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Lipid and protein oxidation in vitro, and antioxidant potential in meat from Charolais cows finished on pasture or mixed diet

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Abstract

In this work, the effect of finishing diet (pasture- or mixed-diet) on lipid and protein oxidation in beef homogenates was evaluated. Oxidation was chemically induced by ferrous iron and hydrogen peroxide. Lipid and protein oxidation were respectively measured by determining TBA reactive substances (TBA-RS) and protein carbonyl groups (DNPH coupling method). Evaluation of the antioxidant status of meat was also performed by measuring vitamin E concentration and antioxidant enzyme activities: superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. Pasture-finishing mode of animals significantly protected lipids in meat from oxidation but diet mode did not affect protein oxidation. Concentration of vitamin E was higher in meat from pasture-fed animals. Different diets had opposite effects on SOD and GPx activities, pasture-finishing mode of animals increasing SOD activity but decreasing GPx activity. No significant effect of diet was noted on catalase activity.

Keywords: Grain; Pasture-feed; Meat; Lipid oxidation; Protein oxidation; Vitamin E; SOD; Catalase; Glutathion peroxidase; Beef; Charolais

1. Introduction

Beef production based on pasture feeding offers good animal welfare and economic advantages compared with grain feeding (Lanari, Brewster, Yang, & Tume, 2002). It also offers dietary improvements to the consumers as pasture feeding can lead to increased concentrations of polyunsaturated fatty acids (PUFA's). (Larick and Turner, 1989; Enser et al., 1998; Yang, Lanari, Brewster, & Tume, 2002b). Saturated fatty acids are considered harmful to human health and, on the contrary, polyunsaturated fatty acids (rich particularly in n-6 and n-3) would play a favorable role in the prevention of some human diseases (cancer, obesity and cardiovascular diseases); so, increasing the proportion of polyunsaturated fatty acids in meat by means of animal diet, is nowadays recommended. Moreover, meat is a source of conjugated linoleic acid (CLA), a component with anticarcinogenic and antiatherogenic activities. A pasture feeding system, produces higher CLA levels in meat than of concentrates (Enser, 2000).

However even if the nutritional value of meat can be improved, its quality must remain acceptable to consumers. Pasture and grain finishing can affect the colour and flavor of meat (Lanari et al., 2002), with some reports showing that pasture feeding produces darker meat (Vestergaard, Oksbjerg, & Henckel, 2000), but the results remain contradictory. The effects of feeding regimes on lipid and, especially, on protein oxidation, is still largely unknown.

Lipid oxidation, which results in the production of free radicals, is a promoter of myoglobin oxidation and leads to the formation of rancid odors and off-flavors. Protein oxidation is responsible for many biological modifications (Decker, Xiong, Calvert, Crum, & Blanchart, 1993), as protein fragmentation or aggregation and decrease in protein solubility, affect the quality of meat and meat products. Oxidation might also play a role in controlling proteolytic activity of enzymes (Starke-Reed & Oliver, 1989) and could be linked to meat tenderness.

The oxidative stability of meat depends upon the balance between anti- and pro-oxidants, including the

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concentration of polyunsaturated fatty acids. Meats containing high contents of polyunsaturated fatty acids, as in pasture feeding, should be more prone to oxidation than those with more saturated ones. But these two diets may also have differences in anti-oxidant content especially vitamins, carotenoids and/or flavonoids which can protect meat against oxidation (Wood & Enser, 1997). These anti-oxidants cannot be synthesized in animals and higher concentrations are found in green herbage compared to cereals (Daly, Youg, Graafhuis, & Moorhead, 1999). Diet can also affect mineral intake and, so, should modulate antioxidant enzyme activities such as SOD, GPx and catalase which need different metals (Cu, Zn, Mn, Fe or Se) as cofactors. It has been also previously demonstrated that dietary fat and vitamin E supplementation can more or less influence these antioxidant enzyme activities in meat (Renerre, Poncet, Mercier, Gatellier, & Metro, 1999).

Meat is complex and, consequently, different model systems have been developped, in vitro, to gain a better understanding of oxidation processes. Many systems exist: muscular extracts, myofibrillar sytems, organelles such as mitochondria or microsomes. Such models involve chemical oxidation induced by transition metals, such as iron or copper, which can generate free radicals. These metal-catalysed oxidation (MCO) systems have been used to enhance both lipid and protein oxidation. In our laboratory, microsomes from bovine muscle have been combined with myoglobin, with or without chemical cofactors (ADP, iron and NADPH), to better understand the relationships between myoglobin and lipid oxidation (Anton, Salgues, Gatellier, & Renerre, 1993). In beef, the process of myofibrillar protein oxidation has been studied in different model oxidation systems (Martinaud et al., 1997). More recently, in turkey, influence of dietary vitamin E supplementation on microsomal lipid and protein oxidation has been performed after a chemical oxidation by H_2O_2 and activated myoglobin (Batifoulier, Mercier, Gatellier, & Renerre, 2002). This vitamin E effect on oxidation has also been studied in raw turkey muscle extracts (Gatellier, Mercier, Remignon, & Renerre, 1996).

The objectives of the present study were to determine the influence of finishing mode (pasture- or mixed diet-finishing) on lipid and protein oxidation in beef homogenates, after a chemical oxidation by a ferrous/hydrogen peroxide system, and to what extent such oxidations are related to antioxidant activities of muscles post-mortem.

2. Materials and methods

2.1. Animals and diets

This study was initiated by Institut Charolais as part of their programme to determine the effect of diet on meat quality in Charolais cattle. Ten Charolais cows were finished for about 100 days outdoors (during summer) and fed almost exclusively grass (pasture group), and ten Charolais cows were finished for the same time indoors (during winter) and fed diets containing predominantly cereals, silage, cattle-cake of different origin (mixed diet group) in many private farms from Bourgogne (France). Animals, from 4 to 12 years old, were slaughtered in the abattoir of Paray le Monial (Saône et Loire). Twenty four hours after slaughter, *longissimus dorsi* (LD) muscle was removed from carcass; 20 g of muscle were removed. The fat was removed and muscle cut into cubes and frozen in liquid nitrogen for transport to INRA. Samples were then kept at −80 °C until use.

2.2. Vitamin E content of muscles

Vitamin E content was determined according to the method of Buttriss and Diplock (1984). After saponification and hexane extraction, all the samples were analysed by normal phase HPLC (Lichrospher Si 60 column from Merk) fitted with fluorimetric detection (excitation 292 nm/emission 330 nm; Kontron, France). The results were expressed as μg vitamin E g^{-1} of muscle.

2.3. Antioxidant enzyme activity measurements

Frozen muscle samples (9 g) were homogeneized in a Waring Blender with 100 ml 50 mM phosphate buffer (pH 7.0) and centrifuged at 1000 *g* for 15 min at 4 °C (Renerre, Dumont, & Gatellier, 1996). Protein concentration was determined by the Biuret method (Gornall, Bardawill, & David, 1949). Total superoxide dismutase activity (Cu-Zn SOD and Mn SOD) was measured according to the procedures of Marklund and Marklund (1974) using inhibition of pyrogallol autoxidation in a basic medium. One unit was taken as the activity that inhibits the pyrogallol autoxidation by 50%. Catalase activity was measured by the rate of disappearance of H_2O_2 at 240 nm (Aebi, 1974) and expressed as nmol of decomposed H_2O_2 min^{-1} mg^{-1} protein. Glutathione peroxidase (GPx) activity was assayed with a GSH reduction coupled to a NADPH oxidation by glutathione reductase (Agergaard & Thode Jensen, 1982) and was expressed as nmol of oxidized NADPH min^{-1} mg^{-1} protein.

2.4. Assay for in vitro oxidation

Frozen meat (1 g) was ground in 10 ml of sodium phosphate 100 mM, pH 7 buffer with a Polytron homogeneiser (Kinematica PT-2100) for 1 min at high speed. This homogenate was then incubated at 37 °C in a dry bath, under agitation, with a mixture of ferrous

sulfate (0.5 mM) and hydrogen peroxide (1 mM) for 0 h, 30 min, 2 h and 5 h. After each incubation time, oxidations were stopped by addition of butylated hydroxytoluene (BHT, to 0.02% final concentration) to aliquots of 2 ml of homogenate, and samples were immediately frozen at −80 °C until analysis.

2.5. Lipid oxidation measurement

Lipid oxidation was measured by the thiobarbituric acid reactive substances (TBA-RS) (Lynch & Frei, 1993). Samples of 0.5 ml of homogenate were incubated with 1% (w/v) 2-thiobarbituric acid in 50 mM NaOH (0.25 ml) and 2.8% (w/v) trichloroacetic acid (0.25 ml) in a boiling water bath for 10 min. After cooling at room temperature over 20 min, the pink chromogen was extracted with *n*-butanol (2ml) and its absorbance measured at 535 nm against a blanck of *n*-butanol. TBA-RS concentrations were calculated using 1,1,3,3 tetraethoxypropane (0–0.8 μM) as standard. Results were expressed as mg MDA per kg of meat.

2.6. Protein oxidation measurement

Protein oxidation was measured by an estimation of carbonyl groups formed for the duration of the experiment (Oliver, Ahn, Moerman, Golstein, & Stadtman, 1987) with slight modifications. Each sample of homogenate was divided into two equal aliquots of 0.5 ml. Proteins were precipitated in both aliquots by 10% trichloroacetic acid (w/v, final concentration) and centrifuged at 2000 *g* for 10 min. One pellet was treated with 1ml of 2 N HCl and the other with an equal volume of 0.2% (w/v) 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl. Both samples were incubated for 1 h at room temperature and stirred regularly. The samples were precipitated with 10% TCA (w/v final concentration) and centrifuged at 2000 *g* for 10 min. The pellets were then washed twice with 1 ml of ethanol: ethyl acetate (1:1) to eliminate traces of DNPH and to make soluble residual lipids. Proteins were finally dissolved in 2 ml of 6 M guanidine HCl with 20 mM sodium phosphate buffer pH 6.5. To remove insoluble fragments, samples were centrifuged 10 min at 2000 *g*. Protein concentration was calculated at 280 nm in the HCl control using BSA in 6M guanidine as standard. Carbonyl concentration was measured on the treated sample by measuring DNPH incorporated on the basis of an absorption of 21.0 mM^{-1} cm^{-1} at 370 nm for protein hydrazones. The results were expressed as nanomoles of DNPH fixed per milligram of protein.

2.7. Statistics

All values are reported as the mean ± standard deviation for ten measurements on each animal group. An unpaired Student's *t*-test was used to determine the levels of statistical significance with $P > 0.05$, NS; $P < 0.05$, * ; $P < 0.01$, ** ; $P < 0.001$, ***. The relationships between the different parameters were assessed by calculation of correlation coefficients.

3. Results and discussion

3.1. Antioxidant status

Table 1 shows an estimation of antioxidant status as measured by the content of vitamin E and by antioxidant enzyme activities in the two diet groups.

3.1.1. Vitamin E content of muscles

Meat from pasture-fed cows had a higher vitamin E content (approximately 17% more) when compared with mixed diet-fed cows, but this difference was not statistically significant ($p > 0.05$). Differences in vitamin E levels, between the two diets, were more pronounced in young steers ($p < 0.05$) and heifers ($p < 0.001$) from Charolais (results not shown). Vitamin E levels, found in this study, were similar to those previously obtained in Charolais cattle fed diet supplemented (1000 mg / kg feed / day, during 111 days) or not (75 mg as basal level) with α-tocopheryl acetate (Gatellier, Hamelin, Durand, & Renerre 2001). In this last study, vitamin E content of LD muscle was 3.57 ppm in control and 5.16 ppm in supplemented animals. The relationship between nutritional background (pasture- and mixed diet-finishing) and vitamin E content in meat has been described in beef (Lanari, Roblin, Brewster, Young, & Tume 2000; Lanari et al., 2002; Descalzo, Insani, Eyrerabide, Guidi & Pensel, 2000; Descalzo, Insani, Margaria, Garcia, Josifovich, & Pensel, 2000; Yang, Brewster, Lanari, & Tyme, 2002; Yang, Lanari et al., 2002) and lamb (Turner, McClure, Weiss, Borton, & Foster, 2002). All

Table 1
Effect of finishing diet on antioxidant status in beef meat[a]

	Vitamin E	SOD	Catalase	GPx
Mixed-diet	3.61 +/− 1.22	0.58 +/− 0.10	2678 +/− 892	197.8 +/− 39.6
Pasture	4.22 +/− 1.33	3.65 +/− 0.68	3527 +/− 1982	63.6 +/− 17.9
S	NS	***	NS	***

[a] Values are means ± standard deviation.

these results showed that grazing animals on good quality pasture resulted in elevated concentration of vitamin E in muscles, similar to those found in meat of mixed diet-fed animals supplemented with high dietary vitamin E levels. This could be partially explained by the higher concentration of vitamin E in green leaf tissue compared to mixed diet. For Yang, Lanari, et al. (2002), beef from cattle raised on good quality pasture had an equivalent amount of vitamin E to that from grain-fed cattle supplemented with 2500 IU α-tocopheryl acetate.

3.1.2. Antioxidant enzyme activities

Table 1 shows that meat from pasture-fed animals had a much higher SOD activity compared with the mixed diet-fed animals ($P < 0.001$). Catalase activity was also greater in the pasture group, when compared with the mixed diet group, but the difference was not statistically significant. The pasture-finishing diet decreased, about three-fold, GPx activity ($P < 0.001$) in agreement with results of Descalzo, Insani, Eyerabide et al. (2000), which showed that SOD and catalase activities were higher in meat from pasture- than those from feedlot-finished steers and that pasture finishing reduced GPx activity. In a recent study, Mouty et al., (2002) also showed in steer muscles that a fresh grass-based diet significantly reduced GPx activity (−70% in RA and −52% in ST) when compared with a maize-based diet. In meat from steers fed a diet supplemented with sunflower oil (particularly rich in n-6 PUFA's), Durand, Gruffat-Mouty, Hocquette, Micol, Dubroeucq et al. (2001) measured no change in SOD activity, in RA and LT muscle, when compared with non-supplemented animals. All these enzyme activities are also mediated by mineral cofactors. For exemple, DeVore and Greene (1982) obtained, in bovine *semitendinosous* muscle (ST), a statistically significant correlation between GPx activity and muscle selenium concentration. Walsh, Kennedy, Goodall, and Kennedy (1993) demonstrated in calves that feeding diets, depleted of selenium, such as forage, reduced GPx activity in muscles. To better understand the effect of diet on enzyme antioxidant activity, determinations of mineral levels in meat are in progress in our laboratory. Further investigations on the relation of muscle mineral values to mineral nutritional status of Charolais cattle would also be of interest but it is also well known that exercise can also increase enzyme antioxidant activities in muscles. So, in our study, the effect of feeding may be confounded with differences in other rearing factors, such as physical activity, between animals allowed to move freely (pasture-finishing) or restricted in feedlots (mixed diet- finishing).

3.2. Lipid oxidation

As shown in Fig. 1, chemical oxidation induced by the Fenton reaction (Fe^{++}/H_2O_2) resulted in an important

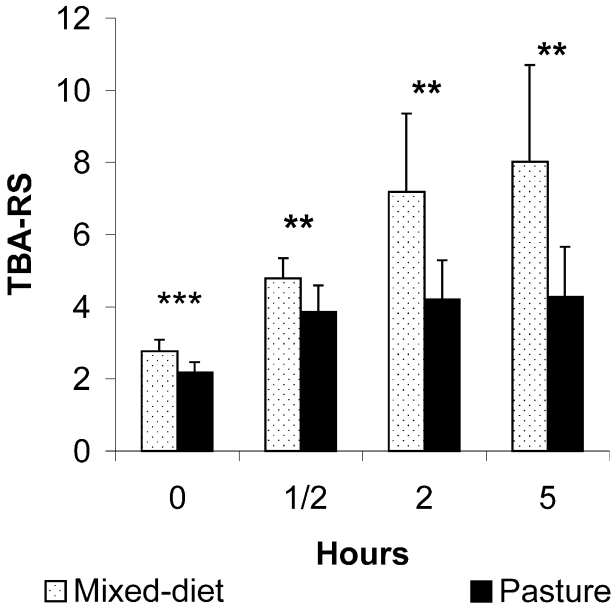

Fig. 1. Effect of finishing diet on TBA-RS level (mg MDA/kg meat) in beef homogenates oxidized by Fe^{2+}/H_2O_2.

increase in lipid oxidation, as measured by TBA-RS values, in beef homogenates. This effect of ferrous iron/hydrogen peroxide system has already been observed in our laboratory and the maximum effect of the oxidative system was observed with a 1/2 iron/H_2O_2 ratio. Excess of H_2O_2 was needed because a quantity of peroxide can be decomposed, before playing a role in Fenton reaction, by proteins with peroxidase activity, such as catalase and myoglobin, present in the homogenate. A ferric iron/ascorbate system, previously used with success in our laboratory on turkey meat extracts (Gatellier et al., 1996), was unable to oxidize both lipid and protein in beef homogenates (results not shown).

Diet considerably affected TBA-RS values. Although meat from pasture-fed groups had higher polyunsaturated fatty acid concentration (results to be published) and, consequently, presented higher potential for lipid peroxidation, homogenates of pasture-fed cows had significantly lower TBA-RS values than mixed diet-fed cows. This effect was already noted before chemical oxidation (0 h) and increased during the 5 h of the experiment. After 5 h, the level of lipid oxidation was approximately twice as high in the mixed diet-fed group compared with the pasture-fed one. Differences were highly significant ($P < 0.01$) whatever the length of oxidation. These results could be partly explained by the fact that, from the two diets, there were some important differences in antioxidant status. Table 1 showed that meat from the pasture fed-group was richer in vitamin E than meat from the mixed diet-fed group, even if values were not significantly different. Measurement of enzyme antioxidant activity also showed a better protection by catalase and, particularly, by SOD in meat of pasture-

fed animals. Although a negative correlation ($r = -0.68$; $P < 0.05$) was obtained between SOD activity and TBA-RS level, after 5 h oxidation, no significant correlation was obtained between catalase and TBA-RS. SOD and catalase are coupled enzymes: SOD removes superoxide anion by forming hydrogen peroxide while catalase reduces hydrogen peroxide to water and oxygen (Aebi, 1974). Some time after the death of an animal, this coupled enzymic system probably acts, in complement of vitamin E protection, to prevent lipid oxidation in the meat homogenate. The effective role of GPx, the activity of which was more pronounced in mixed diet-fed animals, was more ambiguous in lipid oxidation. GPx can act only with selenium as cofactor and glutathione as substrate, and it was previously shown that the level of this last compound decreased rapidly after slaugther (Renerre et al., 1996). Estimation of selenium concentration and glutathione level in meat are in progress in the laboratory to better understand the effect of diet on GPx activity. Protection by other vegetable antioxidants such as carotenoids, phytic acid and flavonoids, particulary abundant in fresh grass (not estimated in this study) could also account in the protection against lipid oxidation in pasture-fed animals. Descalzo, Insani, Margaria, et al. (2000) have demonstrated that both α- and β-carotene were in higher concentration in meat from pasture- than in meat from grain-fed steers. Similar results were observed by Yang, Brewster, et al. (2002), in different steer muscles, for whom pasture-fed animals had significantly more β-carotene than grain-fed animals. Nevertheless, when added as dietary supplements to farm animals, little information is available on whether or not carotenoids can decrease the oxidative stability of meat (Gatellier et al., 2001).

Such a diet effect on lipid oxidation has already been observed in refrigerated meat storage in air (Mercier, Gatellier, & Renerre, 2002): after 6 days storage, it was observed that TBA-RS values were significantly higher (approximately twofold) in mixed diet-fed animals compared to pasture-fed ones. However, it is inappropriate to compare TBA-RS values from the two studies directly. In steers, fed fresh grass or maize, Mouty et al. (2002) showed no changes in lipoperoxidation in RA and ST muscles, in spite of an important n-3 PUFA's increase in grass-fed animals, and concluded that there was a better antioxidant protection (especially by vitamin E) in grass group. In beef from pasture- and grain-fed cattle, with or without vitamin E supplement, Yang, Lanari, et al. (2002) showed that treatments did not affect the lipid stability of fresh meat during aerobic storage.

3.3. Protein oxidation

Fig. 2 showed the time course of protein oxidation in beef homogenates as measured by carbonyl groups accumulation. The initial level of protein oxidation was approximatly 3 nmol of DNPH per mg of protein. This basal level of carbonyl group has been shown in fresh beef meat (Martinaud et al., 1997). The content of carbonyl groups increased during the first 30 min of incubation to reach a maximum level which was approximatly threefold the initial level and then slightly decreased until 5 h oxidation. This decrease of carbonyl groups after a long incubation has already been observed with a different oxidation system (Batifoulier et al., 2002). This phenomena can give rise to protein degradation, fragmentation or aggregation, such as previously described on myofibrillar proteins (Martinaud et al., 1997). Many studies have shown that protein oxidation is linked to lipid oxidation in beef (Mercier, Gatellier, & Renerre, 1995). In microsomes of turkey muscles, oxidized by the metmyoglobin/H_2O_2 system, it was previously demonstrated that vitamin E supplementation in the diet reduced both TBARS formation and carbonyl groups accumulation (Batifoulier et al., 2002). In the present study, although the pasture diet considerably reduced lipid oxidation (Fig. 1), no significant diet effect on protein oxidation was observed. To explain this result, it can be suggested that carbonyl production is limited to only one group of amino-acids and is not representative of the whole oxidation phenomena. Other amino acid groups may be oxidized which do not form carbonyls. Formation of bityrosine, tryptophan oxidation, and loss of protein thiols have also been used as indices of oxidative damage to proteins and could be of great interest to understand the effect of diet on the mechanism of protein oxidation.

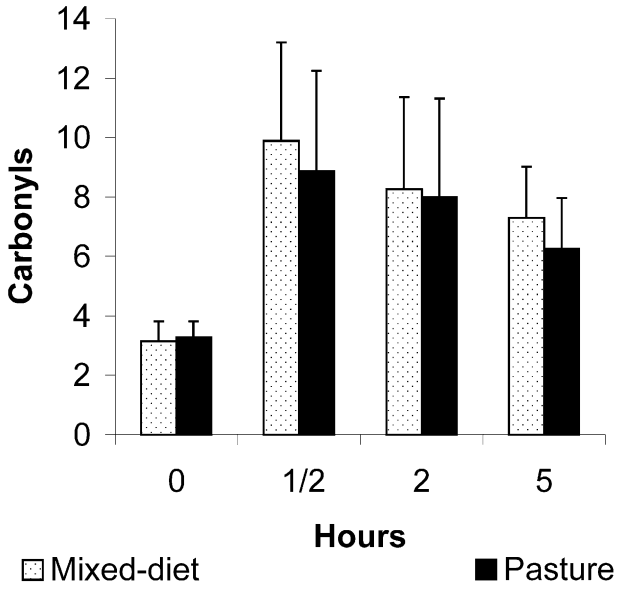

Fig. 2. Effect of finishing diet on carbonyls level (nanomoles DNPH/mg protein)in beef homogenates oxidezed by Fe^{2+}/H_2O_2.

4. Conclusions

According to this study, the finishing diet of cows has an important effect on the antioxidant status of meat, such as vitamin E content and antioxidant enzyme activities. However, it was suggested that a method combining iron/H_2O_2, with TBA-RS measurements, could be suitable to measure the diet influence in meat oxidation processes. According to these results, a pasture-finishing diet presents some advantages over mixed diet-finishing in terms of lipid oxidation, because antioxidant protection compensates the high pro-oxidant effect of PUFA's. Further investigation on the relation of protein oxidation and nutritional status of cattle would be of interest.

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