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THE ACTION OF ERYTHROPOIETIN IS MEDIATED BY LIPOXYGENASE METABOLITES IN MURINE FETAL LIVER CELLS

B.S. Beckman*, M. Mason-Garcia, L. Nystuen, L. King, and J. W. Fisher

Department of Pharmacology,
Tulane University School of Medicine,
1430 Tulane Avenue,
New Orleans, LA 70112

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SUMMARY: Erythroid progenitor cells synthesize 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) when stimulated by erythropoietin (Ep). Maximal stimulation of 12-HETE production occurred at one hour, whereas 15-HETE activity remained constant in response to Ep for 24 hours. Lipoxygenase-selective inhibitors of arachidonic acid metabolism blocked HETE production and Ep-stimulated growth and differentiation of erythroi progenitor cell-derived colonies (CFU-E). On the other hand, specific inhibitors of cyclooxygenase (aspirin and meclofenamate) did not significantly inhibit Ep-induced erythroid colony formation. It is hypothesized that the stimulation of HETE production from arachidonic acid (AA) is an essential step in the mechanism of action of Ep.

Erythroid progenitor cells proliferate and differentiate in response to the glycoprotein hormone, Ep. In cultures of bone marrow or murine fetal liver cells, the first detectable effect of Ep on erythroid cells is an increase in RNA synthesis followed by an enhancement of DNA synthesis and cellular division (1-2). The mode of signal transduction for many growth factors, hormones, and other extracellular messengers utilizes one of two major mechanisms: cyclic AMP as an intracellular mediator, or phosphatidylinositol turnover, leading to calcium mobilization and AA release, and is often accompanied by increases in cyclic GMP (3-5). Examples of hormones utilizing the cyclic AMP mechanism are epinephrine and glucagon; hormones

*To whom all correspondence should be addressed.
Abbreviations: 12-HETE, 12-hydroxyeicosatetraenoic acid; Ep, erythropoietin: HPLC, high performance liquid chromatography, BW755C, 3-amino-1-(m-trifluoro-methyl)-phenyl)-2-pyrazoline; CFU-E, colony forming unit-erythroid; AA, arachidonic acid; HPETES, hydroperoxyeicosatetraenoic acids; NDGA, nordihydroguiaiaretic acid; BHA, butylated hydroxyanisole; phenidone, 1-phenyl-3-pyrazolidone.

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utilizing the phosphoinositol pathway are thrombin, histamine, acetylcholine, and platelet-activating factor.

The intracellular events triggered by the interaction of erythroid growth factors with erythroid cells are not well understood. Previous studies have suggested that AA metabolites, particularly the prostaglandins, can modulate erythropoiesis (6-9). AA can be metabolized via two enzymatic pathways, the cyclooxygenase pathway, which catalyzes the conversion of arachidonic acid to prostaglandins and thromboxanes, and the lipoxygenase pathway, which gives rise to leukotrienes and hydroperoxy-eicosatetraenoic acid derivatives (HPETES) which are further metabolized to hydroxyeicosatetraenoic acid derivatives (HETES). Rabbit, chicken, rat, and dog erythrocytes synthesize low levels of 12-HETE (0.01-0.2 ng/10^6 cells) when stimulated by the calcium ionophore A-23187 as measured by HPLC (10); no cyclooxygenase products were reported although prostaglandins (PGE_2, PGD_2) have been shown to enhance erythroid progenitor cell colony formation in a number of studies (11-13).

We postulate that AA metabolites are intimately involved in erythroid cell proliferative and differentiative functions. In order to define the profile of AA metabolites in erythroid cells, we investigated the effect of Ep (human recombinant) on AA metabolism in murine fetal liver cells, which are predominately (70-80%) erythroid at day 14 of gestation (14). This homogenous erythroid progenitor cell population serves as a useful model for normal erythropoiesis, and is extremely sensitive to the hormone, Ep.

MATERIALS AND METHODS

BW755C was a generous gift from Wellcome Research Laboratories. NDGA, phenidone, BHA, aspirin, A23187, AA and citric acid were purchased from Sigma Chemical Co., St. Louis, MO. Sodium meclofenamate was obtained from Warner-Lambert Co., Ann Arbor, Michigan. All chemicals for HPLC were obtained from J.T. Baker Chemical Co. HETEs standards were purchased from Seragen Inc., Boston, MA. All culture supplies were obtained from GIBCO, Grand Island, NY. The lipoxygenase selective inhibitors were dissolved in absolute ethanol, then further diluted in alpha medium to the desired concentration. The maximum ethanol concentration achieved was 0.1% which had no effect on cell viability or formation. Erythropoietin (TCEpo) was supplied by Amgen Corp. (Thousand Oaks, Calif.).

Erythroid Progenitor Cell Assay:

Cells were suspended in a mixture of 0.8% methylcellulose,15% fetal bovine serum, 10^{-4}M mercaptoethanol, 100 units/ml of penicillin, 100µg/ml of streptomycin and 0.2 units/ml of human urinary erythropoietin (Ep) for CFU-E colonies. Inhibitors were added at the desired concentrations on day 0. One milliliter of methylcellulose mixture was plated in each 10x35mm petri dish and incubated for 2 days (CFU-E) or 7 days (BFU-E) in a humidified atmosphere of 95% air and 5% CO_2. After the dishes were stained with 3,3' diaminobenzidine, according to the method of Ogawa (15), CFU-E

colonies of eight or more cells were counted in two replicate dishes using an inverted microscope. One sixteenth of the dish was counted.

Determination of lipoxygenase metabolites:

Reverse phase high performance liquid chromatography was carried out by method of Eling, et al. (16). Separation of HETEs was achieved isocratically on a Perkin Elmer LC-75 variable wavelength detector connected to a Series 10 pump equipped with a Rheodyne 7125 injector fitted with a 20µl loop. The 25 cm Supelco 5µ LC18 column was fitted with a Supelguard LC18 2cm 5µl guard column. Chromatograms were recorded on a model 252 linear integrating strip chart recorder.

A mobile phase of 56% ACN/44% H_2O (H_2O pH adjusted to 3.5 with glacial acetic acid) was pumped through the system at 2.0 ml/min. Absorbance was set at 234 nm.

Cell culture medium in 1.0 ml volumes was adjusted to pH 3.5 with 4M citric acid, and extracted by strong vortexing for 30 sec. in ethyl acetate (2.0 ml). After centrifugation to insure sharp layering, the ethyl acetate was removed and dried under nitrogen. The residue was reconstituted with 100µl 50% ACN/50% H_2O. Percent recovery for 15-HETE was 76.75%±2.64 and the retention time was 14.7 min.

Unextracted culture medium was also assayed for both 12-HETE and 15-HETE utilizing Seragen radioimmunoassay kits.

RESULTS AND DISCUSSION

Effects of Inhibitors of Arachidonate metabolism on Erythroid Colony Formation:

Erythroid colony formation (CFU-E-derived) was assessed after two days of incubation at 37°C in the presence or absence of $1x10^{-4}$M sodium meclofenamate, aspirin, nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), 1-phenyl-3-pyrazolidone (phenidone), or 3-amino-1-(m-trifluoromethyl)-phenyl)-2-pyrazoline (BW755C). The effects of compounds which inhibit both the cyclooxygenase and lipoxygenase pathways (NDGA, BHA, phenidone, and BW755C), were compared to specific inhibitors of the cyclooxygenase pathway (meclofenamate and aspirin) of arachidonate metabolism. As noted in figure 1 in the presence of 0.2 U/ml recombinant Ep alone, the number of CFU-E derived colonies that appeared at 48 hours of culture (time of peak DNA synthesis) was 2400±110 (n=20). Phenidone, BHA, NDGA and BW755C significantly ($p<0.05$) inhibited colony formation at $1x10^{-4}$M. The most potent inhibitor, BW755C, inhibited Ep-stimulated colony formation to 17.1±6.7% of control, followed by NDGA (21.8±8.3%), BHA (40.6±11.3%), and phenidone (26.0±11.5%). In contrast, the cyclooxygenase-specific inhibitors, aspirin and sodium meclofenamate did not significantly inhibit colony formation induced by Ep.

Identification and Quantification of Lipoxygenase Products of Arachidonate:

Because fetal liver cells at day 14 of gestation are predominantly erythroid, it was felt that the effects of Ep on AA metabolism in fetal liver cells could be interpreted with fewer confounding features than in a more mixed

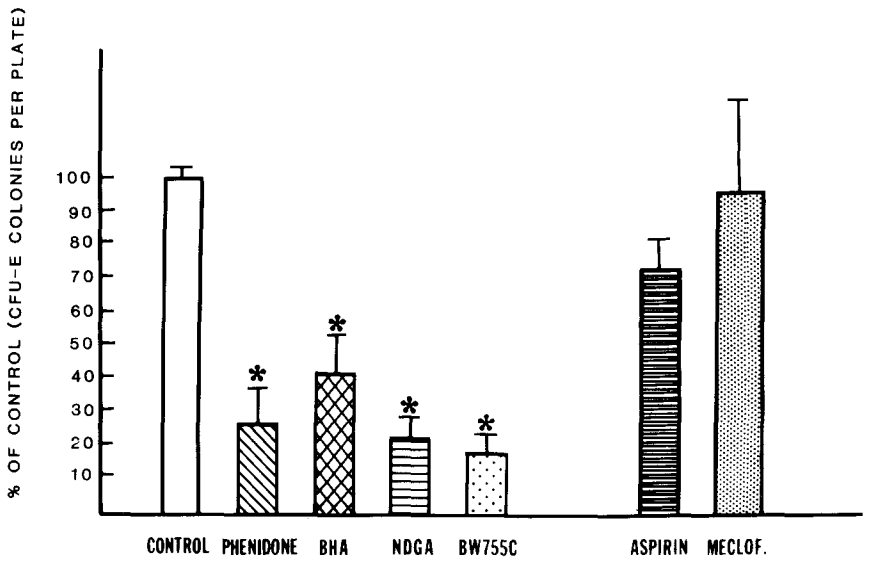

Figure 1. The effect of inhibitors of AA on CFU-E derived colony formation in response to Ep. Phenidone, BW755C, NDGA, BHA, sodium meclofenamate or aspirin ($1x10^{-4}$M) was added to cultures of fetal liver cells ($1x10^5$/ml) plated in a mixture of methylcellulose (0.8%), mercaptoethanol (10^{-4}M), fetal bovine serum (15%), penicillin (100 U/ml), streptomycin (100 µg/ml, and Ep (0.2 U/ml). CFU-E derived colonies composed of 8 or more cells were counted at 48 hr of incubation by staining with 3',3'-diaminobenzidine. Colony counts were expressed as percent of the control number of colonies in the absence of inhibitors (2400±110).
*Indicates significant difference from control ($p<0.05$) by Student's t test.

cell population such as bone marrow. Results with the selective lipoxygenase inhibitors, as well as the observation that mature erythrocytes synthesize HETEs (10), prompted us to perform reverse phase high performance liquid chromatography (HPLC) to determine the lipoxygenase-generated AA metabolite profile in response to Ep stimulation. Figure 2 illustrates the products of AA detected at 0, 15, and 120 minutes after cells ($5x10^6$/ml) were stimulated with Ep (0.2 U/ml). Cells were incubated at 37°C in the presence of AA (65µM) and Ca^{2+} ionophore A-23187 (2µM) without serum. Larger peaks of both 12-HETE and 15-HETE appeared as verified with authentic standards with more prolonged incubation time. Radioimmunoassays (Seragen) were performed in the presence of Ep (0.2µ/ml (Fig. 3) in order to quantify the amount of each product. 12-HETE was 5-7 times more abundant than 15-HETE. In addition, 12-HETE levels increased in response to Ep, whereas 15-HETE levels did not change. In the presence of BW755C, both 12-HETE and 15-HETE levels remained at control levels for 24 hours.

The demonstration of specific lipoxygenase metabolites generated in response to Ep-receptor stimulation in the present studies suggests that Ep probably activates the phosphatidylinositol cascade to elicit erythroid cell

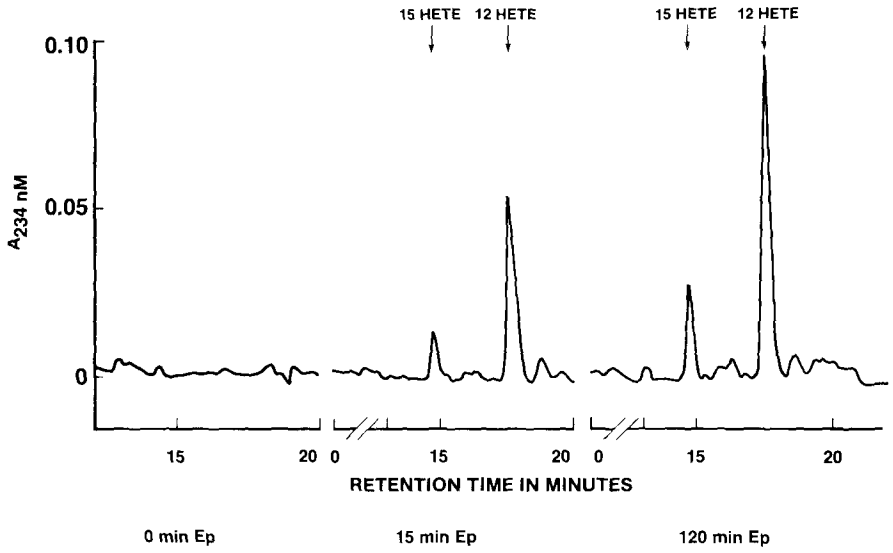

Figure 2. Arachidonic acid metabolites of the lipoxygenase pathway generated in response to Ep. Cells ($5x10^6$/ml) were incubated in the presence of Ep (0.2 U/ml), AA (65μM), and A23187 (2μM) for 0, 15, or 120 min. Cell culture medium was adjusted to pH 3.5 with 4 M citric acid, and extracted by strong vortexing for 30 sec in ethyl acetate. After centrifugation, the ethyl acetate was removed and dried under nitrogen. The residue was reconstituted with 100μl 50% acetonitrile/50% water.

proliferation and differentiation; and that HPETES and perhaps HETES, may function as second messengers. It is generally accepted that the liberation of AA from membrane phospholipids is rate-limiting, and that this reaction is

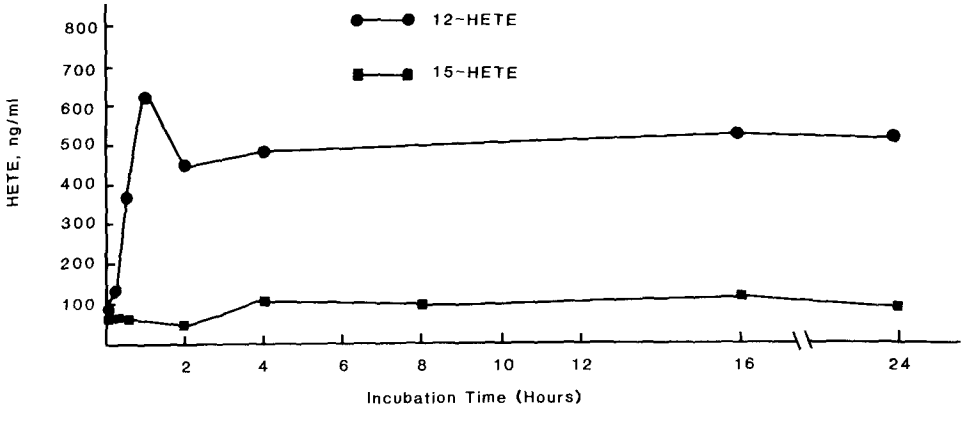

Figure 3. Time-course of HETE production in response to Ep as determined by radioimmunoassay. Fetal liver cells ($5x10^6$/ml) were incubated for 0, 15, 30 min and 1, 2, 4, 8, 16 and 24 hr in the presence of Ep (0.2 U/ml), AA (65μM), and A23187 (2μM). Cells were then centrifuged and the supernatants were collected for radioimmunoassay of 12-HETEs and 15-HETEs (Seragen kits). ■ indicates 15-HETE activity and ● indicates 12-HETE activity determined in duplicates of a representative experiment. Four separate experiments were done with similar results.

regulated by extracellular messengers (3-4). By analogy, our current model is that Ep interacts as an extracellular messenger with a membrane receptor to stimulate phosphatidylinositol turnover resulting in the release of HPETES, which can then activate guanylate cyclase to generate cyclic GMP in concert with Ca^{2+} mobilization. The stimulation of guanylate cyclase by arachidonate, other free fatty acids or by their oxidative derivatives and by hydroxyl radicals is well established in many experimental cell systems (17).

Preliminary experiments from our laboratory indicate that cyclic GMP is essential for erythroid cell proliferation and differentiation in response to Ep (18). Nishisuka et al. (45) have provided evidence that cellular responses, such as proliferation, are controlled by the synergistic effects of protein kinase C activation and Ca^{2+} mobilization. Although each component plays a diverse role in regulating cell function, they must be activated in concert to achieve maximal effects. McPhail et al. (19) found that unsaturated fatty acids can activate protein kinase C in neutrophils and might act as second messengers. They postulate a more general role for AA metabolites as modulators of stimulus-response coupling. Many extracellular messengers, including muscarinic-cholinergic and alpha-adrenergic agonists, insulin, and other peptide hormones, growth factors, histamine, and thrombin, stimulate phosphatidylnositol turnover and the release of AA after binding to their respective receptor (3, 20-21). Whether phospholipid degradation, Ca^{2+} mobilization, AA release, and cyclic GMP are integrated into a single receptor cascade system for Ep must await further study.

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