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RELATIONSHIPS BETWEEN MYOGLOBIN AND MICROSOMAL LIPID OXIDATION. INFLUENCE OF MUSCLE TYPE AND TIME POST-MORTEM

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Please refer to Folio 62.

INTRODUCTION

Autoxidation of oxymyoglobin (MbO_2) to metmyoglobin (MetMb), is the most important factor responsible for discoloration during storage (Renerre and Labas, 1987). In beef, although there are many factors which contribute to increase the rate of myoglobin autoxidation, phospholipid oxidation process is the main one (Anton *et al.*, 1991). Differences between muscles in lipid metabolism could partly explain intermuscular variability of colour stability.

The goal of this study was to better understand the relationships between lipid peroxidation and myoglobin autoxidation in meat stored for eight days at 4°C. In a second part, an enzymic microsomal system was used in the presence of myoglobin. This model system was very attractive for determining the *in vitro* nature of the interactions between the microsomal lipid fraction and myoglobin. The experiment was run with myoglobin and microsomes extracted one hour post-mortem (D0) and after eight days storage at 4°C (D8). This storage period was chosen to determine the consequence of storage on the phospholipid-myoglobin relationships.

MATERIALS AND METHODS

Two muscles with different colour stabilities : *Longissimus lumborum* (LL) : stable and *Psoas major* (PM) : unstable, were taken one hour post-mortem (day 0) from Charolais cull cows and stored at 4°C for colour measurement. Colour stability of the two muscles was measured with an UVIKON 860 spectrocolorimeter between 360 and 760nm, after 1, 3 and 7 days of storage of the two muscles at 4°C. Metmyoglobin percentage at the meat surface was then calculated according to Stewart *et al.* (1965). Concurrently, lipid oxidation was measured by the thiobarbituric assay (TBA) described by Sunderman *et al.* (1985). The results were reported as nmoles of TBA reactive substances per g of wet weight meat.

Oxymyoglobin and microsome fractions were prepared from muscles put in liquid nitrogen one hour post-mortem. Oxymyoglobin was extracted in 10mM TRIS HCl buffer (pH8.4) and precipitated selectively with 70 to 100% ammonium sulfate. The haeminic solution was then passed through a mono-Q column (PHARMACIA) to obtain purified oxymyoglobin (Gatellier *et al.*, 1993). Isolation of the microsomal fraction was done by a procedure described by Apgar and Hultin (1982). After homogenization of meat in a 5mM Histidine, 0.6M KCl buffer (pH6.8), the microsomes were obtained by centrifugation at 100 000g.

The extent of oxymyoglobin (0.66mg/ml) autoxidation in the presence of microsomes was studied in a mixture containing 0.72mg/ml of microsomal proteins and 0.16mM $FeCl_3$, 0.83mM ADP and 0.4mM NADPH to enhance enzymic microsomal peroxidation. The oxidation rate of oxymyoglobin to metmyoglobin was monitored at 580nm with an UVIKON 860 spectrophotometer. Lipid peroxidation levels were measured by TBA test. All measurements were effected for a period of 40 minutes at 37°C. This experiment was done at the two storage times for both muscles (D0 and D8).

The inhibition of oxymyoglobin oxidation and lipid peroxidation by different radical scavengers was followed after the addition of : butylhydroxytoluen (BHT 1mM), vitamin E (8mM), superoxide dismutase (SOD 300 U/ml) and catalase (250 U/ml).

The data are presented as the mean ± standard deviation with statistical significances determined by the t-test after pairing of the data.

RESULTS AND DISCUSSION

Figure 1 shows the metmyoglobin accumulation during eight days storage at 4°C for the LL and PM muscles. Metmyoglobin increases from 15 to 23% for LL and from 18 to 38% for PM muscle. This result is in good agreement with previous observations with nine different bovine muscles (Renerre, 1984). Concurrently, the accumulation of TBA reactive substances increased sharply, for the two muscles, during the same period storage (Figure 2). The rate of lipid peroxidation was more pronounced in PM than in LL muscle.

These results suggest that myoglobin autoxidation is closely linked to lipid peroxidation. Furthermore, these two phenomena appear to be strongest post-mortem in PM than in LL muscle. Consequently, the colour instability of PM muscle could be explained, at least in part, by its greater lipid peroxidation during storage.

In an attempt to determine the relationships between lipid peroxidation and myoglobin autoxidation, we examined the oxidative interactions between the two processes in a model system. The results indicate that, *in vitro*, microsomal phospholipids, peroxidized by an enzymic system (NADPH, $FeCl_3$, ADP), considerably increased the autoxidation of oxymyoglobin to metmyoglobin. Oxymyglobin autoxidation (Figure 3) was faster when MbO_2 is incubated *in vitro* with, rather than without, the microsomal peroxidation system. Concurrently, lipid peroxidation increased rapidly during the first 30 minutes and remain stable thereafter (Figure 3).

The autoxidation process was analyzed by plotting Ln ([MbO2]t / [MbO2]0) against time (Tajima *et al.*, 1987). We observed two different rates : an initial rapid decrease which slowed as the reaction proceeded. This could be explained by the peroxidase activity of myoglobin (Stewart, 1990). It is known that autoxidation of oxymyoglobin generates metmyoglobin and hydrogen peroxide (H_2O_2), through the reduction of superoxide ion product (Gotho and Shikama, 1976). In our model system, in the first stage of the reaction, MbO_2 was autoxidized to MetMb and H_2O_2 which can react with the haeminic iron and increase the rate of the reaction. In the second stage, the MetMb produced can combine with H_2O_2 and the rate of MbO2 autoxidation remains steady.

Using muscles at day zero storage (D0) before myoglobin and microsome extraction, the percentage of myoglobin oxidized after 40 minutes in the model system was similar for the myoglobins of both muscles : 30.1 for LL and 31.6 for PM myoglobin (Figure 4). Furthermore, the values were, on average, three-fold greater in model systems than in the control. Lin and Hultin (1977) showed that enzymic lipid peroxidation of a microsomal fraction prepared from chicken leg muscle led to the oxidation of oxymyglobin, but lipid peroxidation was not controlled. In addition to the spectral measurements of myoglobin autoxidation in our model system at 37°C, formation of thiobarbituric acid reactive substances (TBA-RS) was measured to indicate lipid peroxidation in microsomes. The extent of lipid peroxidation was the same irrespective of the muscular origin of the myoglobin and microsomes (Figure 5).

On the contrary, when myoglobin and microsomes were extracted after eight days storage at 4°C (D8), lipid peroxidation was significantly ($P<0.05$) more pronounced in microsomes of PM than LL muscle (Figure 5) after incubation with NADPH, $FeCl_3$ and ADP. The quantity of thiobarbituric acid reactive substances was ten times higher in the former case (80nmoles TBA-RS/mg protein) than in the latter (8nmoles TBA-RS/mg protein). This result indicates conclusively that, *in vitro*, after eight days storage of muscles, lipid peroxidation occurs much more rapidly in microsomes of PM than in microsomes of LL. In fact, we think that this greater susceptibility of PM microsomes to peroxidation is likely to be the result of the more pronounced lipid peroxidation during storage in this muscle (Figure 2). This contributes to a greater accumulation of peroxidation products in extracted microsomal membranes (results

not shown). Albro *et al.* (1987) have shown that differences in the *in vitro* rate of peroxidation of microsomes prepared by two different methods can be explained by differences in the content of preformed peroxidation products. Consequently, (lipid peroxidation being an autocatalytic chain reaction), PM microsomes, with the greater lipid peroxidation products after eight days storage, are peroxidized more rapidly *in vitro*.

After eight days storage of muscles, in the same mixtures, myoglobin autoxidation was more rapid ($P<0.05$) in PM than in LL sample. As shown in Figure 4, the percentage of oxidized myoglobin after 40 minutes was 1.6-fold improved the in PM mixture compared to the LL mixture. These results suggest a strong relationship, *in vitro*, between microsomal lipid peroxidation and myoglobin oxidation.

To investigate the nature of the oxidative interactions between the two components, we added some antioxidants to the model system. Figure 6 shows the effect of vitamin E and butylated hydroxitoluen (BHT) on TBA-RS accumulation in the mixture. After 40 minutes, these antioxidants halved the extent of lipid peroxidation in comparison with a control without antioxidant. Also, in the same model system, autoxidation of myoglobin declined approximately 25% compared to the control (Figure 7). It must be pointed out that there was no difference in lipid peroxidation and myoglobin oxidation levels between the model systems of the two muscles.

This result provides clear evidence that lipid influence on myoglobin autoxidation is of a radical nature. BHT and vitamin E can deactivate peroxi- (ROOo) and alkoxi- (ROo) radicals in lipid hydroperoxides by giving hydrogen atoms (Tappel, 1973). These radicals may interact with haeminic iron of myoglobin to oxidize it. Nevertheless, the imbalance between levels of inhibition of lipid peroxidation (50%) and myoglobin autoxidation (25%) in the model system suggests that other types of radicals which react with haeminic iron could exist.

To study this, we tested the effect of two free radical scavengers: superoxide dismutase (SOD) and catalase, on microsomal lipid peroxidation and myoglobin autoxidation in our model system. When SOD or catalase are added to the mixture, the rate of TBA-RS accumulation was slightly inhibited (20 %) for the two muscles (Figure 8). This demonstrates conclusively that SOD alone or catalase alone are unable to completely inhibit the peroxidation process. On the contrary, these antioxidants alone significantly decreased myoglobin autoxidation in the mixture for the two muscles (Figure 9). Superoxide dismutase removes superoxide anion (O_2o-) by forming hydrogen peroxide (H_2O_2) (Mc Cord and Fridovich, 1969), while catalase reduces hydrogen peroxide to water and oxygen (Aebi, 1974). Therefore, both superoxide anion and hydrogen peroxide are able to react with haeminic iron and oxidize it. (i.e., free radicals implicated in lipid peroxidation initiation have an oxidative effect on myoglobin *in vitro*).

SOD and catalase, added together, significantly decreased lipid peroxidation (Figure 8) and myoglobin autoxidation (Figure 9). This additive effect shows that the two antioxidants employed together are able to inhibit lipid peroxidation (39% average for the two muscles) with a consequence, in our model system, to decrease myoglobin autoxidation (36% average for the two muscles).

CONCLUSION

This study suggests that, *in vitro*, the processes of lipid peroxidation can enhance the autoxidation of myoglobin. Presumably, the lipid end-products of peroxidation can react with haeminic iron of myoglobin but free radicals are also implicated in the enzymic initiation of microsomal peroxidation. The interdependence between the lipid peroxidation process and myoglobin autoxidation and its nature are evident.

On the basis of the first results of this paper, we have seen that, during the storage at 4°C, PM muscle exhibited a greater lipid peroxidation extent than LL muscle. Consequently, the more pronounced lipid peroxidation in PM muscle gives rise to a far quicker myoglobin autoxidation rate than LL muscle. In other words, difference in rates of discoloration between muscles can be explained by different lipid peroxidation rates during storage.

A question which arises from our analysis is why lipids of PM muscle are more peroxidized during storage than those of LL muscle. Phospholipid composition is similar in the two muscles (Anton, 1993) and this result is essentially the same as those found in the literature. Previous studies in our laboratory intended to determine the pro-oxidant/anti-oxidant balance between the two muscles (Gatellier *et al.*, 1992). We showed that catalase and glutathion peroxidase activities were greater after eight days storage in LL than in PM muscle. This antioxidative prevention in LL muscle could explain the relative stability of lipids during storage. However, further research will be needed to provide an understanding about the mechanism of regulation of lipid peroxidation post-mortem.

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