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Significance of Vitamin E Supplementation, Dietary Content of Polyunsaturated Fatty Acids, and Preslaughter Stress on Oxidative Status in Pig As Reflected in Cell Integrity and Antioxidative Enzyme Activities in Porcine Muscle

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The present study investigates the combined effects of feed-induced increase in polyunsaturated fatty acids (PUFA) content and/or α-tocopherol content in pig muscles and preslaughter stress on cell integrity. Cell integrity was determined by plasma lactate dehydrogenase (LDH) activity, and antioxidative status of muscle was measured by activities of the antioxidative enzymes catalase, superoxide dismutase, and glutathione peroxidase. Preslaughter stress increased LDH activity, reflecting loss in cell membrane integrity independent of increased content of PUFA and/or α-tocopherol. However, feed-induced increase of PUFA decreased the LDH activity in plasma immediately after slaughter. Catalase activity in the muscle tissue increased as a consequence of the high-PUFA diet, which may indicate an increased demand caused by introduction of oxidative labile PUFA.

KEYWORDS: Pig; stress; vitamin E; antioxidative enzymes; LDH; PUFA

INTRODUCTION

Both increased polyunsaturated fatty acids (PUFA) (*1*, *2*) and physical stress (*3*) are known to increase the oxidative susceptibility of lipids in muscle tissue, whereas α-tocopherol has been shown to counteract increased susceptibility (*2*, *4*−*6*). Physical stress (exercise) increases lactate dehydrogenase (LDH) activity in the serum (*7*−*9*). Because LDH is a good marker of cell integrity (*8*), decreased cell membrane integrity may reflect oxidative stress in agreement with the fact that lipid oxidation products have been shown to correlate inversely with membrane fluidity (*10*). The antioxidative status of pig muscles at the time of slaughter is found to be critical for the shelf life and technological quality of pork (*11*, *12*). Consequently, a superior antioxidative status of pigs is a target in production of high-quality pork. The activities of the antioxidative enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) are known to contribute to the sustainment of a delicate oxidative balance in biological tissue upon exposure to stress conditions (*13*). However, at present only little is known about how the antioxidative status of the animal is affected by changes in the oxidative stress for an extended period of time. Diet-induced elevation of PUFA, which are more prone to oxidation, may represent a factor requiring improved antioxidative capacity, as shown by induction of antioxidative enzyme activities in both pig (*9*) and chicken muscles (*14*). Likewise, an elevated tissue level of α-tocopherol may be expected to counteract the oxidative stress response in the muscle tissue by a reduction in the requirement for antioxidative enzyme activity (*14*). The aim of the present study was to investigate the combined effects of increased muscle PUFA content, increased α-tocopherol level, and preslaughter stress on cell integrity, measured as release of LDH activity in serum and the activities of antioxidative muscle enzymes perislaughter.

MATERIALS AND METHODS

A more detailed description of animals, management, biopsy sampling, and slaughter procedure used in the present study is described elsewhere (*15*).

Animals and Management. The 56 female pigs (cross-breeds between Danish Landrace × Danish Yorkshire sows and Duroc boars being noncarriers of the Halothane gene), from 14 four-animal litters, used in the present study were reared at the experimental farm of The Research Center Foulum, The Danish Institute of Agricultural Sciences.

The pigs were allocated to eight different treatments in a 2 × 2 × 2 incomplete balanced block design. The three different factors were (i) diet (see **Table 1**), (ii) vitamin E, and (iii) exercise. The two diets used were a standard grower-finishing diet produced at Research Center Foulum (control diet) and a diet with a high content of inulin (Raftiline HP, Orafti, Belgium), rape seed meal, and animal fat to provide a diet high in PUFA (high-PUFA diet). Two levels of vitamin E were included in the experimental feed: normal levels according to Danish practice

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Table 1. Ingredients and Chemical Composition of Control and High-PUFA Diets

	control[a]	high PUFA
ingredient[b]		
rape seed cake		55.0
soybean meal	18.5	5.0
raw potato starch		6.0
inulin		25.0
barley	57.3	
sugar beet molasses	1.0	
wheat	20.9	
animal and vegetable fat	2.1	8.0
calcium carbonate		0.5
NaCl		0.3
vitamin mineral mixture[c]	0.2	0.2
chemical composition[b]		
gross energy (MJ/kg of DM)	18.7	21.8
net energy[d] (MJ/kg of DM)	9.6	10.8
DM content (%)	89.1	91.9
ash (%)	5.3	5.1
crude protein (N × 6.25) (%)	19.5	20.2
crude fat (%)	5.4	18.2
starch (%)	49.9	5.4
mono- and disaccharides (%)	3.5	3.8
raffinose oligosaccharides (%)	0.9	1.4
inulin (%)		25.4
DF[e] (%)	17.9	21.1
NCP[f] (%)	12.1 (4.8)	11.0 (4.5)
cellulose (%)	3.3	4.5
Klason lignin (%)	2.5	5.5

[a] Supplemented with 0.16% lysine in the form of 40% lysine + 60% wheat bran and 0.04% D/L-methionine in the form of 40% D/L-methionine + 60% wheat bran. [b] Ingredients in percent and chemical composition in percent of dry matter (DM). [c] Solovit Mikro 106 containing 2,500,000 IU of vitamin A, 500,000 IU of vitamin D_3, 30 g of vitamin E, 1.1 g of vitamin K_3, 1.1 g of vitamin B_1, 2 g of vitamin B_2, 1.65 g of vitamin B_6, 5.5 g of D-pantothenic acid, 11 g of niacin, 27.5 mg of biotin, 11 mg of vitamin B_{12}, 25 g of Fe, 40 g of Zn, 13.86 mg of Mn, 10 g of Cu, 99 mg of I, and 150 mg of Se per kg. [d] Determined according to ref *39*. [e] Dietary fiber, the sum of non-cellulose polysaccharides, cellulose, and Klason Lignin. [f] Non-cellulose polysaccharides; values in parentheses denote the content of soluble NCP.

(addition of 60 mg of α-tocopherol/kg of feed) or supranutritional levels (addition of 400 mg of α-tocopherol/kg of feed). The vitamin E used was *all-rac*-α-tocopheryl acetate, silicon dioxide (generously provided by Roche, Hvidovre, Denmark). Finally, the animals were either not exercised or exercised on a treadmill immediately prior to slaughter to simulate preslaughter stress.

The pigs were housed in individual pens from 25 kg and fed the standard grower-finishing diet until a live weight of ~80 kg, when the experimental finishing feeding period was initiated. They were fed semi ad libitum to a standard scale (feed units per day according to live weight of the pig) of Research Center Foulum (*16*, *17*). The experimental feeding was then initiated by a 1-week acclimatization period (days 1−7), gradually changing from the standard grower-finishing diet into 100% of either the control diet supplemented with supranutritional vitamin E levels, the high-PUFA diet, or the high-PUFA diet supplemented with supranutritional vitamin E levels. The diet change was adjusted to the individual pig's eating behavior. Control pigs supplemented with normal levels of vitamin E were given the standard grower-finishing diet during the whole test period (days 1−22). Ingredients and compositions of the diets, analyzed as described in ref *18*, are presented in **Table 1**. Pigs were slaughtered, at an average live weight of 104 kg, on day 22, 15−22 h after feed withdrawal.

The experiment was approved by The Danish Inspectorate of Animal Experimentation, and the pigs were treated in accordance with the guidelines outlined by the same authority.

Muscle Biopsy Sampling. M. longissimus dorsi (LD) biopsy samples (300−500 mg) for determination of antioxidative enzymes were taken using a spring-loaded biopsy instrument (Biotech Ltd. Igor Merheim, Slovakia) on day 1 prior to the diet change (time = 1), on day 21 (time = 2), 1 min post-mortem (time = 3) (moment of death is defined as after bleeding), 4 h post-mortem (time = 4), and 8 h post-mortem (time = 5). The biopsies were taken at the last rib on day 1 and at a site ~5 cm from the first biopsy in the caudal or cranial direction on days 21 and 22. Immediately after sampling, the biopsies were frozen in liquid nitrogen and stored at −80 °C until analysis.

Blood Sampling. Blood was sampled for determination of LDH from the jugular vein into EDTA-coated vacuum tubes on day 1 prior to the diet change (time = 1) and day 21 (time = 2). On day 22, blood was sampled during exsanguination at the abattoir (time = 3). Plasma was prepared by centrifugation at 1000*g* for 15 min at 4 °C and stored at −80 °C until analysis.

Slaughter Procedure. Pigs from the same litter were slaughtered on the same day. One or two litters were slaughtered on the same day. On the day of slaughter, the pigs were transported from the rearing house to the experimental slaughter plant (200 m), where they were penned individually. Immediately prior to slaughter, half of the pigs on each slaughter day were exercised on a treadmill at a continuously increasing speed (up to 7 km/h) until exhaustion (evaluated to be when breathing and stride became uncoordinated). Consequently, the distance and time the pigs exercised varied. The pigs were led to the treadmill or directly to the stunner without mixing with other pigs. The pigs were slaughtered at random, although alternating between exercised and nonexercised pigs. Order of slaughter was not included in the statistical analysis. A biopsy was taken in each pig immediately prior to stunning. The pigs were stunned by 85% CO_2 for 3 minutes, exsanguinated, scalded at 62 °C for 3 minutes, cleaned, and eviscerated within 30 min. The carcasses were placed in a chill room at 4 °C. Forty-five minutes post-mortem a sample of LD (10 g) was taken at the last rib (α-tocopherol analysis), and 24 h post-mortem, a sample (2 g) was taken 3 cm from the last rib in the cranial direction (fatty acid analysis).

Fatty Acid Composition. Fatty acids were determined by gas chromatographic separation and quantification as described (*19*).

α-Tocopherol in the Diet and LD. The α-tocopherol contents in the diets and LD were determined as described (*20*).

LDH Activity. The activity of LDH was determined using pyruvate and NADH as substrates according to principles outlined in ref *21*. LDH activity is expressed as millimoles per liter of plasma.

CAT, GSH-Px, SOD Activities. One hundred milligrams of frozen tissue in 0.8 mL of homogenization buffer (0.05 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose) was homogenized on ice with an Ultraturrax for 5 s at 13500 rpm. The homogenate was centrifuged at 10000*g* for 30 min at 4 °C, and the supernatant was stored at −80 °C until use. The antioxidative enzymes were all assayed in microtiter plates essentially as described: CAT activity was measured spectrophotometrically at 240 nm as a decrease in H_2O_2 concentration (*22*); GSH-Px activity was measured as the rate of NADPH consumption at 340 nm through coupling with added glutathione reductase (*23*); and the activity of SOD as inhibition of xanthine/xanthine oxidase-mediated oxidation of cytochrome *c* was measured at 560 nm (*24*). All samples were measured in triplicate at appropriate dilutions in homogenization buffer to give activities of the enzymes in the linear range of standard curves constructed with pure enzymes (Sigma, St. Louis, MO). Protein content of homogenates was determined using the BCA assay (Pierce, Rockford, IL) and bovine serum albumin as standard. Activities of CAT, SOD, and GSH-Px are expressed as units per milligram of protein homogenate.

Statistical Analyses. The statistical analysis was carried out with the Statistical Analysis System version 8.00 (SAS Institute, Cary, NC). The MIXED procedure was applied when least-squares means and standard errors of all variables were calculated. A model including the fixed effects of diet, exercise, vitamin, and time as well as their interactions, the random effect of litter, and repeated effect of time (times 1−5 depending on the variable) with animal as subject was applied for the variables LDH, GSH-Px, SOD, and CAT. A model including the fixed effects of diet and exercise as well as their interactions and the random effect of litter was applied for fatty acids. To obtain the least-squares means for all of the experimental groups, the statistical models were not reduced in the cases when a fixed effect or an interaction of the fixed effects was found to be nonsignificant.

Table 2. LS Means ($n = 14$) of Fatty Acid Composition (Percent of Total Fatty Acids Identified) and Calculated PUFA Content in M. longissimus dorsi of Pigs Fed Control and High-PUFA Diets[a]

	control		high PUFA		
fatty acid	normal	supra	normal	supra	SEM
C8	0.02	0.02	0.02	0.02	0.001
C10	0.13a	0.13a	0.11b	0.11b	0.003
C12	0.08	0.08	0.08	0.08	0.002
C14	1.36	1.38	1.33	1.31	0.03
C14:1	0.03	0.03	0.03	0.03	0.001
C15	0.05a	0.06a	0.07b	0.07b	0.003
C16	25.98a	26.03a	23.75b	23.78b	0.23
C16:1	3.81a	3.70a	2.94b	3.00b	0.11
C17	0.22a	0.24a	0.31b	0.31b	0.013
C17:1	0.25	0.27	0.25	0.26	0.008
C18	11.12a	11.64b	10.79a	10.92a	0.21
C18:1ω9	43.11a	42.09b	40.55c	41.12c	0.38
C18:1ω7	4.37a	4.22a	3.93b	3.98b	0.07
C18:2	6.26a	6.77a	11.85b	10.75b	0.45
C18:3ω6	0.08a·b	0.09a	0.07b	0.06b	0.006
C18:3ω3	0.35a	0.37a	1.35b	1.27b	0.050
C18:4	0.114a·b	0.118a	0.108b·c	0.105c	0.003
C20	0.11	0.10	0.11	0.11	0.01
C20:1	0.72a	0.66b·c	0.70a·c	0.75a	0.02
C20:2	0.19a	0.20a	0.29b	0.28b	0.01
C20:3ω3	0.06a	0.06a	0.15b	0.15b	0.005
C20:3ω6	0.21a·b	0.23b	0.19a	0.17a	0.01
C20:4	0.93	0.92	0.84	0.78	0.06
C20:5	0.18	0.21	0.17	0.18	0.02
C24	0.27	0.27	0.28	0.29	0.02
PUFA	8.35a	9.01a	15.11b	13.75b	0.93

[a] Within a row, means without a common letter differ ($P < 0.05$).

Least-squares means were calculated and considered to be significantly different if $P < 0.05$.

RESULTS

Fatty Acid Composition and α-Tocopherol Content. Pigs fed the high-PUFA diet did increase PUFA content in muscle tissue (LD) compared to that of control pigs ($P < 0.0001$) (**Table 2**). The fatty acid mainly contributing to this difference was linoleic acid (C18:2). The α-tocopherol content of the supradiets was increased by 5−6-fold compared to control and high-PUFA diets (*15*). In LD from pigs fed the supplemented diets, the α-tocopherol content was significantly increased ($P < 0.0001$) by 38 and 50% in control and high-PUFA diets, respectively (*15*). No effect of exercise on α-tocopherol levels was found.

LDH in Plasma. The LDH activity in plasma was lower in pigs fed the high-PUFA diet compared to the control diet ($P = 0.001$); however, both exercise ($P = 0.04$) and slaughter ($P < 0.001$) increased plasma LDH activities compared to the respective controls (**Table 3**).

Antioxidative Enzymes. The activities of SOD, CAT, and GSH-Px in LD were not systematically different between groups before the change to experimental diets (time 1 in **Table 4**). Independent of diet, supplementation, and exercise, the activity of CAT was the same before initiation of experimental diet (10.6a units/mg of protein) and the day before slaughter (11.1a units/mg of protein), increased significantly during exsanguination (12.5b units/mg of protein), and decreased again 4 h (9.3c units/mg of protein) and 8 h (9.4c units/mg of protein) after slaughter. As a whole, the activity of CAT was higher in tissue from the high-PUFA-fed pigs irrespective of vitamin E supplementation and exercise compared to that of control pigs ($P = 0.01$ for times 2, 4, and 5 in **Table 4**). The activities of SOD and GSH-Px in tissue from pigs was not affected by either feed, exercise, or α-tocopherol.

Table 3. LS Means ($n = 7$) of LDH in Plasma from Pigs Fed either Control or High-PUFA Diets (± α-Tocopherol; Normal/Supra) of Which Half Were Exercised (+ Ex)[a]

	control				high PUFA				
time[b]	normal		supra		normal		supra		SEM
1	966ab	1030a	995ab	895ab	806b	1051a	922ab	880ab	90–94
2	959ab	1068a	1020a	892ab	889ab	902ab	893ab	777b	74–83
	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	
3	1522b	1899a	1369bc	1568ab	1181c	1406bc	1336bc	1501bc	123–133

[a] Within a row, means without a common letter differ ($P < 0.05$). LDH activities are expressed as mmol/L plasma. [b] Data are from blood samples taken the day before initiating the experiment (time = 1), the day before slaughter (i.e., before exercise) (time = 2), and during exsanguination (time = 3).

DISCUSSION

Fatty acid and vitamin E compositions of the diet are directly reflected in the tissue of pigs. Consequently, the content of both α-tocopherol (*2*, *19*) and PUFA (*2*, *19*, *25*, *26*) in the pig diet is reflected in the pork, as also seen in the present study. The diets resulted in two different α-tocopherol levels and two different PUFA levels (mainly due to differences in content of C18:2) in the meat. The preslaughter exercise procedure used in the present study exposed the pigs to physical stress, which is known to increase the oxygen intake ∼10−15-fold above rest (*27*) and increase oxygen flux in active peripheral skeletal muscle fibers 100−200-fold (*28*). Such an elevation in oxygen concentration is assumed to increase the oxidative stress in muscle (*27*, *29*) and serum (*30*), which simultaneously introduce an ischemic-like situation similar to what occurs post-mortem in pig muscle. Both situations are known to increase the oxidative stress through increased formation of reactive oxygen species (*13*, *31*). Exercise-induced reduction in vitamin E levels of rat tissue has been reported (*3*); however, such an effect could not be observed on α-tocopherol levels in muscle from exercised pigs in the present study.

Extended exposure to stressors, for example, training, has previously been reported to affect the antioxidative status of biological fluids and tissue; thus, SOD activities have been reported to increase in the coronary arterioles of pigs (*32*), and CAT activity has been shown to increase in muscle tissue of rats (*33*) upon exposure to training. Similar training-induced responses are most likely to be ascribed to adaptation to the stressor. The hypothesis of adaptation over a longer period of time may be extended to include other stressors, for example, diets containing mildly toxic compounds or high levels of unsaturated fatty acids, which are more prone to oxidation, and may induce comparable changes in the antioxidative status of muscle, even though this area is poorly investigated. The observed increase in CAT activity in muscle with high PUFA content in the present study may be an example of this and support previous observations of increased CAT activity in muscle tissue of pigs (*9*) as well as increased GSH-Px in tissue from chickens (*14*). In both of these studies, high tissue PUFA content was obtained through diets high in PUFA. Similar changes have been observed in antioxidative enzyme activity in erythrocytes of humans eating plant-based materials (*34*), which most probably contain mildly toxic compounds (*35*).

Diets high in PUFA content have been found to increase lipid oxidation in porcine muscle tissue, wheres α-tocopherol supplementation decreases lipid oxidation (*2*, *4*). The reduced oxidative

Table 4. LS Means ($n = 7$) of Activities (Units per Milligram of Protein) of the Antioxidative Enzymes Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (Milliunits per Milligram of Protein) (GSH-Px) in M. longissimus dorsi[a]

	control				high PUFA				
time[b]	normal		supra		normal		supra		SEM
SOD									
1	1.97	1.77	1.86	2.00	1.97	1.84	1.90	2.40	0.27–0.31
2	2.01	2.14	1.92	2.58	2.38	2.56	2.19	2.28	0.27–0.31
	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	
3	2.12	1.86	2.78	2.34	2.12	2.88	2.53	2.37	0.27–0.31
4	2.82	2.61	2.65	2.93	2.49	2.63	3.11	2.73	0.27–0.31
5	2.85	3.12	3.07	2.30	2.38	2.85	2.43	2.50	0.27–0.31
CAT									
1	11.28	11.00	10.16	11.48	10.60	9.75	9.92	10.62	0.76–0.88
2	10.03a	10.12a	10.89ab	10.02a	10.59a	13.36b	12.42ab	11.21ab	0.89–1.02
	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	
3	11.93ab	10.56ab	8.23a	17.93c	11.22ab	12.45ab	14.25bc	13.40bc	1.69–1.94
4	8.43ab	8.25ab	7.97a	8.22ab	10.11bc	9.99abc	10.60c	10.54c	0.70–0.80
5	7.89a	8.48ab	8.43ab	7.61a	10.11bc	10.77c	11.09c	11.21c	0.69–0.79
GSH-Px									
1	6.74	5.76	4.61	5.05	4.57	5.85	4.63	5.32	1.20–1.36
2	8.45	7.48	6.10	5.52	5.50	8.20	11.18	6.84	2.44–2.81
	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	− Ex	+ Ex	
3	11.22	7.68	6.84	11.76	6.64	6.66	13.46	9.41	2.96–3.41
4	7.92	4.68	6.32	4.90	6.90	6.50	13.44	7.79	2.60–2.99
5	7.90	7.04	6.08	3.38	5.47	6.72	6.54	9.20	2.27–2.61

[a] Within a row, means without a common letter differ ($P < 0.05$). [b] Data are from biopsies taken the day before initiating the experiment (time = 1), the day before slaughter (i.e., before exercise) (time = 2), during exsanguination (time = 3), 4 h after slaughter (time = 4), and 8 h after slaughter (time = 5).

stress on animals supplemented with α-tocopherol for an extended period may also affect the antioxidative status of the animal. However, in this study no effect of α-tocopherol was observed, which is in line with ambiguous results from previous studies of activities of various antioxidative enzymes (*4*, *14*, *36*).

CAT activity decreased continuously post-mortem; however, the decrease was less in tissue/meat from pigs fed the high-PUFA diet. A previous study, however, has indicated that increased CAT activity in tissue the day before and immediately after slaughter, from muscle with high PUFA content, cannot counteract PUFA-induced increases in oxidative susceptibility of meat (*9*).

The lower LDH activity in plasma from pigs on the high-PUFA diet has been reported previously (*9*) and may reflect the increase in the degree of unsaturation of the membrane lipids and the concomitant increase in membrane integrity, considering LDH activity is a good marker of muscle cell integrity (*8*). In the present study a dramatic increase in LDH activity in plasma was obtained upon slaughter, as also has been reported previously (*9*, *37*). This can most probably be explained by the ongoing muscle damage (spasms), which is triggered by the CO_2 stunning procedure (*38*).

Preslaughter stress resulted in an additional increase in LDH activity in the plasma compared to respective levels in non-stressed pigs. This corresponds well with previous studies that also found increased LDH activity in blood upon exposure to a physical stressor (exercise) (*8*, *9*). α-Tocopherol supplementation did not affect the exercise-induced increase of LDH activity in pigs on the high-PUFA diet.

In conclusion, increased content of unsaturated fatty acids in the muscle decreased the LDH activity in plasma, possibly due to increased membrane integrity. CAT activity in the muscle tissue increased as a consequence of the high-PUFA diet, which may indicate an increased demand caused by introduction of oxidative labile PUFA.

ABBREVIATIONS USED

CAT, catalase; GSH-Px, glutathione peroxidase; LD, M. longissimus dorsi; LDH, lactate dehydrogenase; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase.

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