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In Vivo Chemotaxis of Bovine Neutrophils Induced by 5-Lipoxygenase Metabolites of Arachidonic and Eicosapentaenoic Acid

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The 5-lipoxygenase metabolites of arachidonic (AA) and eicosapentaenoic acid (EPA), 5S,12R-dihydroxy-6,8,10,14-eicosatetraenoic acid (LTB_4), 5-hydroxyeicosatetraenoic acid (5-HETE), 5S,12R-dihydroxy-6,8,10,14,17-eicosapentaenoic acid (LTB_5), and 5-hydroxyeicosapentaenoic acid (5-HEPE), were injected intradermally into the ear skin of steers to assess their* in vivo *potency as chemotactic factors for bovine neutrophils. A dose of 30 picomoles of LTB_4 was required to elicit a significant extravascular dermal accumulation of neutrophils ($P < 0.05$). In contrast, 1.0 nanomole of LTB_5 was required to achieve a cellular influx equivalent to that elicited by 30 picomoles of LTB_4. Nearly five times as many neutrophils were present in the bovine dermis injected with 1.0 nanomole of LTB_4 compared with sites given the equivalent dose of LTB_5 (245 cells/sq mm vs. 52 cells/sq mm). Six nanomoles of either 5-HETE or 5-HEPE were required before a significant neutrophil accumulation occurred. These results show clearly that 5-lipoxygenase metabolites can initiate the extravascular accumulation of bovine neutrophils* in vivo. *However, the chemotactic potency of the EPA metabolites is much reduced when compared with that of the homologous AA lipids. The results obtained support the premise that modification of the inflammatory response, including control of cellular influxes, by dietary supplementation with EPA is feasible. (Am J Pathol 1989, 134:671–676)

Oxidative metabolites of arachidonic acid are potent pro-inflammatory mediators. The oxidative metabolism of arachidonic acid (AA) by the 5-lipoxygenase pathway results in the release of leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE), lipids which have been shown to have profound pro-inflammatory effects in a variety of species. These effects include neutrophil chemotaxis, degranulation, and superoxide production.[1] It has been reported that *in vitro*, however, LTB_4 is not chemotactic for bovine neutrophils,[2] although the *in vitro* chemotaxis assays may not reflect precisely *in vivo* conditions. The *in vitro* biologic activities of the eicosapentaenoic (EPA) metabolites are generally reduced when compared with their AA derived homologues.[3–5] We have previously examined the comparative metabolism of AA and EPA by bovine neutrophils.[6] In this paper we extend our observations to include a comparison between the *in vivo* chemotactic activities of 5-lipoxygenase metabolites of AA and EPA for the bovine peripheral blood neutrophil.

Materials and Methods

Animals

Conventionally reared Holstein steers weighing approximately 350 kg with no clinical signs or history of respiratory disease were housed outdoors in cement floored covered stalls bedded with wood shavings and fed alfalfa cubes and water ad libitum.

Chemicals

LTB_4 (5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid) and 5-HETE (5S-hydroxy-6-trans-8,11,14-cis eicosatetraenoic acid) were purchased from Seragen Inc.,

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Boston, MA. EPA (5,8,11,14,17-eicosapentaenoic acid), PGB_2, and A23187 were purchased from Sigma Chemical Co., St. Louis, MO. All solvents used were high performance liquid chromatography (HPLC) grade (Burdick and Jackson, Muskegon, MI). Formic and acetic acid were purchased from J. T. Baker (Phillipsburg, NJ).

Generation and Purification of EPA Metabolites

Bovine blood was collected from unanesthetized steers by jugular venipuncture into acid citrate dextrose. The citrated blood was centrifuged at 850*g* for 15 minutes and the plasma and buffy coat discarded. The remaining cells and leukocytes were washed (2×) in isotonic phosphate buffered saline (PBS), pH 7.4, and centrifuged at 600*g* for 10 minutes. Hypotonic lysis of red cells used the addition of 45 ml of distilled water. Lysis was terminated after 45 seconds by addition of 5 ml 10X PBS. Following centrifugation at 600*g* for another 10 minutes, the lysis and centrifugation steps were repeated, and the final cell pellet was resuspended in Hanks balanced salt solution containing 0.4% egg albumin (HBSS-OA). Cells were quantitated using a hemocytometer and cell viability determined by trypan blue exclusion. Typical yields were 85 to 95% neutrophils, with greater than 90% cell viability. The cells were placed into polypropylene tubes and incubated with 50 μM EPA for 10 minutes at 37 C. The cells were then exposed to a 10 μM concentration of the calcium ionophore, A23187, for 10 minutes, and the reaction was terminated by the addition of 10 μl formic acid. After this incubation, the cells were centrifuged at 400*g* for 10 minutes, and the supernatant decanted for extraction of lipid metabolites as described previously.[7] After the final extraction, the lipids were resuspended in 100 μl of HPLC buffer, and injected onto a HPLC column for separation of the lipoxygenase metabolites. The recovery of authentic standards for LTB_4, LTB_5, 5-HETE, and 5-HEPE using this methodology routinely exceeded 85%.

High-performance liquid chromatography was performed using an Altex-Beckman system, with dual model 114M pumps, and a variable wavelength detector (Model 165, Beckman Instruments, Richmond, CA).

Lipoxygenase metabolites of EPA were separated, identified, and quantified using a solvent system of $MeOH:H_2O:CH_3COOH$/70:30:0.1. The aqueous mobile phase contained 3 mM Na_2EDTA. The flow rate was 0.8 ml/min for 25 minutes, then the rate was increased to 1 ml/min over 1 minute for the remainder of the run. At 25 minutes the organic phase was increased to 75%, and EPA eluted from the column by increasing the solvent strength to 95% MeOH at 63 minutes. Biologically generated LTB_5 and 5-HEPE eluted from the column, were reextracted with 1 ml water, 10 μl formic acid, and 1 ml chloroform, dried under N_2, resuspended in absolute ethanol, and stored at −70 C before use. Samples of each preparation were rechromatographed by the above system to assure purity (>99%). LTB_5 and 5-HEPE were identified and quantified on the basis of molar extinction coefficients, retention times, UV absorption spectra, and by gas chromatography/mass spectrometry.[7]

At the time of use, samples were dried under N_2 and resuspended in pyrogen-free phosphate buffered saline. Recovery of the dried metabolites was greater than 95%. The resuspended metabolites were kept chilled on ice until intradermal injection.

Intradermal Injection of Metabolites

The outer pinna of both ears of steers were clipped and then cleansed with 70% isopropyl alcohol and 10% povidone-iodine. LTB_4, LTB_5, 5-HETE, and 5-HEPE were diluted in pyrogen-free phosphate buffered saline and loaded into pyrogen-free tuberculin syringes. A volume of 0.1 ml of each solution, containing known doses of each metabolite, were injected intradermally into the ear skin with 27-gauge needles, forming a distinct bleb. Total metabolite doses ranged from 6.0 picomoles to 6.0 nanomoles. The margins of the skin thickening were encircled with indelible ink. A volume of 0.1 ml of phosphate buffered saline and 0.1 ml zymosan activated serum[8] were injected concurrently as negative and positive controls respectively. All samples were endotoxin free as assessed by the *Limulus* amoebocyte lysate assay (Sigma Chemical Company, St. Louis, MO).

Collection and Processing of Samples

Four hours after injection, the periphery of each injection site was infiltrated with 2% Lidocaine (Gibco/Invenex, Chagrin Falls, OH). A 6-mm circular biopsy of the epidermis and dermis, centered around the marked injection site, was taken with a disposable punch biopsy (Baker Cummins, Miami, FL). The tissues were immediately placed into a 10% neutral buffered formalin solution. After 24 hours fixation, the biopsies were hemi-sectioned through the injection site, embedded in paraffin, sectioned at 4 μ, and stained with hematoxylin and eosin (H & E).

Examination of Histologic Sections

A modification of the technique described by Humphrey et al[9] was used to enumerate neutrophils within the skin

biopsies. The samples were coded and examined in a blind fashion. The cells on the face of each hemisection were counted. Two by two contiguous fields of view at ×250 (0.45 sq mm/field), centered over the injection site, and bordering the epidermal surface were examined. Extravascular neutrophils were counted in the four fields, and the average number of cells per square millimeter was calculated for each hemisection. Hemisection counts were averaged, and the number of neutrophils per square millimeter for each biopsy was used to calculate the means and SEM. Areas were calibrated with a slide micrometer. The coded slides were then identified, and the results correlated with the dose of lipid injected.

Statistical Analysis

The statistical test used for analysis of the results was the Mann-Whitney U-test. Statistical significance was established at the $P < 0.05$ level.

Results

Extravascular neutrophils were rare in saline control injections (4.8 ± 0.4, mean ± SEM per sq mm, N = 3, Figure 1a). Dose-response curves for the chemotactic activity of each of the four metabolites are presented in Figure 2. The number of neutrophils within the two superficial fields of view in a biopsy were consistently 5 to 20% of the number of cells within the deeper fields of view. Values obtained from the deep fields from each face of a biopsy were within 10 to 15% of each other. Neutrophil numbers declined dramatically in tissue deeper than two fields of view from the skin surface and away from the site of injection. Significant ($P < 0.05$) accumulations of neutrophils were observed in dermis injected with 30 picomoles of LTB_4 (36.1 ± 2.5 per sq mm, N = 3). Neutrophil numbers increased rapidly with increasing amounts of LTB_4 injected (Figure 2). At 1.0 nanomole of LTB_4, 245.0 ± 27.3, (N = 3) neutrophils per sq mm were present within the dermis (Figure 1b). Neutrophil accumulation was essentially maximal at this dose. On the other hand, at all doses where neutrophil accumulation was significant, macrophages and lymphocytes were scarce. At the highest dose of LTB_4 used, modest numbers of eosinophils (less than 25 per sq mm) were randomly scattered amongst the neutrophils.

At least 1.0 nanomole of LTB_5 was required to cause a significant accumulation of neutrophils above that of controls (52.0 ± 4.3 per sq mm, N = 3, Figure 1c). Neutrophil numbers were further increased at a dose of 3.0 5-HETE at doses ranging from 0.03 to 1.0 nanomole did not induce a significant neutrophil accumulation. At 3.0 nanomoles, neutrophil numbers began to increase (17.4 ± 7.2 per sq mm), with a marked increase in the dermis observed at 6.0 nanomoles (97.8 ± 27.7 per sq mm, Figure 2).

At least 6.0 nanomoles of 5-HEPE were required to cause a significant neutrophil accumulation in the bovine dermis (32.0 ± 15.8 per sq mm, Figure 2).

Zymosan activated bovine serum provided a strong chemotactic stimulus for bovine neutrophils (301.3 ± 14.0 per sq mm, Figure 1d), and the cellular influx was accompanied by marked edema, a feature that was not observed after injection of the lipid mediators.

Discussion

Extravascular neutrophils are rare in normal bovine skin, indicating the potential of the bovine skin for use as an *in vivo* chemotaxis assay system. This system allows an opportunity to test the relative *in vivo* potency of various chemotactic agents within the same animal. The broad and thin skin surface of the ear is easily injected and sampled.

Under-agarose chemotaxis assays of bovine neutrophils have demonstrated only trace locomotion of the cells in the presence of 0.1 to 100 ng of LTB_4.[2] In contrast, our *in vivo* studies showed LTB_4 to be a potent and reliable intradermal chemotactic agent at doses as low as 30 picomoles (10.1 ng). Our results clearly indicate that the bovine neutrophil can respond to this lipid *in situ*. The reason for the discrepancy between our results and those of Dunn et al[2] is not known, but may well relate to the different sensitivities of the two assays employed. Our results correlate well with those obtained in other *in vivo* studies, including rabbit and human skin. In these cases, marked neutrophil accumulation was observed at doses of 10 to 100 ng.[10,11]

Lipoxygenase metabolites may be indirectly responsible for the influx of neutrophils within bovine skin. LTB_4 may be acting on another cell type that produces factors that are themselves directly chemotactic for, or enhance the intravascular adhesion of, bovine neutrophils. LTB_4 increases the adhesion of human neutrophils to cultured bovine endothelium,[12] but does not induce adhesion of bovine neutrophils to endothelium.[2] Macrophages may be stimulated by LTB_4 to release interleukin-1 or platelet activating factor,[13] either of which could act as the mediator of neutrophil chemotaxis. Alternatively, interleukin 1 has been shown to enhance the adhesion of neutrophils to endothelial cells,[14] and to stimulate the production of platelet activating factor by endothelial cells,[15] again a fea-

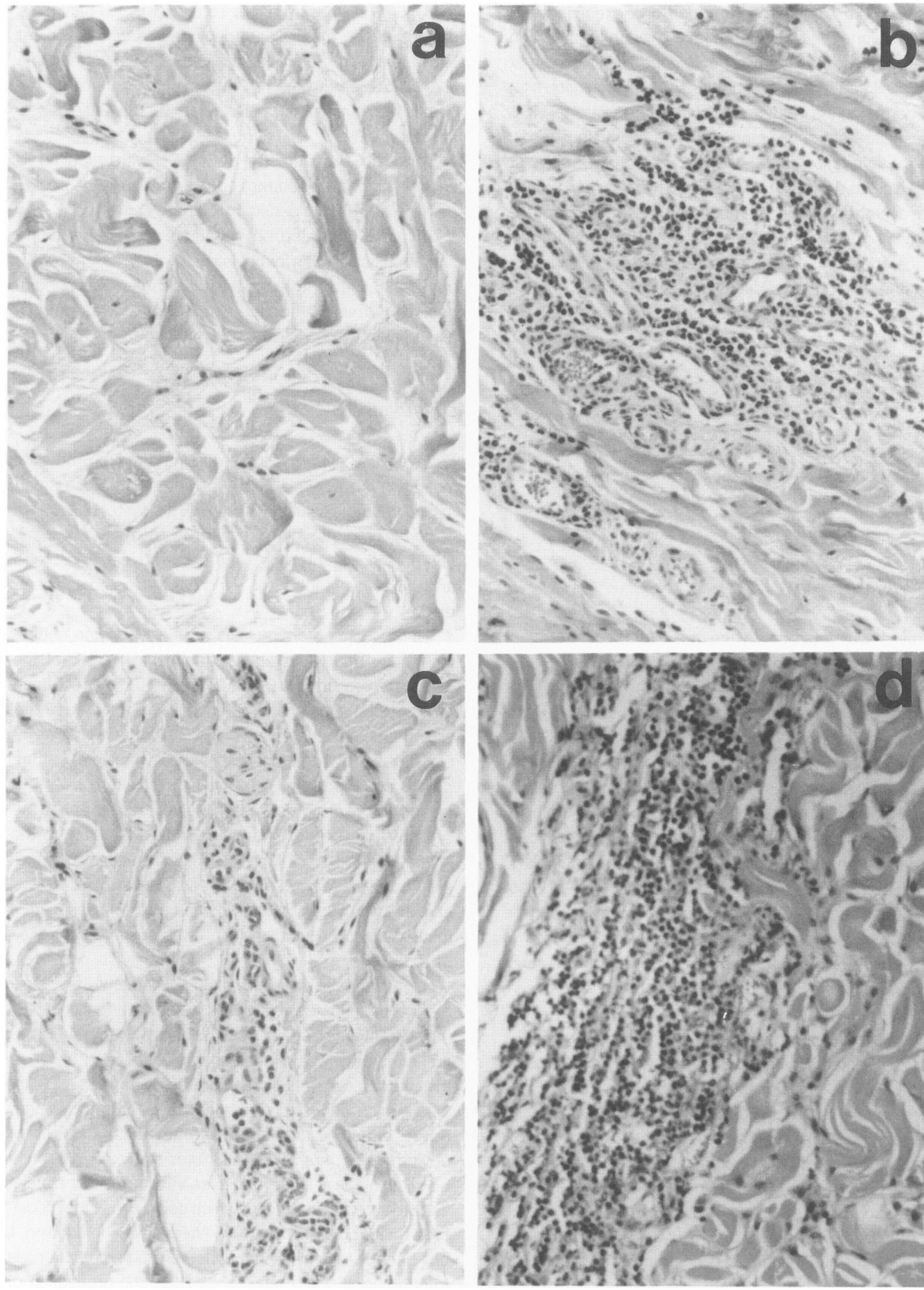

Figure 1. *Photomicrographs of stained skin biopsies taken four hours after injection of* **a**: *saline (4.8 ± 0.3, mean number of neutrophils ± SEM per sq mm);* **b**: *1 nanomole LTB_4 in saline (245.0 ± 27.3);* **c**: *1 nanomole LTB_5 in saline (93.7 ± 6.3), and* **d**: *zymosan activated serum (301 ± 13.9). Total injection volume was 0.1 ml (hematoxylin and eosin, ×225).*

sues. In addition, platelet activating factor has been shown to be a potent chemotactic agent for eosinophils, suggesting a mechanism for their presence within the skin biopsies.

In vitro studies of human neutrophils have indicated the lowest effective dose of LTB_4 inducing neutrophil chemotaxis is 1 ng/ml, and the lowest effective dose of 5-HETE is 10 ng/ml,[16] a 10-fold difference in relative potency. The maximum effective dose of LTB_4 *in vitro* was 30 ng/ml, compared with 1000 ng/ml for 5-HETE, a 30-fold difference in relative potency.[16] We did not reach a maximum effective dose for 5-HETE. However, the minimal *in vivo* effective dose of LTB_4 was 30 picomoles (10.1 ng), and that of 5-HETE was 3.0 nanomoles (960 ng), nearly a 100-fold difference in minimal effective dose (Figure 2). From another perspective, examination of the effective doses at which neutrophil numbers within the dermis are 25% of the maximum number seen with LTB_4 (ED_{25}), we see that while 0.04 nanomoles of LTB_4 are required to achieve the ED_{25}, approximately 4.0 nanomoles of 5-HETE are required, again a 100-fold difference in relative potency (Figure 2). The ED_{25} of LTB_5 is approximately 1.5 nanomoles, or 37.5 times that of LTB_4. The ED_{25} of 5-HEPE was not achieved at 6 nanomoles, the highest dose injected.

LTB_5 has been observed to possess from 3 to 10% of the chemotactic activity of LTB_4 using *in vitro* chemotaxis assays with human neutrophils.[17,18] In our *in vivo* experiments, LTB_5 had 3 to 10% of the chemotactic potency of LTB_4 towards bovine neutrophils depending on the dose used. These results confirm the reduced chemoattractant nature of this 5-lipoxygenase metabolite of EPA when compared with LTB_4 in both humans and cattle. The variation between relative potencies of the homologous metabolites observed in different species may reflect actual sensitivities of respective cell populations, or may simply reflect differences in the assays used.

Further support of the reduced biological activity for the EPA metabolites is evidenced by the reduced capacity of 5-HEPE to serve as a chemotactic mediator when compared to 5-HETE. However, this difference is not nearly as marked as that observed with LTB_4 and LTB_5. The 5-HETE and 5-HEPE data are not suprising in light of the marginal chemotactic activity of the HETEs.[19] 5-HETE has been shown to possess approximately 2% of the chemotactic activity of LTB_4 *in vitro*.[4] Interestingly enough, this difference in potency is approximately the same as that which we achieved in our *in vivo* experiments. However, *in vitro* chemotaxis of human neutrophils with 5-HETE was maximal at a concentration of 10^{-7} molar, whereas the first evidence of *in vivo* chemotaxis of bovine neutrophils was not achieved until concentrations of 2 $\times 10^{-5}$ molar (2.0 nanomoles) were used. No chemotaxis was observed with 5-HEPE in the *in vitro* studies using

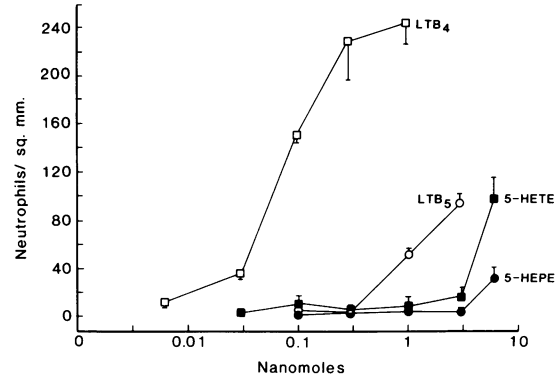

Figure 2. *In vivo chemotaxis of bovine neutrophils within the ear skin of steers. 5-lipoxygenase metabolites of arachidonic acid and eicosapentaenoic acid were injected intradermally in a total volume of 0.1 ml. Skin biopsies were harvested 4 hour after injection and each point represents the mean of three separate experiments. Abcissa: total amount of metabolite injected (nanomoles). Ordinate: Mean number of neutrophils per square millimeter. Bars represent standard error of the mean. Saline control = 4.8 neutrophils per square millimeter ± 0.4 SEM.*

human neutrophils,[4] and 5-HEPE had only about 0.5% of the chemotactic potency of LTB_4 in our *in vivo* assay using bovine neutrophils.

The accumulation of eosinophils in response to the injection of 1.0 nanomole of LTB_4 into bovine skin was not entirely unexpected. Others have shown that the injection of LTB_4 (0.3 nanomoles) into human skin resulted not only in the accumulation of significant numbers of neutrophils, but also low numbers of eosinophils.[11] Our results would suggest a possible role for LTB_4 in the chemotactic attraction of eosinophils *in vivo* in cattle as well as humans.

Zymosan activated serum provided a strong positive control for these *in vivo* studies. This *in vivo* observation complements *in vitro* studies that have shown activated bovine serum induces neutrophil chemotaxis.[20]

We have shown that the 5-lipoxygenase metabolites of AA, LTB_4, and 5-HETE provide a chemotactic stimulus for bovine neutrophils *in vivo*. This responsiveness of the bovine neutrophil to these lipids indicates a potential role for arachidonate metabolites in neutrophil-mediated inflammatory responses in this species. Moreover, we have observed the reduced chemotactic potency of the homologous metabolites of EPA, namely LTB_5 and 5-HEPE. These latter results confirm the reduced inflammatory capacity of LTB_5 and 5-HEPE, and provide further evidence for modulation of inflammatory response by the use of dietary EPA.

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