Effect of Anesthesia and Hypoxia - Reoxygenation on Xanthine Oxidase Levels in Mouse Brain and Plasma

Nail M. Hasan

Department of Basic Sciences, College of Medicine, King Saud Bin Abdulaziz University for Health Sciences, Riyadh 11426, Saudi Arabia

Abstract: The conversion of xanthine dehydrogenase to the free radical producing xanthine oxidase was investigated in mouse brain and plasma after exposure of mice to low oxygen concentration (10%) followed by reperfusion (100% oxygen) under different anesthetic conditions. Results showed that there was no significant change in XO/XD levels in tissues tested after hypoxia/reoxygenation treatment of whole mice or dissected brain slices. Only mice exposed to anesthetics in 10% or 100% oxygen have shown changes in XO/XD ratios. Intravenous injection of sodium pentobarbital was also shown to induce changes in plasma XO/XD ratios. These results indicate that interference of anesthesia with xanthine oxidase need to be taken into consideration when investigating ischemia reperfusion injury.

Key words: Xanthine Oxidase • Anesthesia • Hypoxia and Reoxygenation

INTRODUCTION

Ischemia - reperfusion is an important clinical condition that could cause tissue damage under many conditions such as stroke, myocardial infarction and cardiopulmonary bypass, intestinal ischemia, surface air after deep diving (decompression sickness), kidney and heart grafting and many other conditions.

There is a great deal of evidence supporting the role of XO-derived reactive oxygen species and beneficial effects of XO inhibitors against ischemic-reperfusion injury of the heart, brain, intestine, liver, kidney, lung and other tissues [1-4] that has emerged after the introduction of the concept of ischemia-reperfusion injury [5, 6]. It is widely believed that the production of oxygen free radicals by the increasing xanthine oxidase levels due to the conversion of xanthine dehydrogenase to the oxidase form is one of the major causes that lead to various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases and chronic heart failure [7-9].

Xanthine oxidoreductase is distributed throughout various organs including liver, gut, lung, kidney, heart and brain as well as blood plasma. The enzyme is normally present \textit{in vivo} as an NAD-dependent cytosolic dehydrogenase (XDH), incapable of producing oxygen radicals. It is accepted that XDH activity converts by sulfhydryl oxidation or limited proteolysis to an oxidase that produces superoxide and hydrogen peroxide. It is worth noting that both XO and XDH can oxidize NADH, with the concomitant formation of reactive oxygen species [10, 11]. Agents like lipopolysaccharides and hypoxia are known to regulate the expression of XO and XD by pre- and posttranslational modifications (i.e. phosphorylation) [12]. It is noteworthy to mention that there are differences between the XDH/XO system of various experimental animals and the XDH/XO system present in humans [13-15]. Nevertheless, both the conversion of XDH to XO and the presence of circulating XO have been also been confirmed in human experiments.

The use of hyperbaric oxygen is used for delivering increased oxygen dissolved in plasma to body tissues. Hyperbaric oxygen therapy (HBOT) is a form of treatment in which a patient breathes 100% oxygen at higher than normal atmospheric pressure that is greater than 1 atmosphere absolute (ATA). This type of therapy is given in special therapeutic chambers, which were earlier used primarily to treat of deep sea divers [16-18].

Brain tissue was mainly chosen in this investigation because of our interest in possible effects on the nervous system in divers suffering from decompression sickness.
and the damage which could be caused by free radical production by the xanthine oxidase system upon hyperbaric oxygen therapy.

**MATERIALS AND METHODS**

**Hypoxia / Reoxygenation:** 3 months-old BALB/c mice in the usual cages (5 mice each) containing water/food were put in a plastic box that has an inlet-outlet system and was tightly sealed. The box was flushed with the required oxygen concentration at room temperature. The flow rate of 300 ml/minute was adjusted so that a tube from the outlet inserted in a beaker full of water resulted in continuous but slow bubbling. Mice were under 12 hours light and 12 hours dark schedule at all times.

**Sample Preparation:** Mice were decapitated by guillotine after each experiment. Brain tissue was extracted immediately and put on ice for processing. Blood was collected and plasma prepared for analysis.

All exposures and treatment of mice including the decapitation procedure were approved by the local ethical committee.

**Xanthine Oxidase Assay:** 1 g of brain tissue was homogenized in 2ml of 50mM phosphate buffer, pH 7.4 containing 10 mM of freshly prepared dithiothreitol, 0.1 mM EDTA and 0.2mM phenylmethylsulfonyl fluoride (PMSF) diluted before use from a 100 mM stock solution. The homogenate was centrifuged and the supernatant passed through a prepacked 9 X 2 cm Sephadex G-25 column (Pharmacia) to remove any endogenous low molecular weight inhibitors. The activity of XO/XD was assayed by a fluorimetric method [19] using SLM-8000 fluorimeter set to 345 nm excitation and 390 nm emission. Mouse brain slices obtained by decapitation of mice and slicing the excised brain were incubated in artificial cerebrospinal fluid at 37°C and exposed to 95% N₂, 5% CO₂. An increase in XO ratio was not observed after hypoxic exposure of up to 6 hrs. Nor did reperfusion of hypoxic slices with 95% O₂, 5% CO₂ produced any change in XO/XD ratio.

**RESULTS**

XO activity in untreated mouse brain tissue was found to be 25% of total XO and XD present. The measured combined activity in the brain tissue was 0.1 µM/g/ 10 min. Exposure of mice to hypoxia (10% oxygen, 90% N₂) up to 48 hrs, 100% Oxygen up to 3 days, 95% O₂, 5% CO₂ for up to 3 days, 10% O₂ followed by 95% O₂, 5% CO₂ had no significant effect on the conversion of XD to XO in any of these conditions as shown in Table 1. The determined enzyme ratio being within the limits of experimental error, as for untreated mice breathing atmospheric air. The total enzyme activity also remained constant.

Mouse brain slices obtained by decapitation of mice and slicing the excised brain were incubated in artificial cerebrospinal fluid at 37°C and exposed to 95% N₂, 5% CO₂. An increase in XO ratio was not observed after hypoxic exposure of up to 6 hrs. Nor did reperfusion of hypoxic slices with 95% O₂, 5% CO₂ produced any change in XO/XD ratio.

XO activity increased in mice brain tissue when mice were killed by inhalation of CO₂ and the carcass of whole mice incubated at 37°C. XO activity increased from 25% to 40% after 1 hour of death and remained the same up to 4 hours.

XO (and XD) activity was also measured in mouse plasma. Exposure of mice to 10% and 100% O₂ resulted in changes of plasma and XO ratios. Mice exposed to 2% halothane in 100% or 10%O₂ showed changes in the

---

**Fig. 1:** A typical trace showing the measurement of XO/XD activity

1. Enzyme solution alone, 2. Pterin (10µl) added to measure XO activity, 3. Methylene blue (10µM) added to terminate the reaction and 5. Isoxanthopterin (0.05uM) added as internal standard
Table 1: Effect of different oxygen concentrations on mouse brain XO ratio

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hrs)</th>
<th>% XO (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0</td>
<td>24±1.98</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>23±1.20</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>25±0.89</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>24±1.25</td>
</tr>
<tr>
<td>100% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>24</td>
<td>23±1.34</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>26±0.98</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>25±0.83</td>
</tr>
<tr>
<td>10% followed by 100%</td>
<td>24 hrs followed by 24 hrs</td>
<td>26±1.87</td>
</tr>
</tbody>
</table>

Table 2: Effect of 2% halothane on brain and plasma XO levels in mice breathing 10% or 100% O<sub>2</sub> (%XO = XO/XO+XD, total activity is expressed in µmoles of Isoxanthopterin produced/g or ml/10min). Number of animals for each determination is 5

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Brain XO%</th>
<th>Total activity</th>
<th>Plasma* XO%</th>
<th>Total activity</th>
<th>Brain XO%</th>
<th>Total activity</th>
<th>Plasma* XO%</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24±1.1</td>
<td>0.09±0.01</td>
<td>47.2±1.6</td>
<td>0.074±0.006</td>
<td>24.1±1.1</td>
<td>0.09±0.01</td>
<td>47.2±1.6</td>
<td>0.074±0.006</td>
</tr>
<tr>
<td>0.5</td>
<td>39.0±1.6</td>
<td>1.07±0.02</td>
<td>46.0±1.8</td>
<td>0.098±0.012</td>
<td>22.4±2.5</td>
<td>0.12±0.02</td>
<td>32.5±1.7</td>
<td>0.122±0.02</td>
</tr>
<tr>
<td>1.0</td>
<td>36.3±3.9</td>
<td>0.07±0.01</td>
<td>41.4±0.9</td>
<td>0.067±0.007</td>
<td>23.1±2.0</td>
<td>0.085±0.007</td>
<td>25.7±0.8</td>
<td>0.117±0.009</td>
</tr>
<tr>
<td>1.5</td>
<td>24.1±2.0</td>
<td>0.22±0.02</td>
<td>35.5±3.5</td>
<td>0.147±0.013</td>
<td>P&lt;0.874</td>
<td>P&lt;0.411</td>
<td>P&lt;0.001</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>2.0</td>
<td>21.0±2.2</td>
<td>0.088±0.02</td>
<td>34.5±4.0</td>
<td>0.084±0.012</td>
<td>P&gt;0.001</td>
<td>P&gt;0.001</td>
<td>P&gt;0.001</td>
<td>P&gt;0.005</td>
</tr>
</tbody>
</table>

Table 3: Effect of 2% isoflurane on brain and plasma xanthine oxidase levels in mice breathing 10% or 100% O<sub>2</sub> (%XO = XO/XO+XD, total activity is expressed in µmoles of Isoxanthopterin produced/g or ml/10min)

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Plasma XO</th>
<th>Total activity</th>
<th>Plasma XO%</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>47.2±1.6</td>
<td>0.074±0.006</td>
<td>47.2±1.6</td>
<td>0.074±0.006</td>
</tr>
<tr>
<td>0.5</td>
<td>41.2±2.14</td>
<td>0.12±0.02</td>
<td>36.4±1.82</td>
<td>0.12±0.009</td>
</tr>
<tr>
<td>1.0</td>
<td>37.5±3.14</td>
<td>0.12±0.03</td>
<td>33.9±2.45</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td>P&lt;0.005</td>
<td>P&lt;0.45</td>
<td>P&lt;0.005</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

No change in brain XO % was observed under the above conditions

Table 4: Effect of intravenous injection of sodium pentobarbital (45mg/kg) on XO ratio

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Plasma XO ratio</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>47.2±1.6</td>
<td>0.074±0.006</td>
</tr>
<tr>
<td>0.5</td>
<td>27.0±2.10</td>
<td>0.1±0.003</td>
</tr>
<tr>
<td>1.0</td>
<td>25.0±3.5</td>
<td>0.095±0.002</td>
</tr>
<tr>
<td>P&lt;0.05</td>
<td></td>
<td>P&lt;0.5</td>
</tr>
</tbody>
</table>

No change in brain XO % was observed under the above conditions

proportion of XO in both brain and plasma and also in total activity (Table 2). Isoflurane (2%) in 100% O<sub>2</sub> or 10%O<sub>2</sub> also brought changes in plasma but not brain XO/XD ratio (Table 3). Table 4 also shows that intravenous injection of sodium barbital also changed plasma but not brain XO/XD ratio.

Finally we compared XO/XD in blood plasma of human, pigs, rats and mice. XO/XD could not be detected in plasma of humans and pigs. This is in contrast with mice and rats who has a high XO/XD activity (~ 0.1 µM/ml/min).

**DISCUSSION**

It was previously shown by Hasan et al. [19] and others [20] since, that hypoxia-reoxygenation of cultured cells will result in the conversion of xanthine dehydrogenase to the free radical producing xanthine oxidase. The main idea from this investigation is to see whether such a treatment of mice resembling the treatment of people suffering from decompression sickness by hyperbolic oxygen will affect the xanthine oxidase /dehydrogenase system. Initial results show
that treatment with different concentrations of oxygen had no significant effect on the xanthine oxidase system (Table 1).

The absence of changes in XO/XD ratio in brains of mice exposed to hypoxia (10% O\textsubscript{2}) and reoxygenation (100% O\textsubscript{2}) shows that 10% O\textsubscript{2} might not be sufficient to the extent of inducing biochemical changes which lead to the activation of cellular processes responsible for producing XO. The effect of hypoxia on the activity of mouse brain proteases that could lead to production of XO has not been specifically investigated and we are not aware of any comparable study on any related system.

Previous reports of conversion of brain XD to XO had only occurred in rats or mice which received cerebral ischemia by blocking blood supply to the brain by carotid artery occlusion [21]. According to these studies XO increased from 15% to 40% of total activity which certainly involves a more drastic induction of hypoxia- reoxygenation than animals breathing 10% O\textsubscript{2}. This procedure involves marked nutrient deprivation of brain tissue and will cause extreme biochemical effect.

The absence of XO increase in mouse brain slices exposed to hypoxia and reoxygenation which resembles cultured cells experiments is probably due to the fact that brain slices cannot be kept intact long enough in culture to carry out extended experiments.

It was noted that procedures in ischemia reperfusion experiments in animals involve the administration of anesthesia, a treatment that leads to alteration in cerebral enzymes [22]. Therefore we decided to introduce anesthesia in our experiments. Data in Tables 3 and 4 show that the presence of an anesthetic agent can cause changes in XO% in mice brain and plasma. It is not clear why halothane affects xanthine oxidase in mouse brain while isoflurane and pentobarbital do not. It has been reported that halothane generates reactive free radicals in biological tissues [23] and this could influence the endothelial system indirectly. It was also shown that halothane treatment has elevated XO levels in rat serum [24].

The effects of the different anesthetics on the plasma XO ratios are similar although the effect of pentobarbital is the most marked in this case. Although pentobarbital has been shown to inhibit purified XO (our observation), it is unlikely in view of the low concentration that the influence of anesthetics observed are due to direct inhibition of the enzyme in mouse tissue. However, it is interesting to note that in the case of pentobarbital at least there is a significant demonstration of interaction between anesthetics and proteins, one of the suggested mechanisms of anesthetic action [25, 26]. This is not surprising in view of the structural similarities between the pyrimidine ring in xanthine and pentobarbital. The presence of high levels of protease inhibitors in blood [27] may also account for the lack of efficient conversion of XO to XO in mouse plasma.

Anesthetic agents can cause the release of histamine in blood [28] and this could be the cause of changed XO/XD ratio in mouse brain and plasma since it has been reported that histamine enhances XO activity in cultured endothelial cells and in rat plasma exposed to thermal injury [29, 30]. Xanthine oxidase derived superoxide radicals exert their actions in the overall context of various endogenous oxidant and antioxidant systems. For example, systems that lead to production of antioxidants such as nitric oxide (NO) can act as endogenous suppressors of XO activity [31-33].

Therefore, it is of high importance to investigate the xanthine oxidase system separately under the different conditions before making any general views.

REFERENCES