Superoxide dismutase responds to hyperoxia in rat hippocampus.

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Freiberger J, Coulombe K, Suliman H, Carraway M, Piantadosi C.  Superoxide dismutase responds to hyperoxia in rat hippocampus.  Undersea Hyperb Med 2004; (31)2:227-232. The brain’s anti-oxidant response to highly elevated oxygen (O2) partial pressures is poorly understood. In this study we hypothesized that hyperbaric O2 (HBO2) would stimulate superoxide dismutase (SOD) transcription in the oxidative stress-sensitive rat hippocampus and measured the time course and extent of the changes in hippocampal mRNA for all three SOD isoforms and total SOD enzyme activity.  Comparisons were made between exposures to 2 hours of 1 atmosphere pressure normobaric oxygen (NBO); 2 hours of 3 atmospheres HBO2; and room air.  Hyperoxia (HBO2 > NBO) was associated with statistically significant increases in transcript levels of the antioxidant enzymes SOD2 (MnSOD) and SOD3 (EC-SOD) at 6 and 18 hours but not SOD1 (Cu, Zn SOD) respectively. Hyperoxia, however, did not affect total hippocampal SOD activity measured at 6 and 24 hours, indicating that the mRNA responses were necessary to maintain the anti-oxidant enzyme activity after oxidative stress.

INTRODUCTION

Hyperoxia is well known to stimulate the production of anti-oxidant enzymes in several tissues including the lung (5, 10), but the brain’s anti-oxidant response to elevated O2 partial pressures is less well characterized. Superoxide dismutase (SOD) is a critical antioxidant enzyme that catalyses the dismutation of superoxide radical to molecular O2 and hydrogen peroxide (H2O2). SOD occurs in three distinct isoforms, SOD1 or copper-zinc superoxide dismutase (Cu,ZnSOD), SOD2 or manganese superoxide dismutase (MnSOD), and SOD3 or extracellular superoxide dismutase (EC-SOD). These enzymes form an important part of the cellular antioxidant defense mechanism. Hyperbaric hyperoxia (HBO2) has been reported to up-regulate superoxide dismutase (SOD) when used in doses shown to precondition the brain to resist subsequent ischemia (14, 15), but this effect has not been found in all HBO2 neuro-preconditioning models (9). In addition, where SOD up-regulation has been demonstrated, the time course and isoenzyme patterns have not been carefully defined. To address this issue we measured changes in mRNA expression for SOD1 (Cu,Zn SOD), SOD2 (MnSOD) and SOD3 (EC-SOD) as well as total SOD enzyme activity after hyperoxia in the oxidant-sensitive rat hippocampus.

METHODS

We exposed rats to hyperoxia as single treatments of 2 hours duration of either NBO at 1 atmosphere absolute pressure (atm abs) or HBO2 at 3 atm abs. To define the effects, the time
profile of changes was followed over a 36 hour period for SOD mRNA and over a 24 hour period for total SOD activity. The timing of the enzyme activity measurements was chosen to correspond to the estimated time of peak SOD translation based on the transcription peaks observed in the first section of the experiment.

All animal protocols were carried out in accordance with the National Institutes of Health guidelines for the use of laboratory animals and approved by the Duke University Animal Care and Use Committee. Forty adult male SD rats (300-400 g) were randomly assigned to one of three groups: air, NBO or HBO2. Animals from the air group were handled and marked in the same manner as the hyperoxia-exposed animals. These animals (n=8) served as controls for all three isoenzyme comparisons. The hyperoxia groups each received two hour exposures of 100% O2 at either 1.0 atm abs (101.1 kPa) or 3.0 atm abs (303.3 kPa). Four animals from each hyperoxia group were euthanized at 6, 18, 24 and 36 hours after the onset of the NBO or HBO2 to measure any change in SOD mRNA or total SOD enzyme activity that might occur over time. The 36 hour measurement was made in order to exclude a late or secondary response that was not apparent in the expected 24 hour response window after HBO2 exposure. At the appropriate times, anesthesia was induced with 5% Halothane in air and a catheter inserted into the left ventricle through which the brain was flushed with 0.9% NaCl at room temperature. The brains were immediately removed, placed on ice and the subcortical (hippocampal) tissue extracted by sharp dissection. Tissue samples were snap frozen in liquid nitrogen and stored at – 80°C. Total time from induction of anesthesia to sample freezing was approximately 3 minutes.

The method of semi-quantitative reverse transcriptase polymerase chain reaction for analysis of the three SOD isoforms was as follows. RNA was extracted with TRIzol® Total RNA isolation kit (GIBCO BRL, Gaithersburg, Maryland, USA) and RNA concentration determined optically at 260 nm. One µg of RNA from each sample was reverse-transcribed into cDNA, using oligo (dT) as a primer. PCR amplification was carried out in a thermal cycler for 25 cycles for glyceraldehyde phosphate dehydrogenase (GAPDH), 27 cycles for rat Cu,ZnSOD, and 25 cycles for MnSOD and EC-SOD. The number of cycles was determined by titration to achieve visible products on ethidium bromide stained gels during the exponential phase of the PCR. The cycling parameters were: denaturation at 95°C for 30 s, annealing at 56°C for GAPDH and at 59°C for Cu,ZnSOD, MnSOD and EC-SOD for 45 s, and extension at 72°C for 45 s. Primers specific for rat GAPDH, Cu,ZnSOD, MnSOD and EC-SOD were used (Table 1) The amplified products (10 µl) were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and viewed under ultraviolet light. Quantification of the amplification product was done by densitometry of ethidium bromide stained gels using ImageQuant (Version 1.1; Molecular Dynamics, Cambridge, Massachusetts, USA). The GAPDH signals were used to control for variation in the efficiency of RNA extraction, RT, and PCR.

Table 1. Primer sequences for glyceraldehyde phosphate dehydrogenase (GAPDH), SOD1 (Cu,Zn SOD), SOD2 (MnSOD), and SOD3 (EC-SOD)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5′-AACCTTTGCCATGGTGGAAAGG-3′</td>
<td>5′-ACACATTGGGGGTAGGAACA-3′</td>
</tr>
<tr>
<td>CuZn (SOD1)</td>
<td>5′-CGGATGAAGAGACGAGCA TGT-3′</td>
<td>5′-CAATCAGACCCACACCGAAG-3′</td>
</tr>
<tr>
<td>MnSOD (SOD2)</td>
<td>5′-CAGTGTGGCTAGCTTTGTG-3′</td>
<td>5′-CAAAGCAATTCAAGCTCT-3′</td>
</tr>
<tr>
<td>ECSOD (SOD3)</td>
<td>5′-GACCTTGGAGATCTGGAGAAG-3′</td>
<td>5′-GTGGTTGAAGGTGTTCTGCT-3′</td>
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In a separate group of 20 rats (4 controls, 8 HBO2, 8 NBO) total SOD activity per gram of hippocampal tissue was measured at 6 and 24 hours. All rats were exposed and tissue
Harvested as described for the PCR analysis above. Hippocampal tissue samples were weighed and homogenized in 10 volumes of ice-cold 50 mM potassium phosphate buffer (pH 7.4) with 0.3 M KBr, and a set of antiproteolytic agents (0.5 mM phenyl methylsulfonyl fluoride, 3 mM diethylentriaminepentaacetic acid, 90 mg of aprotinin l\(^{-1}\), 10 mg of pepstatin l\(^{-1}\), 10 mg of chymostatin l\(^{-1}\), and 10 mg of leupeptin l\(^{-1}\)). The homogenate was sonicated and SOD activity assayed by measuring the decrease in the rate of cytochrome c reduction in a xanthine / xanthine oxidase superoxide generating system using the method of Crapo, et al (1). In this assay one unit of SOD activity is the amount of enzyme required to produce 50% inhibition of the rate of cytochrome c reduction at the conditions specified (pH 7.8, 25 °C, at 0.025 OD min\(^{-1}\) rate of increase in absorbance, at 550 nm).

To analyze the results, mRNA transcription for the three SOD isoforms was expressed as the fold change compared to air control. Total SOD activity was expressed in units x mg\(^{-1}\) protein. For mRNA expression a multivariate analysis of variance was performed with time and treatment as the main effects. Because the time = 0 group served as the pre-treatment air control, a priori orthogonal contrasts between time = 0 and the four later time periods were done. The contrasts compared time = 0 (pre-treatment air control) to each post-treatment time group based on the null hypothesis that the values before O\(_2\) exposure (time = 0) would not be significantly different from those after treatment (time = 6, 18, 24 and 36 hours). This was confirmed in pairwise comparisons using Tukey’s post hoc HSD. The single dependent variable, total SOD activity, was compared in a similar fashion using a two-way analysis of variance also with time and treatment as the main effects. All analysis was done using SPSS v 8.0 for Microsoft Windows and p values of < .05 were considered statistically significant.

RESULTS

Figures 1-3 show the fold increase for mRNA expression of each SOD isoform over the 36 hour interval categorized by treatment group (HBO\(_2\) or NBO). We found significant increases in MnSOD (p = .03) and EC-SOD (p = .02) expression after both NBO and HBO\(_2\) exposure. Although the enzymes’ mRNA expression during HBO\(_2\) tended to be higher earlier, the only statistically significant difference between NBO and HBO\(_2\) treatments was for MnSOD at 6 and 18 hours (HBO\(_2\) > NBO p=.03). MnSOD mRNA transcripts showed an early response to HBO\(_2\) that peaked at a 4 fold increase after 18 hours and then declined. HBO\(_2\) exposure induced increased mRNA expression for EC-SOD compared to other SOD isoenzymes at 6 and 18 hours. Neither HBO\(_2\) nor NBO significantly changed Cu,Zn SOD mRNA expression (p= .183).

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**Fig. 1.** Fold Increase in MnSOD mRNA compared to GAPDH over 36 hours after NBO or HBO\(_2\), measured by semi-quantitative reverse transcriptase polymerase chain reaction. Values are presented as means ± SEM for the 4 rats in each group. * p< .05 for mean difference in comparison with time = 0. **p< .05 for mean difference in comparison with time = 0 and between HBO\(_2\) and NBO groups.
Fig. 2. Fold Increase in EC-SOD mRNA compared to GAPDH over 36 hours after NBO or HBO2, measured by semi-quantitative reverse transcriptase polymerase chain reaction. Values are presented as means ± SEM for the 4 rats in each group. * p< .05 for mean difference in comparison with time = 0 (Note that the 36 hour period near statistical significance with p=.058).

Fig. 3. Fold Increase in Cu,Zn SOD mRNA compared to GAPDH over 36 hours after NBO or HBO2, measured by semi-quantitative reverse transcriptase polymerase chain reaction. Values are presented as means ± SEM for the 4 rats in each group.

Figure 4 shows the mean enzyme activity of the HBO2 and NBO treatment groups at the two time points that were measured (6 and 24 hours). After these single hyperoxia exposures, total SOD enzyme activity did not change. Neither treatment (HBO2 or NBO), time after treatment (6, 24 hours), nor their interaction were significant predictors of total SOD activity.

Fig 4. Total SOD activity in units mg⁻¹. There was no statistically significant effect of either time or treatment

DISCUSSION

In this study we have shown that a brief exposure to elevated O₂ partial pressures can induce MnSOD and EC-SOD gene expression in the rat brain. The study also suggests, but does not conclusively show, that this effect is dose related for MnSOD and EC-SOD with a greater response to the higher O₂ partial pressure. These findings support the existing knowledge that increases in antioxidant enzyme expression may be a mechanism for ischemia tolerance in preconditioning models (2, 15, 18). HBO2 has previously been shown to increase immunoreactive MnSOD at 24 hours (15), but data at earlier time points has not been available. In this study anti-oxidant gene expression was detected as soon as 6 hours after the O₂ stimulus and it lasted more than 24 hours for MnSOD and EC-SOD. In addition, mRNA expression of MnSOD did not peak for 18 hours
or more. This late peak is compatible with the so-called “second window” phenomena observed with ischemic preconditioning (19).

In our study neither HBO2 nor NBO increased total anti-oxidant enzyme activity above that of control. This finding agrees with previous whole forebrain SOD measurements by Harabin (4) and raises the possibility that in addition to transcriptional activation, translational and/or post-translational regulation of SOD expression regulates brain SOD activity after O2 exposure. Another possibility involves enhanced generation of oxidants such as H2O2 in the brain under hyperoxic conditions. H2O2 has been shown to inactivate SOD through modification of the active site of the enzyme (11) and estimated brain H2O2 concentrations during HBO2 are as much as 2-7 times normoxic values (8).

The time course of changes in hippocampal catalase and glutathione peroxidase (GPx), the major scavengers of H2O2, were not measured in this study. However, different reports have shown that hyperoxia can decrease or delay the increase of the activity of catalase and GPx in brain tissues (7, 12). Furthermore, in our non-selective SOD assay system up to 90% of the enzyme activity can be ascribed to Cu,ZnSOD. This means a lack of a significant change in total SOD activity may be reflecting the stability of Cu,ZnSOD mRNA. Although MnSOD and EC-SOD mRNA were found to be upregulated by exposure to O2, their activities may not have increased sufficiently to influence total SOD activity. Alternatively, inactivation of these proteins, e.g. by oxidation or proteolysis could explain increased transcriptional activity without increased enzymatic activity. These possibilities will require more sensitive enzyme assay methods suitable for evaluation of very small tissue samples.

The main importance of these observations involves phenomena of HBO2 preconditioning. HBO2 has been reported to precondition neurological tissues to resist subsequent ischemia, e.g. as reported by Wada in gerbil CA1 hippocampal neurons (13) and confirmed by Ward, Prass and Dong (3, 9, 16). In both HBO2 and other preconditioning models the protective effect is theorized to require reactive oxygen species (ROS) generated during the preconditioning stimulus that incite a subsequent burst of protective anti-oxidant enzyme synthesis. Administration of ROS scavengers before preconditioning consistently inhibits this effect (3, 6, 14, 15, 17). Because HBO2 is relatively free of dangerous side effects, HBO2 preconditioning may have important applications where neurological ischemia is anticipated as in surgery of the thoracic aorta or during cardiopulmonary bypass. However, the optimal dose and time interval between the preconditioning stimulus and the subsequent ischemic insult are unknown, and augmentation of SOD transcription is likely only one part of the overall cell signaling process put into motion by HBO2. The stable enzyme activity at 6 and 24 hours in these study periods may or may not accurately reflect the fate or the biological importance of the SOD that was translated. As mentioned above, HBO2 induced generation of H2O2 could rapidly inactivate SOD making enhanced SOD activity less likely to be found in vitro. Finally, the effect of multiple HBO2 treatments, such as in a clinical setting, may influence the regulation of anti-oxidant enzymes in an as yet unknown manner.

In summary, our results show that the three SOD isoforms respond differently in the hippocampus to the oxidative challenges of HBO2 and NBO. Further studies are needed to correlate this response to hyperoxia preconditioning-induced neuroprotection.

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REFERENCES


