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INCREASED 12-HETE PRODUCTION IN BOVINE LYMPHOCYTES DURING SELENIUM DEFICIENCY

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When peripheral blood lymphocytes were incubated with arachidonic acid in the presence of Ca^{++} ionophore (A23187), the cells from the selenium-deficient dairy cows produced significantly greater quantities of 12-hydroxyeicosatetraenoic acid (12-HETE) than the cells from the selenium-supplemented animals. The major product of reaction was verified as 12-HETE by cochromatography with a 12-HETE standard on an HPLC and structural analysis by GC-MS. Additionally, concanavalin A-stimulated lymphocyte proliferation was significantly decreased in cells from the Se-deficient cows. Furthermore, 12-HETE generated by the A23187-stimulated lymphocytes inhibited lymphocyte proliferation when added to Se-supplemented cell cultures. These observations suggest that self-regulation of lymphocyte proliferation might be mediated by 12-HETE production, especially during an altered nutritional state such as Se deficiency.

In previous studies from this lab, we have shown that selenium-dependent glutathione peroxidase (Se-GSH-Px) plays an important role in controlling the peroxide tone of cells, especially in relation to the arachidonic acid cascade (1,2). The role of selenium (Se) in enzymatic oxidation of arachidonic acid and the effect of eicosanoids in immune cell regulation have received great attention recently. Lymphocytes are known to produce and respond to eicosanoids *in vitro* (3,4) and Se has been shown to affect the immune response in many different species and has been the subject of recent review (5); however, the interaction between Se and arachidonic acid metabolism in immune cell function is still not well understood. In the present study, we examined the effect of Se nutrition on arachidonic acid metabolism in bovine lymphocytes, and in turn, the effect of the lipoxygenase pathway products generated on lymphocyte proliferation.

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Abbreviations: 12-HETE: 12-hydroxy-5,8,10,14, eicosatetraenoic acid, Se-GSH-Px: selenium-dependent glutathione peroxidase, Con A: concanavalin A, LTB_4: 5(S),12(R)-dihydroxy-6Z, 8E, 10E, 14Z-eicosatetraenoic acid, ^{3}H-TdR: [^{3}H]-thymidine.

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MATERIALS AND METHODS

Materials: The following materials were purchased from the company shown in parentheses. Arachidonic acid, calcium ionophore A23187, Histopaque-1077, Hepes buffer, cytochalasin B and gentamycin (Sigma Chemical Co., St. Louis, MO); [^{3}H]-thymidine with a specific activity of 6.7 Ci/mmol (ICN, Cleveland, OH); Con A (ICN Immuno Biologicals, Lisle, IL); RPMI 1640 (Cellgro Inc., Herndon, VA); HPLC grade methanol, hexane and 2-propanol (J.T. Baker, Inc., Phillipsburg, NJ); LTB_4 standard (Biomol Research Lab., Plymouth Meeting, PA); all other diHETE and mono-HETE standards (prepared by Dr. Vladimir Garkov in our laboratory).

Animals and Diets: Twelve Holstein cows were fed a standard dairy ration of grains and forages, grown in Se-poor soil, which provided a Se concentration of approximately 0.05 mg/kg dry matter. Six of these cows were fed an additional 2 mg Se (as sodium selenite) per cow per day to increase their dietary Se intake to approximately 0.14 mg/kg dry matter. Blood Se and Se-GSH-Px were monitored in all cows on a monthly basis according to the methods of Olson *et al* (6) and Paglia and Valentine (7), respectively. Animals were maintained on experimental diets for at least 6 months before use in this study.

Isolation of lymphocytes: Peripheral blood was collected from the jugular veins of both Se-supplemented and Se-deficient cows and lymphocytes were isolated as described previously (8). This technique typically yields 70-80% T-cells, 10-20% B-cells, 2-8% monocytic macrophages and <5% red blood cells. Cells were resuspended in Hepes buffer to a concentration of $1x10^8$ cells/ml for arachidonic acid metabolism, or in RPMI 1640 medium to a concentration of 2 to $5x10^6$/ml for proliferation studies.

Arachidonic acid metabolism and analysis: Lymphocytes were suspended in 1 ml Hepes buffer with 5 μg/ml cytochalasin B and incubated with 150 μM arachidonic acid at 37°C for 15 min and were then stimulated with 10 μM A23187 for 5 min. Two ml of cold methanol were added to stop the reactions and samples were stored overnight at -20°C. Arachidonic acid metabolites were extracted using diethyl ether and isolated by HPLC as described previously by the methods of Borgeat and Samuelsson (9) and Haines *et al* (10). A Zorbax ODS C18 column (5 μm particle size, 4.6 mm x 25 cm, Dupont Instruments) was used to isolate the mono-HETEs. Elution was carried out using methanol/water/acetic acid (750:250:0.1, v/v/v, pH 5.6) at a flow-rate of 1.0 ml/min.

Lymphocyte proliferation: Cells were cultured in 200 μl of RPMI 1640 medium supplemented with 10% bovine serum, 0.1% gentamycin and 5 μg/ml Con A. Cultures were pulsed with ^{3}H-TdR for 12 h and harvested at different time intervals on filter paper by an LKB-auto harvester and radioactivity determined in an LKB-liquid scintillation counter. All cultures were performed in triplicate. It has been previously verified that ^{3}H-TdR incorporation reflects blastogenesis and proliferation (11).

Statistical analyses: The Student's t test was employed for data analysis and significance was established at $P<0.05$.

RESULTS

Se status: The Se-deficient cows had a significantly lower mean blood Se concentration (0.04 μg/ml) and Se-GSH-Px activity (17.4 mUnits/mg Hb) than the Se-supplemented cows (0.18 μg/ml and 57.4 mUnits/mg Hb) at the time of the study.

Effects of dietary Se status on generation of lipoxygenase pathway products: Lymphocytes incubated with exogenous arachidonic acid and stimulated with A23187

Table 1. Effects of Se deficiency on generation of lipoxygenase products by A23187-stimulated lymphocytes

Se Status	Lipoxygenase Products (nmol/1x10^6 cells/5 min)			
	12-HETE	5-HETE	LTB_4	5S,12S-diHETE
+Se	84 ±10	20 ±7.2	6.0 ±2.4	4.7 ±1.0
-Se	105 ±22*	8.5 ±3.5*	2.4 ±1.1*	1.5 ±1.2*

Lymphocytes were incubated in the presence of 150 μM AA for 15 min at 37°C, then stimulated with 10 μM A23187 for 5 min. Values are means ±SD of three individual experiments. *Indicates value is significantly different from +Se ($P<0.05$) for the same product. Data were analyzed using the Student's t test.

generated both mono- and diHETEs, whereas unstimulated cells did not generate any detectable diHETEs and yielded only small quantities of mono-HETEs. A comparison of Se-supplemented with Se-deficient cells shows no qualitative difference in HPLC profiles of arachidonic acid products generated, but the quantities of the various compounds were different (Table 1). On HPLC analysis, the major product peak, which co-eluted with 12-HETE standard, was approximately 30% larger in the cells from Se-deficient cows than in the cells from Se-supplemented cows. Other peaks, which co-eluted with 5-HETE, LTB_4, and 5S,12S-(E,Z,E,Z)-diHETE standards, were much smaller, on average, in the Se-deficient cells than in the Se-supplemented cells. Moreover, all cells generated 4 to 70X less of each of these products than 12-HETE (Table 1). All of the mono- and diHETEs displayed characteristic UV spectra.

The major lipoxygenase product isolated from HPLC was identified as 12-HETE from its mass spectrum, with the prominent ion of the trimethylsilyl-methyl ester at m/z 295 [M - 111, loss of $\cdot CH_2\text{-}CH{=}CH\text{-}(CH_2)_4\text{-}CH_3$]. The fragmentation pattern of this product was essentially identical to a 12-HETE standard (Fig. 1).

To confirm that the lipoxygenase pathway products were generated exclusively from lymphocytes, contaminating macrophages were separated from lymphocytes by adherence to plastic flasks and passage through a cotton column (12). Macrophage contamination dropped from 8% to 2%, as determined by esterase staining (13), but the quantities of lipoxygenase pathway products did not change significantly.

Effects of Se on lymphocyte proliferation: No significant difference in ^{3}H-TdR incorporation was observed during the first 36 h of incubation. However, after 36 h, while the quantity of ^{3}H-TdR incorporated continued to increase in the cells from the Se-supplemented cows through 60 h, it levelled off in the cells from the Se-deficient cows and was significantly lower than the Se-supplemented cells from 48 to 96 h of incubation (data not shown).

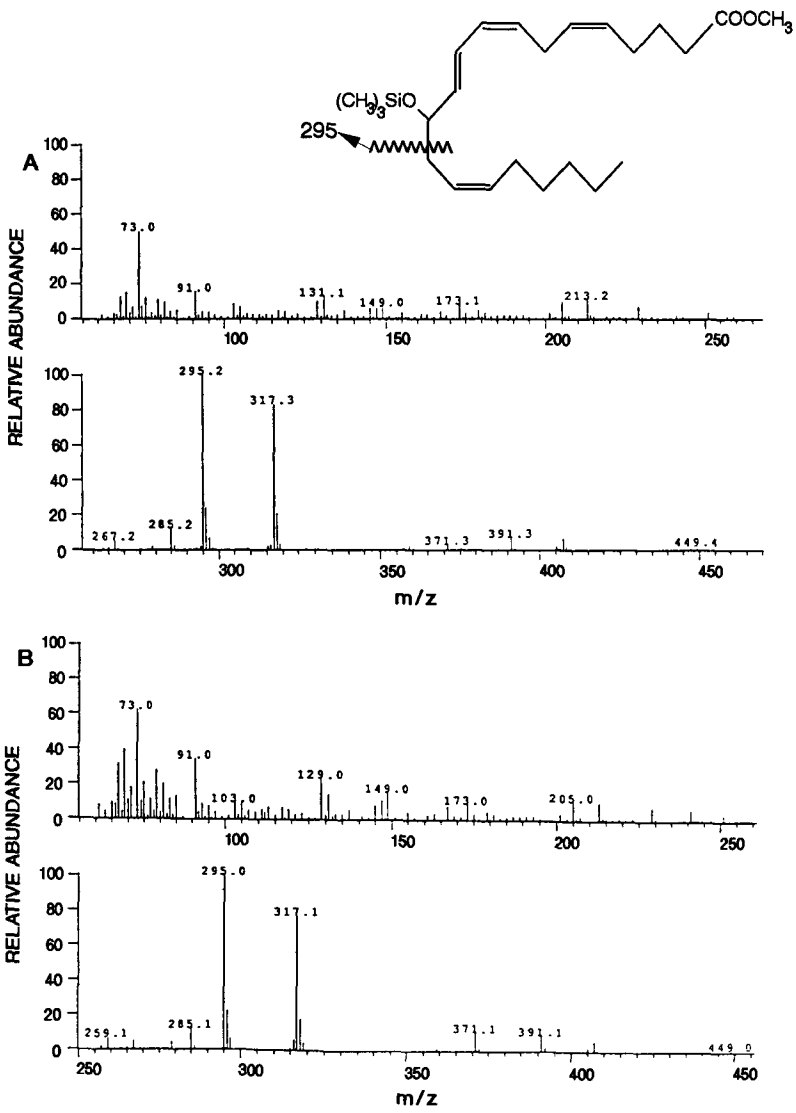

Figure 1. Mass spectra of the Me_3Si-methyl ester of (A) 12-HETE standard and (B) the major lipoxygenase pathway product generated by stimulation of bovine lymphocytes with A23187.

Suppression of lymphocyte proliferation by 12-HETE: As shown in Table 2, 12-HETE was found to cause a dose-dependent suppression of ^{3}H-TdR incorporation when added to cultured cells. 12-HETE alone had no effect on ^{3}H-TdR incorporation in unstimulated lymphocytes and did not alter cell viability at these concentrations.

DISCUSSION

Previously, 12-lipoxygenase studies focused mainly on platelets, and the enzyme located in platelets has been well characterized (14). However, a wider distribution of 12-

Table 2. Effects of 12-HETE addition to Con A-stimulated lymphocyte proliferation

12-HETE Concentration (M)	% Inhibition
10^{-8}	5 ±4
10^{-7}	10 ±4
$2x10^{-7}$	16 ±6*
10^{-6}	24 ±8*
$2x10^{-6}$	33 ±7*

The 12-HETE was added upon the initiation of culture. After incubation at 37°C for 48 h, cells were ^{3}H-TdR labeled for 12 h. Results are expressed as mean percent inhibition of proliferation ±SD, as compared with a control culture with no 12-HETE added. *Indicates significant inhibition ($P<0.05$). Data were analyzed using the Student's t test.

lipoxygenase has since been demonstrated in various tissues (15-17). The 12-lipoxygenase product of arachidonic acid, 12-HETE, has many physiologic activities, including: promotion of endothelial cell growth (18), induction of cGMP production (19), enhancement of tumor cell attachment to endothelial cells (20), inhibition of 5-lipoxygenase (14), regulation of prostaglandin and leukotriene production (21), and enhancement of mononuclear procoagulant activity (22). However, the regulation of lymphocyte proliferation by changes in 12-lipoxygenase activity and, in turn, to Se nutrition has not been previously documented.

The present study demonstrates a significant increase in 12-HETE production in A23187-stimulated lymphocytes from Se-deficient cows. Concurrently, this study reveals that Con A-stimulated proliferation was significantly decreased in lymphocytes from Se-deficient cows. These data suggest a possible relationship between the increase in 12-HETE and the inhibition of lymphocyte proliferation in the Se-deficient cells. When exogenous 12-HETE was added to cell cultures, it clearly indicated that 12-HETE elicits a dose-dependent suppression of lymphocyte proliferation. Therefore, we postulate that the enhanced production of 12-HETE in Se-deficient animals could result in diminished lymphocyte proliferation and, thus, affect the immunologic status of these animals.

Because Se-GSH-Px has been shown to regulate intracellular hydroperoxide levels (1,2), the mechanism of enhanced 12-HETE production in lymphocytes is possibly mediated by higher concentrations of intracellular hydroperoxides in the Se-deficient state. It has

been shown that fatty acid hydroperoxides are potent stimulators of lipoxygenase activity in low concentrations, but can cause inhibition at high concentrations (23,24). Recently, it was reported that ebselen (2-phenyl-1,2-benzisoselenazol-3-(2H)-one), an anti-inflammatory seleno-organic compound with GSH-Px-like activity, inhibits the formation of 12-HETE in ocular tissues and platelets (17), therefore, the selective influence of Se-GSH-Px activity on 12-HETE concentration appears to occur in cells other than lymphocytes. However, it is not understood why the 12-lipoxygenase pathway is selectively stimulated, while 5-lipoxygenase activity appears diminished. It is possible that there are subtle differences in the hydroperoxide sensitivity of the various lipoxygenases causing selective activation or inhibition. These hypotheses merit further investigation. Regardless of the mechanism involved, this study yields interesting new evidence in revealing the connections among Se, the arachidonic acid metabolites, and immune function.

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