 4 minute time period, the method of calculation is believed to accurately reflect changes in SOD activity between samples. Protein concentration was measured in lung homogenates using a hand-held refractometer (Atago Co. Ltd., Japan) standardized with albumin. SOD activity was expressed as units SOD/gram lung homogenate protein or as units SOD/right lung.

At the reactant concentrations used 1 unit of SOD activity in lung homogenates was found to equal 1 unit of activity in Sigma SOD (Sigma Chemical Co., St. Louis, Mo.). Boiling abolished SOD activity in both homogenized lung tissue and Sigma SOD.

TABLE I. Parameters showing protection of fasted adult rats against hyperoxia by endotoxin or by serum from donors treated with endotoxin (ES) 3 hours prior to serum collection[a].

Group	60 hour survival	Changes in body temp. (°C)[b]	Pleural effusion vol. (ml/kg)	Hematocrit (%)
AIR/SALINE (i.p.)	100% 5/5	-0.3 ± 0.3[c] (5)	0.2 ± 0.06[c] (5)	44.9 ± 0.5[c] (5)
OXYGEN				
Endotoxin (i.p.)	100% 14/14	-0.9 ± 0.2[c] (14)	1.7 ± 1.0[c] (14)	45.4 ± 1.1[c] (14)
ES[d] (i.v.)	100% 14/14	-1.0 ± 0.2[c] (14)	1.7 ± 0.4[c] (14)	48.3 ± 0.9[c] (13)
Serum (i.v.)	77.8% 14/18	-4.2 ± 0.3 (16)	35.8 ± 1.7 (18)	70.4 ± 2.7 (16)
Saline (i.p.)	74.1% 20/27	-4.9 ± 0.3 (21)	35.6 ± 1.1 (25)	68.1 ± 1.8 (21)
Residual Endotoxin[e] (i.v.)	100% 4/4	-4.2 ± 0.5 (4)	30.9 ± 3.8 (4)	63.1 ± 2.8 (4)

[a] All values are means ± SEM and were obtained from rats surviving the 60 hour exposure period or at the time of death. Numbers in parenthesis indicate the number of rats.

[b] Post-oxygen exposure colonic temperature minus pre-oxygen exposure colonic temperature.

[c] Differences from comparable values for Serum, Saline, or Residual Endotoxin groups are significant at $p < 0.005$ by the unpaired, group t-test.

[d] Group receiving serum from donors treated with endotoxin (1 mg/kg body weight) at a dose of 15 ml serum/kg body weight.

[e] Group receiving endotoxin dissolved in saline (2, 4, 6, or 8 µg/ml) at a dose of 15 ml saline/kg body weight.

Freeze/thawing did not affect the activity of Sigma SOD but did produce a slight (about 10%) increase in lung homogenate SOD activity. This increase in SOD activity probably resulted from a greater release of MnSOD by mitochondria during freeze/thaw induced cell lysis.

Residual endotoxin in serum: Endotoxin levels in serum were determined using the limulus amebocyte lysate (LAL) assay (E-toxate research kit, Sigma Chemical Co.). Prior to performing the endotoxin assay possible LAL inhibitor(s) in serum samples were inactivated by dilution-heating (9).

RESULTS. Serum from rats injected with endotoxin 3 hours prior to serum collection protected recipients from oxygen toxicity (Table I, ES group). Treatment with endotoxin alone was also

TABLE II. Superoxide dismutase (SOD) activity in lung homogenates from air and oxygen-exposed rats[a].

Group	Units SOD / Right lung	Units SOD / gram protein
AIR/SALINE	394 ± 44 (5)	7,127 ± 823 (5)
OXYGEN		
Endotoxin	734 ± 60[b] (12)	11,450 ± 840[c] (12)
ES	423 ± 24 (8)	7,228 ± 371 (8)
Serum	513 ± 41 (11)	7,925 ± 750 (11)
Saline	517 ± 31 (13)	7,299 ± 482 (13)

[a]All values are means ± SEM and represent the same groups as in Table I. Numbers in parenthesis indicate the number of rats.

[b]Differences compared to the other groups in the column are significant at $p < 0.001$ by the unpaired, group t-test.

[c]Differences compared to the other groups in the column are significant at $p < 0.005$ by the unpaired, group t-test.

protective. Residual endotoxin levels in ES serum (administered i.v.) were 1.3 ± 0.3 µgrams/ml (mean ± SEM, n = 4). This level of endotoxin was not protective when administered i.v. at a dose equal to (or up to 6 fold greater than) the total amount introduced with protective sera (Table I). Protection was not due to a transfer of SOD since SOD activity was not detected in protective sera by our assay.

The lungs of ES recipients, although protected from oxygen toxicity, did not exhibit SOD induction. Lung SOD activity for protected ES rats did not differ significantly from values for unprotected Saline or Serum control rats (Table II). Endotoxin alone (i.p.) did induce SOD during hyperoxia reconfirming work by others (2-4). The observed lack of SOD induction in the protected ES group was not a lung edema or homogenate protein artifact because post-exposure right lung weights and homogenate protein values for ES and endotoxin rats did not differ significantly ($p > 0.05$).

DISCUSSION. This study reports that serum from endotoxin treated rats protects recipients from oxygen toxicity without SOD induction. Previously, Block et al. (10) reported that endotoxin prevents oxygen toxicity in cultured endothelial cells without SOD induction. Phan et al. (11) have also found that SOD induction does not occur during endotoxin protection from possible free radical injury during bleomycin-induced pulmonary fibrosis.

Recently, White et al. (12) protected rats from hyperoxia by combined treatment with tumor necrosis factor/cachectin and interleukin 1 (TNF/C + Il 1) and again observed protection without increases in lung SOD activity. The results of this study are supported by the work of these investigators and suggest that the role of SOD induction in endotoxin protection from oxygen toxicity may require reevaluation.

The observation that serum from endotoxin treated rats prevents hyperoxic lung injury supports the hypothesis that cells of the reticuloendothelial system (RES) play a role in endotoxin protection from oxygen toxicity. Earlier work in our laboratory (1) suggesting that endotoxin lessens the ability of phagocytes to release free radicals in vivo, also supports a possible RES involvement.

Work by others on endotoxin protection from whole body irradiation may provide insights into the identity of possible serum mediators. Behling et al. (6) detected high levels of colony stimulating factor (CSF) in radioprotective sera and proposed that this RES secretory product may confer protection. Addison and Berry (5) have confirmed that endotoxin-triggered sera is radioprotective but have questioned a central role for CSF. Work showing that Il 1 is radioprotective (13) suggests that this cytokine may be involved. The recent report by White et al. (12) further strengthens a putative role for Il 1 in protection from hyperoxia although, interestingly, Il 1 did not provide protection unless given with TNF/C. These reports, and the results of this study, support the hypothesis that endotoxin may generate a RES secretory factor(s) which confers protection from oxygen toxicity. Whether this factor(s) is CSF, Il 1, TNF/C, or a different (possibly unidentified) RES secretory product remains to be determined.

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