Short communication: Effect of vitamins E and C on cortisol production by bovine adrenocortical cells in vitro

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ABSTRACT

The aim was to determine if vitamins E and C inhibit the release of cortisol from bovine adrenocortical cells when stimulated with ACTH in vitro. A factorial arrangement of treatments was used to culture bovine adrenocortical cells with different concentrations of vitamins E and C [(+)- α -tocopherol at 0, 2.3, and 16 μ M and L-ascorbic acid at 0, 15, and 50 μ M]. After 3 and 7 d of vitamin treatments, cell cultures were stimulated with ACTH (1 nM) for 24 h and the culture medium extracted to measure cortisol released by the cells using HPLC with UV detection. Vitamin E, vitamin C, or their combination did not affect the amount of cortisol released by the adrenal cultures to the media. Cortisol released by the adrenal cultures ranged from 33.6 ± 6.85 to 49.7 ± 8.01 nmol per 10^7 cells. The modulation effect of vitamins E and C on the stress response does not take place at the cortex of the adrenal gland.

Key words: cattle, cortisol, vitamin C, vitamin E

Short Communication

Animals can be stressed by either psychological or physical stressors (Grandin, 1997). The hypothalamic-pituitary-adrenocortical (HPA) axis is mainly activated when the animal perceives a harmful or potentially harmful stimulus (Von Borell, 2001). Activation of both HPA-axis and non-HPA-axis events during stress results in dehydration, ion depletion, energy depletion, and protein catabolism in the animal (Schaefer et al., 2001). Common management practices such as milking may induce a stress response characterized by an increase in plasma cortisol (de Passillé et al., 2008; Komara and Marnet, 2009). Furthermore, producers may have no control over the incidence or intensity of some stressors (social interactions, weather); consequently, reducing

the response of the animal to unavoidable stressors might be helpful in reducing the effects of stress.

Dietary compounds with antioxidant activity reduce activation of the HPA axis in animals (Butterweck and Winterhoff, 2004; Tsiakitzis et al., 2005; Dhanalaskshmi et al., 2006). Studies suggest that vitamin E supplementation or injection may reduce cortisol concentration in serum or plasma of cattle (Gupta et al., 2005; Larraín et al., 2008). Vitamin C is a potent antioxidant, and it reduces stress indicators in vivo (Ayo et al., 2006). Furthermore, vitamin C and the α -tocopheryl radical can interact, resulting in regeneration of vitamin E (Packer et al., 1979). Thus, supplementation of both vitamin E and C may have synergistic effects (de la Fuente et al., 1998; Fischer et al., 2004).

To the best of our knowledge, no information is available on target tissues for antioxidants as modulators of stress response in ruminants. In other species, modulation of HPA axis activity by antioxidant supplementation seems to occur in both the pituitary and the adrenal glands: a reduction in ACTH concentration in plasma or serum from antioxidant-supplemented animals was observed by Butterweck and Winterhoff (2004) in rats. Taniguchi et al. (2001) observed that broiler chickens supplemented with vitamin E and injected with ACTH had reduced corticosterone content in the adrenal glands. The aim of the current study was to determine whether vitamins E and C inhibit the release of cortisol from bovine adrenocortical cells when stimulated with ACTH in vitro.

Two sets of cell cultures were established about 6 mo apart, each set consisting of cells coming from 5 different adrenal glands ($n = 10$). Cells from each gland were cultured in 9 wells, and each well received 1 of 9 combinations of different concentrations of vitamins E and C in the media. A factorial arrangement of treatments was used, consisting of 3 concentrations of each vitamin in the media: vitamin E [(+)- α -tocopherol] at 0, 2.3, and 16 μ M, and vitamin C (L-ascorbic acid) at 0, 15, and 50 μ M. Medium and high concentrations of vitamins E and C were selected based on concentrations measured in plasma of un-supplemented and

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supplemented cows or steers (vitamin E: Arnold et al., 1993; Liu et al., 1996; vitamin C: Padilla et al., 2007; Tanaka et al., 2007) and assuming that their respective concentration in the culture media was negligible. Vitamin E was first dissolved in ethanol and then diluted in water. Each well received 42 μ L of a 0.95% aqueous ethanol solution containing vitamin E to reach the desired concentration.

Sampling and culture of bovine adrenocortical cells were based on the methodology described by Hornsby and McAllister (1991) and Williams et al. (1989). Bovine zone fasciculate/reticularis adrenocortical cells were extracted from adrenal glands of steers and heifers less than 30 mo of age, slaughtered in a local slaughterhouse (Frigorífico O'Higgins S.A., Santiago, Chile). One adrenal gland from each animal was sampled within 20 min postmortem. After removal of fat and other foreign tissues, the capsules were removed and the glands were briefly washed in 70% ethanol and rinsed with PBS. Slices of the gland were put in dispersion medium (growth medium containing 1 mg/mL type I collagenase and 0.1 mg/mL DNase I), cut into 1- to 2-mm pieces, and incubated at 37°C and 5% CO₂ for 2 h. Dispersed cells were centrifuged at 300 $\times$ *g* and room temperature for 10 min, and then suspended in growth medium (1:1 mix of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% fetal calf serum, 50 IU/mL penicillin, 50 μ g/mL streptomycin, and 5.6 μ g/mL amphotericin B). Suspended cells were counted using an improved Neubauer hemocytometer and an inverted microscope (Olympus CKX41SF, Tokyo, Japan) and plated out in 6-well plates (Corning CellBIND, Wilkes Barre, PA). Nine wells were plated with about 50,000 cells each, coming from each sampled gland. Cells were cultured in a 5% CO₂ atmosphere at 37°C, and growth medium was replaced every 48 h after rinsing twice with PBS. When the 9 wells with cells from the same adrenal reached around 70% confluence (100,000 to 200,000 cells/well), vitamin C and vitamin E were added to the growth medium. After 3 and 7 d of vitamin treatments, cell cultures were stimulated with ACTH (1 nM) for 24 h, and the culture medium was extracted to measure the cortisol released by the cells. Previous work in our laboratory showed that cortisol in the medium was below the limit of detection when no ACTH was added.

Cortisol was measured by HPLC according to Scott and Dixon (1979). Culture medium from each well was recovered, and 300 μ L of NaOH (0.25 M) and 7 mL of dichloromethane were added. Tubes were vortexed for 30 s and centrifuged at 1,300 $\times$ *g* for 5 min at room temperature to separate the aqueous and organic phases. Six milliliters of the dichloromethane phase were recovered and dried on a water bath at 40°C under an

air current. Dry samples were stored at -20°C until analysis. The dry samples were dissolved in 1 mL of 45% methanol in water and filtered through a 0.45- μ m regenerated-cellulose filter before injection into the HPLC. The HPLC system was a PerkinElmer Series 200 UV/Vis HPLC System (PerkinElmer, Waltham, MA). An autosampler was used and 72 μ L of each sample was injected. The mobile phase was 45% methanol and the column used was a Hypersil Octadecysilane, 150 mm $\times$ 4.6 mm, 5- μ m particle size (Phenomenex, Torrance, CA). Ultraviolet detection was used at 239 nm, and prednisone (250 nM) was used as the internal standard.

Statistical analysis was conducted using SAS 9.1 software (SAS Institute Inc., Cary, NC) using the GLM procedure. Cortisol released was expressed as nanomoles per 10⁷ cells. The average of cortisol released to the medium on d 3 and 7 was used for the analysis. A 3 $\times$ 3 factorial arrangement of treatments was used, with concentrations of vitamins E and C as factors. Culture set and adrenal gland within culture set were used as classification variables in the model, and concentrations of vitamin E, vitamin C, and their combined doses were considered as continuous variables. Effect of vitamin concentrations and their interaction was considered significant when ANOVA *P* $\leq$ 0.05.

Cortisol released by the adrenal cultures ranged from 33.6 $\pm$ 6.85 to 49.7 $\pm$ 8.01 nmol per 10⁷ cells (Table 1). Neither vitamin E (*P* = 0.30) or vitamin C (*P* = 0.78) affected the amount of cortisol released by the adrenal cultures to the medium, and no interaction was observed between vitamins (*P* = 0.36). In vivo, several studies showed that plasma cortisol was decreased after injection or supplementation of vitamin E and other antioxidants in bovines (Reddy et al., 1987; Gupta et al., 2005; Larraín et al., 2008). In vitro, we observed no changes in cortisol released by bovine adrenocortical cells cultured with different concentrations of vitamins E and C. These observations suggest that in cattle, vitamin E and other antioxidants might have an effect in reducing cortisol in plasma by modulating the activity of tissues other than the adrenal gland. Other tissues that play a central role on modulating the activity of the HPA axis are the hypothalamus and the

Table 1. Cortisol released to the media (nmol per 10⁷ cells $\pm$ SE) by bovine adrenocortical cells cultured with different concentrations of vitamins E and C

Vitamin E (μ M)	Vitamin C (μ M)		
	0	15	50
0	43.1 $\pm$ 5.3	43.8 $\pm$ 3.8	45.5 $\pm$ 6.1
2.3	41.7 $\pm$ 4.5	43.0 $\pm$ 3.3	46.1 $\pm$ 5.3
16	33.6 $\pm$ 6.9	38.4 $\pm$ 5.0	49.7 $\pm$ 8.0

pituitary gland, so these organs might be target tissues for antioxidant compounds as modulators of the stress response *in vivo*.

In conclusion, cortisol production in cultured adrenocortical cells did not change with different concentrations of vitamins E and C. The modulation effects of vitamins E and C on stress response does not take place at the cortex of the adrenal gland.

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