Lipid peroxidation products inhibit Glutathione based protection of Oxymyoglobin; a Model system

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Abstract

This study was performed as an *in vitro* model system to investigate the antioxidant role of glutathione in protecting oxymyoglobin from oxidation in competition with its role in detoxifying intracellular toxins such as 4-Hydroxynonenal (HNE). Briefly, the model consisted of a control solution (0.15mM oxymyoglobin solution), a positive control (0.2mM reduced glutathione (GSH) and glutathione peroxidase activity) and test samples (added HNE at 0.1, 0.2 and 0.4, 0.6 and 0.8mM). All samples were incubated for 300 minutes at pH 5.6 and 25^{o}C. Rapid oxidation of oxymyoglobin occurred in the control solution, while in the positive control, strong antioxidant activity was observed over the entire incubation period. Antioxidant activity was observed up to 240 mins in the presence of 0.1mM HNE, while in the presence of 0.2-0.6mM HNE antioxidant activity was observed for no more than 60 mins before the GSH antioxidant activity was apparently inactivated. A complete loss was observed at HNE concentrations of 0.8mM and above. At molar ratios of HNE to GSH greater than 1:1 the antioxidant activity of GSH seems to be inhibited. The level of HNE in meat are likely to exceed this ratio, thus it seems doubtful that GSH would be an important antioxidant in post mortem muscle.

Introduction

Browning of meat during retail display costs the US meat industry $1 billion each year (Williams *et al.*, 1992). Browning of meat is caused by the oxidation of the red pigment, oxymyoglobin, to metmyoglobin. This brown metmyoglobin form is visually undesirable and consumers will quickly discriminate based on visual appearance.

This oxidation can be slowed by antioxidants, such as glutathione (Tang *et al.*, 2003), which occur naturally in muscle tissue. Glutathione is an intracellular tripeptide with a free sulfhydryl group and is involved in the detoxification of hydrogen peroxide and other organic peroxides, catalysed by the enzyme glutathione peroxidase (Stryer, 1975). Hydrogen peroxides are potent oxidants of lipids and proteins such as myoglobin (Morey *et al.*, 1973), glutathione plays an antioxidant role by removing these cytotoxic species. Glutathione also plays an important role in detoxification of intracellular toxins by forming conjugates with its free sulfhydryl group.

4-hydroxynonenal (HNE) is a cytotoxic product of lipid peroxidation derived from omega -6-polyunsaturated fatty acids such as arachidonic and linoleic acid (Esterbauer *et al.*, 1991). HNE is known to covalently bind to oxymyoglobin, readily forming metmyoglobin (Faustman *et al.*, 1999), however this can be prevented by glutathione which forms conjugates with HNE (Alin *et al.*, 1985; Esterbauer *et al.*, 1991). Thus accelerated formation of HNE will rapidly deplete intracellular glutathione concentrations (Siems & Grune, 2003). Therefore the interaction of glutathione with cellular toxins such as HNE, is likely to impact on its antioxidant activities.

This study tested the hypothesis that glutathione will not protect oxymyoglobin from oxidation in the presence of HNE.

Materials and methods

This experiment was performed as an in-vitro model system. The model consisted of 0.15mM oxymyoglobin (prepared from commercial metmyoglobin), 0.4mM EDTA, 1 unit glutathione peroxidase, 0.2mM reduced glutathione (GSH) and 0.1, 0.2, 0.4, 0.6 and 0.8mM HNE. Controls contained ethanol (in the same amount used to deliver HNE) and oxymyoglobin. The model had a total volume of 1ml and was buffered to pH 5.6 (sodium citrate), to represent the conditions of post-mortem muscle. HNE and GSH were pre-incubated at 25^{o}C for 90 minutes before oxymyoglobin was added, after which the rate of formation of metmyoglobin was monitored spectrophotometrically over 5 hours at 25^{o}C. The percentage metmyoglobin was calculated using the revised krzywicki method (Tang *et al.*, 2004). GSH was replaced by 0.2mM N-

acetyl cysteine (NAC), to determine the selectivity of HNE when forming conjugates. NAC is often used as a GSH "mimic" as it contains a single sulfhydryl group and can ready conjugate with cytotoxic toxins.

Results and discussion

The control solution represents oxymyoglobin with no antioxidant capacity and demonstrated rapid conversion to the metmyoglobin form (figure 1a). A solution with oxymyoglobin and GSH demonstrated the same rapid conversion to metmyoglobin as the control (data not shown). Compared to this, GSH and glutathione peroxidase together showed the strongest antioxidant activity by slowing the oxidation of oxymyoglobin more than any other treatment ($P<0.05$). This result supports that found by Tang *et al.* (2003), who suggested that the antioxidant effect of GSH on bovine oxymyoglobin was dependent on glutathione peroxidase as a cytosolic component.

Antioxidant activity of GSH was observed for no more than about 60mins in samples pre-incubated with greater than 0.2mM HNE (molar ratio 1:1) after which the GSH antioxidant activity was apparently inactivated (figure 1a). A complete loss of antioxidant activity was observed in samples pre-incubated at HNE concentrations above 0.8mM (figure 1a) and resembled that of the control samples. Antioxidant activity was observed for up to 240 mins when GSH was pre-incubated with 0.1mM HNE (molar ratio of 2:1), 4 times longer than the 0.2mM HNE concentration.

HNE was pre-incubated with glutathione peroxidase, a component required for the antioxidant activity of GSH, before its addition to oxymyoglobin to determine whether HNE was inactivating the enzyme or in fact conjugating with GSH (results not shown). This incubation had no effect on the activity of glutathione peroxidase and thus it could be concluded that HNE was in fact exhausting GSH stores resulting in more rapid formation of metmyoglobin. Moreover, the addition of the GSH "mimic", NAC, to the system resulted in about 1 hour more observed antioxidant activity (Figure 1 b). NAC was not a substrate for glutathione peroxidase as NAC and glutathione peroxidase alone did not show any antioxidant capacity. However, this result demonstrates that HNE also formed conjugates with the sulfhydryl group in NAC and spared the antioxidant activity of GSH.

These results suggest a possible toxin threshold, where HNE will inhibit the antioxidant activity of GSH at molar ratios greater than 1:1. At this threshold, GSH-HNE conjugates readily form. Oxidative stress in post-mortem muscle is extremely high and additional cytotoxic products resulting from lipid peroxidation are likely to exceed this 1:1 molar ratio (Esterbauer *et al.*, 1991) thus overwhelming the antioxidant capacity of GSH. Thus it seems unlikely that GSH would be an important antioxidant in post mortem muscle under normal conditions.

Reddy *et al.*(1982) showed that the inhibitory effect of GSH on lipid peroxidation in rat microsomes was dependent on the presence of vitamin E. Glutathione is known to regenerate oxidized vitamin E by donating protons and in turn forming a disulfate. However, other possible interactions may exist, such that vitamin E can inhibit lipid peroxidation (Wulf *et al.*, 1995) which will lower the amount of cytosolic HNE, thus sparing the antioxidant activity of GSH. This mechanism is yet to be proved and further investigation is required.

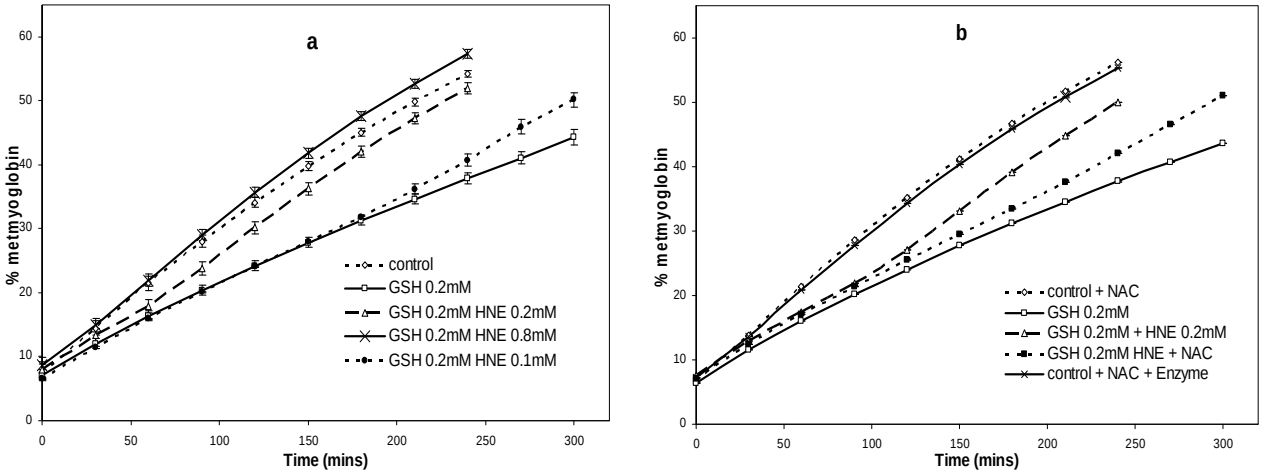

Figure 1. Percent metmyoglobin formed pH 5.6 and 25^{o}C ±sem, in a) samples of oxymyoglobin plus differing levels of HNE and in b) samples with the addition of NAC.

Conclusions

The antioxidant activity of GSH slows the formation of metmyoglobin in an *in vitro* system, and is dependent on the activity of the enzyme glutathione peroxidase. However when HNE is present in a molar ratio of greater than 1:1 the antioxidant activity will be almost completely inactivated. GSH will assume its detoxifying role and thus the oxidation of oxymyoglobin will proceed. This antioxidant activity of GSH is unlikely to impact on the colour stability of meat under normal display conditions, as the molar ratio of GSH to HNE, or other toxins, is likely to exceed that of 1:1. However, possible interactions may exist where multiple antioxidants can coexist and in turn protect the GSH antioxidant activity, but a further understanding of these mechanisms need to be addressed.

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