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Title: High feed dose levels of alpha-tocopherol induce the liver tocopherol associated protein (TAP) and the pregnane X receptor (PXR) in cattle.

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Introduction.
The meat industry has accepted that feeding high dietary doses of vitamin E at approximately 10-fold higher than maintenance dose, from 10 IU/d to 1000 IU/d, will improve oxidative stability, color, and overall meat quality in cattle (Greer et al.1998). However, feeding doses of alpha-tocopherol 10-fold above maintenance typically give only a 4-fold increase in liver content and a 3-fold increase in muscle (Arnold et al. 1993). The absorption of tocopherols occurs in the upper small intestinal tract and then transported to the liver (Herrera & Barbas, 2001). Accumulation of the D-alpha form occurs in the liver due to selection by the alpha-tocopherol transfer protein (alpha-TTP) and selective breakdown of the other forms by CYP4F2 (Sontag & Parker, 2002). Non-ruminant studies, have determined that the absorbed alpha-tocopherol is then either, distributed from the liver, or metabolized (Doring et al., 2004). Liver catabolism is regulated by the pregnane X receptor (PxR) which activates the cytochrome P450 xenobiotic enzyme CYP3A4. In humans, the alpha-tocopherol is distributed throughout the body by, very low density lipoproteins (VLDL), chylomicrons, lipoprotein lipase (LPL) multidrug resistance protein 2 (MDR2) and a specific carrier, the tocopherol associated protein (TAP) (Zimmer et al., 2000).

This research project was performed to determine if the extra dietary alpha-tocopherol was affecting gene expression and possibly increasing alpha-tocopherol metabolism in the cattle livers which would explain the 10-fold diet to 4-fold liver concentration difference. Metabolic activity related to transport was measured according to alpha-tocopherol transfer protein (alpha-TTP) and tocopherol associated protein (TAP) genes. Catabolic activity was measured using the pregnane X receptor (PxR) and CYP3A4 genes (Traber 2004: Greger et al., 2006).

Materials and Methods.
Beef cattle (*n*= 30) were fed barley based diets supplemented with 0, 500 IU, or 1000 IU of dl-alpha-tocopherol acetate (1 IU = 1 mg dl-alpha-tocopherol acetate) per day, for 130d, to an average slaughter weight of 586.2 kg. Cattle were slaughtered according to Canadian Council of Animal Care (1993) procedures at the Lacombe Research Station Meat abattoir. Livers were collected and frozen at -80C, 1hr after slaughter. Hepatic vitamin E (alpha-tocopherol) was determined using the HPLC method of Thompson and Hatina (1979) using a fluorescent detector. Total RNA was isolated from 100 mg of liver using a TRIzol solution (GIBCO-BRL, Burlington, ON, Canada). Liver cDNA, was made using total RNA (5 ug) primed with 0.5 oligo-dT and random hexamers primers in a MMLV Reverse Transcriptase Kit (Sigma-Aldrich, Oakville, ON, Canada). The 435bp bovine version of the alpha-TTP cDNA was cloned using a consensus sequence of human (Gb# D49488), mouse (Gb# NP_056582) and rat alpha-TTP (Gb# D16339) mRNAs, with the CLUSTA W program (Chenna et al. 2003). Sequencing was performed using an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analysis on a CEQ 8000 machine (Beckman Coulter, Fullerton, CA).

Quantitative Real Time-PCR (Spencer and Christensen, 1999) was performed on liver cDNA to measure gene expression of alpha-Tocopherol Transfer Protein (alpha-TTP; Gb# AF185291), tocopherol associated protein (TAP; Gb# AF487977), the pregnane X receptor (PxR; Gb# AY789647), cytochrome P450-3A4 enzyme (CYP3A4; Gb# Y10214) and the internal standard house-keeping genes, cyclophilin (GenBank accession no. L41692) and beta-actin (Gb# DQ017256). Quantitative RT-PCR primers to be used for gene expression analysis were designed using the Primer3 program (Rozen & Skaletsky, 2000). The estimate of gene expression was measured using a Quantitech SYBR green PCR master kit (Qiagen, Mississauga, ON) run on a Mx4000 Stratagene machine (Stratagene, La Jolla, CA).

Data was analyzed using the Mixed model procedures and Spearman's Rank Correlation procedures (SAS Institute Inc. 1985).

Results and Discussion.

There was a very good correlation between the diet and the concentration of alpha-tocopherol in the liver of the cattle $r^2 = 0.832$, $P < 0.0001$; $n = 34$. However, there was a large variation in hepatic vitamin E content between individual animals within each of the 3 diets (Figure 1). The average hepatic level of vitamin E in the non-supplemented diet was 4.24 ± 0.28 ug/g (Table 2). A vitamin E liver concentration below 12 ug /gm is usually correlated with a *longissimus dorsi* (LD) muscle concentration below 3.3 ug /gm (Arnold et al. 1993). Therefore, the results from this trial indicate that all cattle fed the non supplemented control diet for 130d would not contain enough vitamin E for good meat quality. In the 11 animals supplemented with 1000 IU/d, 4 of the animals had a hepatic tocopherol concentration below the liver 12 ug vitamin E/gm, cut–off value (Figure 1). The large variation in cattle hepatic tocopherol content within each diet group indicates that vitamin E uptake or retention is being altered, possibly by gut microbiology or host genotype. To investigate if, the variation between dietary vitamin E and hepatic content was caused by differences in catabolic activity, the bovine PxR and CYP3A4 mRNAs were measured (Table 1) using PCR primers reported by Greger et al. (2006). PxR was significantly increased with the higher level of dietary vitamin E. The effect on CYP3A4 was not significant between diet groups. There was a significant correlation between hepatic liver content and PxR expression and between PxR and TAP mRNA ($r > 0.6$; $P<0.01$).

Conclusions. These experiments gave significant evidence that altering the level of dietary vitamin E can affect cattle liver gene activity *in vivo*. There was an increase in transfer activity as indicated by the TAP gene and in catabolic activity as indicated by the PxR gene. It was also observed that individual animals vary considerably in the amount of vitamin E retained within their livers, when fed the same high dietary concentrations of vitamin E.

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Table 1. Bovine hepatic genetic response to 3 dietary levels of dl-alpha-tocopherol acetate after ~130d in feedlot. An equal number of animals were tested per feeding group CON, 500 IU and 1000IU (*n*=30).

	***Diet*Δ**			
	Control 0 IU/d	Diet B 500 IU/d	Diet C 1000 IU/d	*P* value
Liver alpha-tocopherol [ug/gm of liver] †	*4.23 ± 0.28*^{A}	*11.06 ±0.77*^{B}	*14.29 ±1.10*^{C}	*0.0001*
alpha-TTP mRNA ‡	100 ± 12^{A}	86 ± 76^{A}	81 ± 37^{A}	0.43
TAP mRNA ‡	100 ± 10.4^{A}	171± 78^{AB}	480 ± 140^{B}	0.02
PxR mRNA‡	100 ± 21^{A}	211 ± 78^{B}	335 ± 90^{B}	0.015
CYP3A4 mRNA‡	100 ± 51^{A}	130 ± 38^{A}	110 ± 42^{A}	0.41

$^{A-C}$ Means with same letters are not significantly different (*n* = 30). Duncan's multiple range test. (*P*<0.05).
† Liver alpha-tocopherol concentration expressed as ug /g of liver.
‡ mRNA values are expressed as the %difference from the control group level.
Δ Basal diets estimated to contain approximately 10 IU of endogenous alpha-tocopherol acetate /kg of feed contributing approximately 100 IU tocopherol/animal/d before adding dl-alpha-tocopherol acetate supplement as outlined in material and methods.

Fig. 1. The liver tocopherol concentrations [ug of alpha-tocopherol/g of liver] in beef cattle (*n* = 34) grouped according to the three dietary levels of alpha -tocopherol acetate supplement (*Distribution/diet*). Note the non-linear distribution of liver tocopherol concentrations, within the same vitamin E dietary groups of 500 IU/d and 1000 IU/d.