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Effect of dietary lipid and vitamin E supplementation on free radical production and lipid oxidation in porcine muscle microsomal fractions

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The effect of dietary oxidised lipid and α-tocopherol on iron-induced free radical production and on lipid oxidation in porcine muscle was investigated. Free radical production and lipid oxidation were significantly lower in muscle microsomes from pigs fed an α-tocopherol-supplemented diet (200 mg α-tocopherol acetate/kg diet) compared to microsomes from pigs fed a control diet (10 mg α-tocopherol acetate/kg diet). The susceptibility of pork lipids to oxidise during refrigerated storage was also significantly lower in chops from pigs fed the α-tocopherol-supplemented diet. The inclusion of oxidised corn oil (peroxide value, 4·5 meq/kg diet) in diets did not have a significant effect on the oxidative stability of muscle lipids.

INTRODUCTION

Oxidation of lipids in foods is receiving increased attention as evidence emerges of possible adverse health effects related to consumption of oxidised lipids (Pearson *et al.*, 1983; Frankel, 1984; Sevanian & Peterson, 1986; Addis & Park, 1989). Previous research on muscle foods has focused on the organoleptic aspects of lipid oxidation occurring during refrigerated and frozen storage (Gray & Pearson, 1987; Mottram, 1987). However, greater consumer awareness of the possible health risks associated with consumption of lipid oxidation products (Blume, 1987; Addis, 1990) has given impetus to research into the occurrence of lipid oxidation products, particularly cholesterol oxides, in fresh or processed meats (Dawson & Gartner, 1983; Higley *et al.*, 1986; Pie *et al.*, 1991).

The rate of lipid oxidation in meat products depends on a number of factors, including the polyunsaturated fatty acid content of the muscle (Allen & Foegeding, 1981; Tichivangana & Morrissey, 1985), the presence of prooxidants, notably haem and nonhaem iron (Rhee & Ziprin, 1987; Johns *et al.*, 1989) and ferritin (Decker & Welch, 1990; Kanner & Doll, 1991), and the presence of antioxidants (Enser, 1987). While the identity of the initiators of oxidation in biological systems remains unclear (Asghar *et al.*, 1988; Hsieh & Kinsella, 1989), the free radical nature of lipid oxidation once initiated is long established (Uri, 1961; Smith, 1981).

In muscle, the membrane phospholipids are believed to be the major contributors to lipid oxidation (Igene *et al.*, 1980; Pikul *et al.*, 1984). The high polyunsaturated fatty acid content of phospholipids makes them particularly susceptible to oxidation. Oxidation of muscle cholesterol, 60–80% of which is associated with the subcellular membranes (Hoelscher *et al.*, 1988), may be initiated by free radicals generated during the oxidation of neighbouring polyunsaturated fatty acids (Smith, 1981). Vitamin E (α-tocopherol) functions as a free radical quencher in biological cells (Machlin, 1984); its localisation within the phospholipid bilayer of cell membranes provides a means of controlling lipid oxidation at a likely initiation site (Hafeman & Hoekstra, 1977). Animal tissues respond to dietary α-tocopherol levels (Bieri, 1972; Machlin *et al.*, 1979; Jensen *et al.*, 1988) and α-tocopherol supplementation of pig diets

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has been shown to elevate, significantly, the α-tocopherol content of the subcellular membranes of porcine muscle (Monahan *et al.*, 1990).

The primary objective of the present study was to investigate the effect of inclusion of oxidised lipid or α-tocopherol in pig diets on free radical production in the microsomal membrane fractions of porcine muscle using electron spin resonance (ESR) techniques. The effect of these dietary treatments on lipid oxidation in pork chops during refrigerated storage was also examined.

MATERIALS AND METHODS

Reagents

α-(4-Pyridyl-1-oxide)-*N*-tert-butylnitrone (4-POBN) and 2-thiobarbituric acid were purchased from Sigma Chemical Co. Ltd, St Louis, MO. All other chemicals were of 'AnalaR' grade obtained from Sigma Chemical Co. Ltd, St Louis, MO; Fisher Scientific, Fair Lawn, NJ or E. M. Science, Gibbstown, NJ.

Animals and diets

Eighteen Yorkshire × Landrace pigs, approximately 4 months old and averaging 30 kg in weight, were divided into three groups of six. One group was fed a control diet (Table 1). The other groups received diets which differed from the control in either the degree of oxidation of the dietary corn oil (oxidised oil diet) or in the concentration of dietary α-tocopherol (α-tocopherol-supplemented diet). Pigs were given feed and water *ad libitum*. Before slaughtering, blood samples were taken from each pig into 10 ml heparinised tubes.

Table 1. Composition of diets

Ingredient	Percentage of diet		
	Control	Oxidised oil	α-tocopherol supplemented
Ground shelled corn	70·9	70·9	70·9
Soybean meal	22·5	22·5	22·5
Corn oil (fresh)[a]	3·0	—	3·0
Corn oil (oxidised)[a]	—	3·0	—
Mono-dicalcium phosphate[b]	1·5	1·5	1·5
Calcium carbonate	1·1	1·1	1·1
Sodium chloride	0·3	0·3	0·3
Vitamin-trace mineral mix	0·5	0·5	0·5
Selenium 90 premix[c]	0·15	0·15	0·15
Vitamin E (BASF 50%)[d]	0·002	0·002	0·04
Aureomycin 50	0·05	0·05	0·05

[a] All diets contained 3% corn oil. Oxidised corn oil was prepared by bubbling air through the fresh oil (peroxide value, 2 meq/kg oil) at 80°C until a peroxide value of 150 meq/kg oil was reached.
[b] Mixture of mono- and dicalcium phosphate salts and contains 18% calcium and 21% phosphorous.
[c] Supplied 0·3 mg Se/kg diet.
[d] α-Tocopherol acetate was obtained from BASF Corporation, Parsippany, NJ.

The blood was centrifuged within 30 min (15 min, 1500 × *g*, 4°C) and the plasma fraction was stored at −20°C. The pigs were slaughtered after twelve weeks on their respective diets at a commercial slaughtering facility. The average weight of the pigs was 98 kg. The carcasses were chilled overnight and one loin was removed from each carcass. The *Longissimus dorsi* muscle was isolated and stored at −20°C until required.

Preparation of muscle microsomes

Muscle microsome fractions were obtained as previously described (Kalyanaraman *et al.*, 1979). Microsomes were not purified after the final ultracentrifugation step.

Determination of α-tocopherol

Concentrations of α-tocopherol in plasma and in muscle microsomes were determined using the extraction procedures of Bieri *et al.* (1979) and Buttriss and Diplock (1984), respectively, and quantitation by HPLC (Monahan *et al.*, 1992).

Electron spin resonance (ESR) studies

Microsomal suspensions, at a protein concentration of 14·0 ± 0·1 mg/ml, were prepared in a total volume 0·6 ml 0·1M potassium phosphate buffer, pH 7·4, containing 100 mg/litre POBN and 1 mg/litre $FeCl_2$ and incubated at 37°C. Following the addition of $FeCl_2$, 0·3 ml aliquots were removed from the incubation medium at 30 min intervals over a 90 min period. ESR spectra were recorded at room temperature (21·0 ± 0·5°C) in a flat quartz cell with a Varian E-line Century Series, Model 112, X-band spectrometer operating under the following conditions: gain 2·5 × 10^4, microwave power, 20 mW; modulation amplitude, 0·8 G, modulation frequency, 100 KHz, time constant, 0·5 s; scan time, 4 min. The spectra were recorded over a 100 G sweep range. The spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), was used for sweep calibration. Microsomal lipid oxidation was monitored by measuring 2-thiobarbituric acid reactive substances (TBARS) (Buege & Aust, 1978) in incubations identical to those used in the ESR studies except that the spin trap was omitted.

Lipid oxidation in pork

Pork chops were stored in refrigerated display as described previously (Monahan *et al.*, 1992). Lipid oxidation in pork chops was assessed by the 2-thiobarbituric method of Ke *et al.* (1977). TBARS were expressed as mg malonaldehyde per kg muscle.

Statistical analysis

Statistical significance of the difference between mean values was determined using Fisher's LSD test (Steel & Torrie, 1980).

RESULTS AND DISCUSSION

α-Tocopherol levels in plasma and muscle microsomal fractions

Dietary α-tocopherol supplementation significantly increased the α-tocopherol content of microsomal cell membranes ($P < 0.05$) (Table 2). The α-tocopherol content of muscle microsomes from pigs receiving the supplemented diet was almost 7-fold higher than that of pigs fed the control diet. Previous studies with pigs (Monahan *et al.*, 1990) and chickens (Asghar *et al.*, 1989) have shown that dietary α-tocopherol supplementation significantly increases the α-tocopherol content of muscle microsomal membranes. The α-tocopherol content of plasma and muscle of pigs fed the oxidised dietary oil was lower than that of pigs fed the control diet but mean values were not significantly different ($P > 0.05$) as reported earlier (Monahan *et al.*, 1992).

ESR studies

The effect of dietary α-tocopherol supplementation on formation of the 4-POBN-free radical adduct is shown in Fig. 1. The rate of free radical formation was higher in microsomes from pigs fed the control diet (10 mg α-tocopherol acetate/kg diet) compared with pigs fed the supplemented diet (200 mg/kg). Mean values of signal height were significantly different ($P < 0.05$) after 60 and 90 min of incubation. Figure 2 shows typical spectra obtained from microsomes prepared from the muscle of control and α-tocopherol-supplemented pigs. The spectra consisted of a triplet of doublets, the basic triplet arising from the interaction of the unpaired electron with the α-nitrogen of 4-POBN and the secondary splitting from interaction with the β-hydrogen. The hyperfine splittings were $a = 15.8$ G and $a = 2.7$ G for α-nitrogen and β-hydrogen, respectively. The ESR spectra obtained in this study are similar to those obtained in earlier studies in which liver microsomes were incubated with NADPH (Rosen & Rauckman, 1981) or lipoxygenase (Kalyanaraman *et al.*, 1979) and in linoleic acid/lipoxygenase systems (Connor *et al.*, 1986).

Table 2. Effect of dietary oxidised lipid and α-tocopherol supplementation on α-tocopherol levels in porcine plasma and muscle microsomes

Dietary treatment	α-Tocopherol concentration	
	Plasma ($n = 6$) (μg/ml)	Microsomes ($n = 3$) (ng/mg protein)
Control	0·53	15·41
Oxidised oil diet	0·20[a]	9·11[a]
α-Tocopherol-supplemented diet	3·54[b]	102·3[b]

[a] Mean values are not significantly different from the control ($P < 0.05$).
[b] Mean values are significantly different from the control ($P > 0.05$).

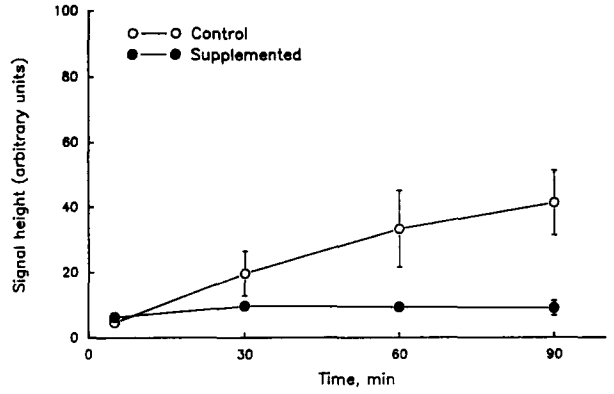

Fig. 1. Effect of dietary α-tocopherol supplementation on $FeCl_2$-induced free radical production in muscle microsomes.

Identification of free radical species trapped by nitrones, such as 4-POBN, is often difficult because radicals add to the carbon adjacent to the nitrogen, rather than directly to nitrogen as is the case with nitroso spin traps, with the result that the spectral line pattern is the same for different free radical species (Davies, 1987). The magnitude of splitting is determined by the size and electrophilicity of the particular free radical and by the nature of the solvent in which it is dissolved. In the present study, trapping of hydroxyl ($OH^{\cdot}$) or superoxide ($O_2^{\cdot-}$) can be excluded on the basis of hyperfine splitting constants (Finkelstein *et al.*, 1980) and because of the short-lived nature of these spin trapped adducts (Finkelstein *et al.*, 1979). Free radical adducts of 4-POBN with hyperfine splittings similar to those observed in this study have, in the past, been assigned to either a lipid dienyl- (Poli *et al.*, 1987) or lipid peroxy- (Rosen & Rauckman, 1981; Miyazawa *et al.*, 1985) adduct of 4-POBN. However, the assignments of Rosen and Rauckman (1981) and Miyazawa *et al.* (1985) have been disputed by Connor *et al.* (1986). The results of isotopic substitution studies (Connor *et al.*, 1986) and the instability of peroxy radical adducts of α-phenyl-*N*-*tert*-butylnitrone (PBN) (Niki *et al.*, 1983), which is structurally similar to 4-POBN, suggest that the free radical species trapped during the oxidation of unsaturated lipids is more likely to be a carbon-centred alkyl or lipid dienyl radical than a lipid peroxy radical. However, the possibility that the free radical derives from a protein or other molecules in the microsomal

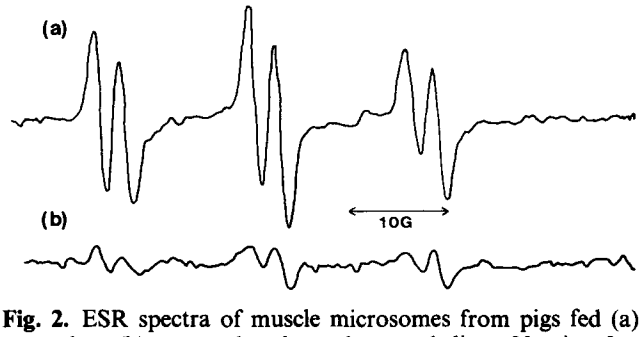

Fig. 2. ESR spectra of muscle microsomes from pigs fed (a) control or (b) α-tocopherol-supplemented diets, 90 min after addition of $FeCl_2$.

preparation cannot be ruled out. In addition, the detection of a particular 4-POBN-free radical in this system does not mean that this radical species is the most significant in quantitative terms, merely that it reacts preferentially with the spin trap under the particular experimental conditions. On the other hand, the relatively narrow line pattern of the spectra obtained in this study is indicative of a nitroxide label with a good degree of rotational mobility (Knowles *et al.*, 1976). Rotational correlation times, τ, calculated from spectral line widths and line heights (Keith *et al.*, 1970), were of the order of 0·3 ns for the 4-POBN-free radical adduct. This suggests that the spin-trapped radical is less likely to be buried within a protein structure because such binding would lead to greater immobilisation of the nitroxide group and a longer rotational correlation time (Knowles *et al.*, 1976).

Despite the uncertainty regarding the nature of the free radical species trapped in the present experiments, the results show that in muscle microsome fractions, dietary α-tocopherol supplementation led to a suppression in the production of free radicals which have the potential to initiate lipid oxidation or facilitate the propagation of lipid oxidation once initiated (Aust & Svingen, 1982; Halliwell & Gutteridge, 1990).

The time course of lipid oxidation as monitored by the formation of TBARS is illustrated in Fig. 3. In agreement with the ESR results, muscle microsomes from control pigs were more susceptible to oxidation than those from α-tocopherol-supplemented pigs having significantly higher ($P < 0{\cdot}01$) levels of TBARS after 30, 60 and 90 min of incubation.

Compared with the control pigs, free radical production appeared to be higher in muscle microsomes from pigs receiving the oxidised oil (Fig. 4). Mean signal height values were only significantly different ($P < 0{\cdot}05$) after 90 min of incubation. Oxidised dietary lipid did not affect the production of TBARS when microsomes from pigs fed the oxidised corn oil-containing diets were compared with those from pigs receiving the control diet (Fig. 5). The results of previous studies on feeding oxidised lipids to animals have often been inconclusive and it appears that the toxic effects of dietary peroxides, particularly their effect on tissue lipid

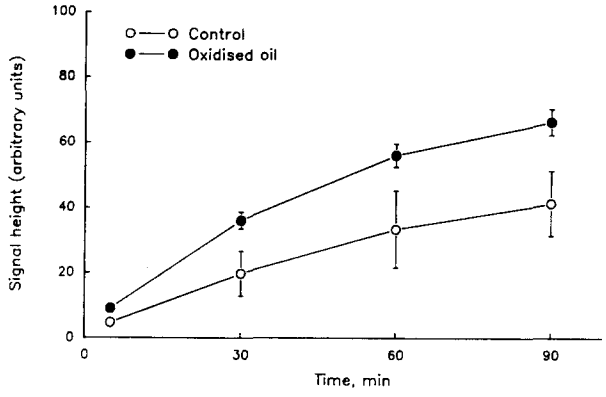

Fig. 4. Effect of oxidised dietary lipid on $FeCl_2$-induced free radical production in muscle microsomes.

peroxidation *in vivo*, may depend on the type of oxidised lipid which is fed and on the degree to which it has been oxidised (Kubow, 1990). It has been found that the effect of feeding oxidised dietary fats to pigs on lipid oxidation in post-mortem muscle depends on the peroxide value of the dietary fat (Buckley *et al.*, 1989; Monahan *et al.*, 1992).

Lipid oxidation in pork

The data in Table 3 show the effect of the various treatments on lipid oxidation in uncooked pork chops during refrigerated storage. TBARS values were significantly higher ($P < 0{\cdot}01$) in pork chops from pigs fed the control diet compared with pigs fed the α-tocopherol supplemented diet after 4 and 8 days of storage. TBARS values of pork chops from pigs fed the control diet and oxidised oil-containing diet were not significantly different.

The free radical quenching capability of α-tocopherol demonstrated in this study may effectively retard the oxidation of membrane phospholipids which are significant contributors to lipid oxidation in meats (Pikul *et al.*, 1984). Since muscle cholesterol is closely associated with membranal phospholipids, and its susceptibility to oxidation is believed to be influenced by oxidation of the surrounding lipids (Smith, 1981), dietary α-tocopherol may also offer significant protection

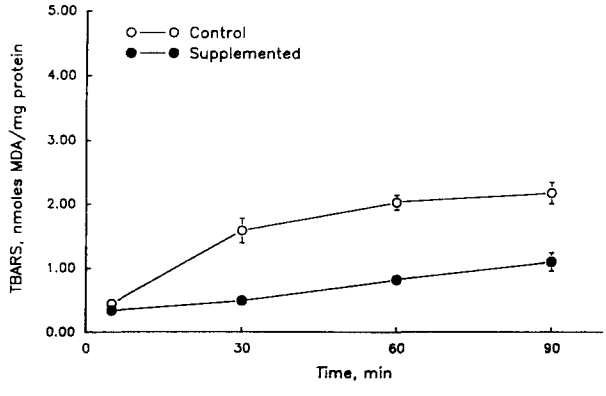

Fig. 3. Effect of dietary α-tocopherol supplementation on $FeCl_2$-induced lipid oxidation in muscle microsomes.

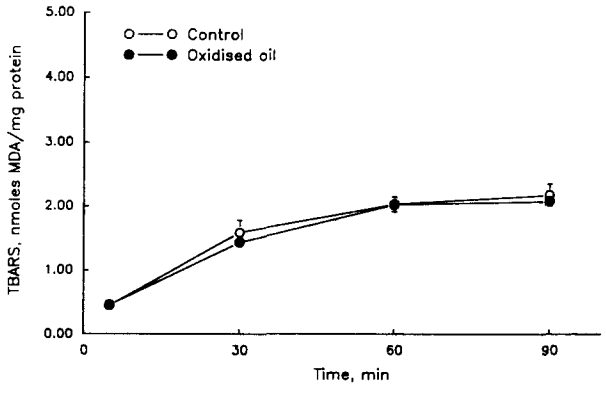

Fig. 5. Effect of oxidised dietary lipid on $FeCl_2$-induced lipid oxidation in muscle microsomes.

Table 3. Effect of dietary oxidised lipid and α-tocopherol supplementation on TBARS development in uncooked pork chops stored at 4°C for 8 days

Dietary treatment	TBARS		
	Day 0	Day 4	Day 8
Control	0·19	0·36	0·74
Oxidised oil diet	0·23[a]	0·49[a]	0·75[a]
α-Tocopherol-supplemented diet	0·16[a]	0·19[b]	0·16[b]

[a] Mean values are not significantly different from the control ($P > 0.05$)
[b] Mean values are significantly different from the control ($P < 0.05$).

against cholesterol oxidation, particularly when meats are exposed to processing conditions that promote oxidation, for example, grinding or cooking. Higley *et al.* (1986) demonstrated that processed meats contain significant levels of cholesterol oxides. In support of the contention that membranal α-tocopherol may suppress oxidation of cholesterol, cholesterol oxide formation was found to be lower in muscle from pigs fed α-tocopherol supplemented diets (Monahan *et al.*, 1992).

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