Vol. 92, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1980 Pages 268-276

ALTERED LIPOXYGENASE METABOLISM AND DECREASED GLUTATHIONE PEROXIDASE ACTIVITY IN PLATELETS FROM SELENIUM-DEFICIENT RATS

Robert W. Bryant and J. Martyn Bailey

Department of Biochemistry, George Washington University
School of Medicine and Health Sciences,
Washington, D.C. 20037

Received October 26,1979

SUMMARY: Washed platelets from selenium-deficient and control rats were incubated with [1-^{14}C]-arachidonic acid and the lipoxygenase and cyclooxygenase products were identified by gas chromatography/mass spectrometry. Platelets from selenium-deficient rats showed a three to four-fold increased synthesis of the lipoxygenase-derived isomeric trihydroxy fatty acids, 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid and 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid. A major reduction in glutathione peroxidase activity was also observed in platelets from deficient rats. These results support the interpretation that these trihydroxy fatty acids arise from breakdown of the primary platelet lipoxygenase product L-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) under conditions in which its reduction to the L-12-hydroxy product (12-HETE) by a selenium-dependent glutathione peroxidase is limited. Furthermore, these results indicate a specific function for selenium in platelet metabolism of essential fatty acids.

Platelets contain an active lipoxygenase (1,2) which converts arachidonic acid to L-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE). Platelets efficiently reduce 12-HPETE to L-12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) in a reaction which has been suggested to be catalyzed by a glutathione peroxidase (5). Recently, other platelet lipoxygenase products of arachidonate have been identified (3,4,5) namely the isomeric trihydroxy fatty acids 8,9,12-trihydroxy, 5,10,14-eicosatrienoic acid and 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid, collectively termed THETE, which are probably formed by an intramolecular rearrangement of 12-HPETE (4). We speculated that (5) THETE synthesis occurred when some 12-HPETE escaped reduction by a glutathione peroxidase, thus being available for rearrangement to THETE.

Abbreviations: 12-HPETE, L-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, L-12-hydroxy-5,8,10,14-eicosatetraenoic acid; THETE, a mixture of 8,9,12-trihydroxy-5,8,10,14-eicosatrienoic acid and 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid; TXB_2, thromboxane B_2; ME, methyl ester; TMS, trimethylsilyl; GSH-Px, glutathione peroxidase.

0006-291X/80/010268-09$01.00/0

Glutathione peroxidase activity has been shown to occur in both selenium dependent (6) and selenium independent forms (7). This paper reports experiments which indicate that selenium deficiency in the rat markedly lowers platelet glutathione peroxidase activity and stimulates THETE synthesis from added arachidonic acid.

MATERIALS AND METHODS

Weanling male rats (Fisher 344 in the first experiment and Sprague Dawley in the second experiment) were housed in hanging wire cages and were fed 5-6 weeks ad libitum either a selenium deficient basal diet (8) or the basal diet supplemented with 0.5 ppm of Se as Na_2SeO_3 to serve as the control. The basal diet also contained 500 ppm dl-α-tocopherol acetate and 0.3% dl-methionine with the exception that dl-methionine was omitted from the diet of the control animals in the first experiment.

Rats were anesthetized with sodium pentobarbital (6.5 mg/100 g I.P.) and blood (4-7 ml) was obtained by cardiac puncture and then added to 1/10 volume of 90 mM EGTA, 0.35% NaCl anticoagulant. One half volume of 0.9% NaCl was added to increase platelet yield (9). Red cells were sedimented at 250xg for 10 min and the platelets were then sedimented at 720xg for 10 min. The platelet pellet was resuspended in 2 ml of 12 mM Tris HCl, 1.5 mM EDTA, 135 mM NaCl buffer (10), pH 7.4 containing 5 mM D-glucose and resedimented at 720xg for 10 min. The washed platelets were finally resuspended in modified Krebs-Henseleit buffer (10) without calcium but containing 5 mM D-glucose. Platelet yields from deficient animals were similar to controls. Aliquots (0.25 ml) of the platelet suspensions (5 to $8x10^5$ platelets/ul) from individual rats were incubated at 37° C for 10 min in siliconized tubes with 0.1 uCi of [1-^{14}C]-arachidonic acid (Amershame-Searle Corp.) which had been diluted to a specific activity of 6 uCi/umole with unlabelled arachidonic acid (Sigma Chemical Co.). Arachidonate metabolites were extracted from the acidified incubation with ethyl acetate and analyzed by thin-layer chromatography using solvent system I, iso-octane:ethyl acetate:acetic acid:water, 50:110:20:100 by volume (organic phase), as previously described (5).

Radioactive plates were scanned with a Vanguard model 940 Radiochromatogram Scanner and radioactive peaks for TXB_2, HETE and arachidonic acid were identified by reference to unlabelled carrier TXB_2, ricinoleic acid and arachidonic acid respectively, which were added to the incubation extracts. These reference compounds were detected on the plates by phosphomolybdic acid spray reagent. For quantitative determination of metabolite synthesis, radioactive bands were first located exactly by autoradiography (11) and then were scraped into scintillation vials containing 5 ml of Hydromix (Yorktown) and counted.

Gas chromatography/mass spectrometry analyses were performed on a Hewlett-Packard 5992A GC/MS (5) fitted with a 0.91 M x 2 mm I.D. column packed with 3% SP2100 (Supelco). Methyl ester, trimethylsilyl ether derivatives were prepared by successive treatment of samples with etheral diazomethane and trimethylsilyl imidazole (5). Authentic reference 8,9,12- and 8,11,12-trihydroxy-eicosatrienoic acid (THETE) was prepared as previously described (5).

Platelet glutathione peroxidase activity was assayed according to Karpatkin and Weiss (12). Aliquots of the washed platelet suspensions in the

modified Krebs-Henseleit previously described were sonicated (4 x 10 sec) and centrifuged at 105,000 x g for 1 hr at 4°C. The 105,000 x g supernatant (200 ul) was added to a cuvette containing 64 umole sodium phosphate, pH 7.0, 3 umole EDTA, 3 umole NaN_3, 5 umole GSH, 0.2 umole NADPH and 1 unit of glutathione reductase to give a volume of 980 ul. After 5 min of preincubation at 27°C, 20 ul, (0.35 umole) of t-butyl hydroperoxide was added. A linear decrease in absorbance at 340 mu was observed between 1 and 4 min. Non-enzymatic oxidation of glutathione by t-butyl hydroperoxide, assayed by substituting buffer for the platelet supernatant, was less than 10 per cent of the platelet supernatant activity from selenium supplemented rats. This non-enzymatic "blank" activity was subtracted from the enzymatic values. The assays were done in duplicate and were in the linear portion of the activity versus enzyme addition curve. The GSH-Px activity of platelet poor plasma (50 ul aliquots) was determined by the same assay with inclusion of slightly more sodium phosphate (76 umole), EDTA (4 umole) and NaN_3 (4 umole). The GSH-Px activity of red cells was assayed according to Thomson et al. (13). Student's t-test was used to compare means. Glutathione reductase, reduced glutathione and NADPH were obtained from Sigma Chemical Co. and t-butyl hydroperoxide was supplied by Curtin Matheson Scientific, Inc.

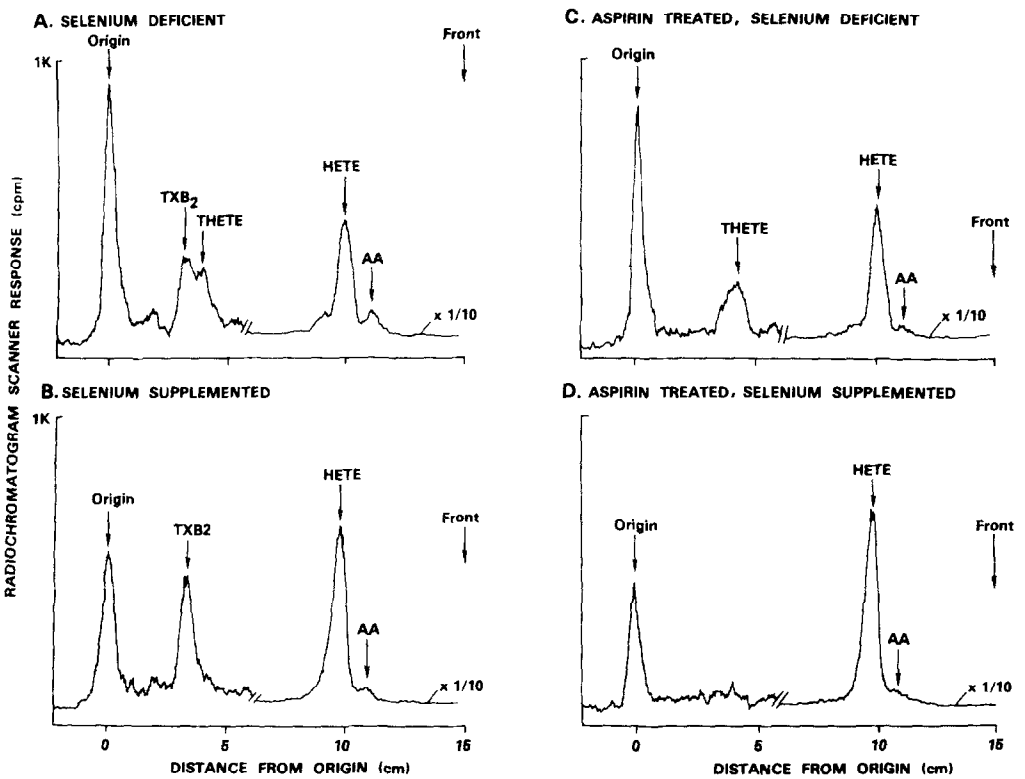

Fig. 1. Stimulation of THETE Synthesis from [1-^{14}C]-Arachidonic Acid in Platelets from Selenium Deficient Rats. Washed platelets isolated from selenium deficient (panels A and C) or selenium supplemented control (panels B and D) rats were incubated with [1-^{14}C]-arachidonic acid and analysed by radioscanning TLC as described in Materials and Methods. Platelets in panels C and D were pretreated for 15 min. with aspirin (100 ug/ml). This figure is representative of duplicate incubations with platelet preparations (unpooled) from three rats (Fisher 344) in each dietary group.

RESULTS

Washed platelets were prepared from weanling rats (strain Fisher 344, 3 animals per group) which were fed 5 to 6 weeks on either a selenium deficient or selenium supplemented diet. Platelets from the two groups were incubated with [1-^{14}C]-arachidonic acid and the products were analyzed by radioscanning thin layer chromatography (Fig. 1). A distinctive and reproducible difference between the two groups was observed in the arachidonate metabolite pattern in that platelets from selenium deficient rats produced significant amounts of the isomeric trihydroxy fatty acids 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid and 8,11,12-trihydroxy-5,9,14-eicosatrienoic acids (THETE) which migrated above TXB_2 on the TLC plate (Fig. 1A). The stimulation of THETE synthesis with platelets of selenium deficient rats was more striking in incubations pretreated with aspirin (Fig. 1C), since the closely migrating TXB_2 radioactive peak was then eliminated. THETE synthesis was barely detectable in selenium supplemented controls (Figs. 1B and 1D).

The identity of THETE synthesis in platelets from deficient rats was positively confirmed by selected ion monitoring GC/MS as shown in Fig. 2. Derivatized samples were analyzed by monitoring the mass ions (m/e) 213, 243 and 569 which are prominent in the mass spectra of the THETE-ME-$(TMS)_3$ derivatives (4,5). Platelets from deficient rats (Fig. 2A) gave a mass ion chromatographic peak for THETE-ME-$(TMS)_3$ with a retention time (3.3 min) and mass ion ratios (100:71:0.2 for m/e 213/243/569) very similar to those for an authentic reference sample (Fig. 2B) of THETE-ME-$(TMS)_3$ which had a retention time of 3.3 min and mass ion ratios of 100:79:0.2. The identity of 12-HETE and TXB_2 was also confirmed by GC/MS in platelets from selenium deficient and control rats.

Table 1 (Experiment #1) summarizes the quantitative changes in arachidonic acid metabolism of aspirin-treated platelets of the selenium deficient rats. There was a moderate drop in HETE formation (30%) and a 4-fold stimulation in THETE synthesis. Table 1 also shows the results (Experiment #2) of

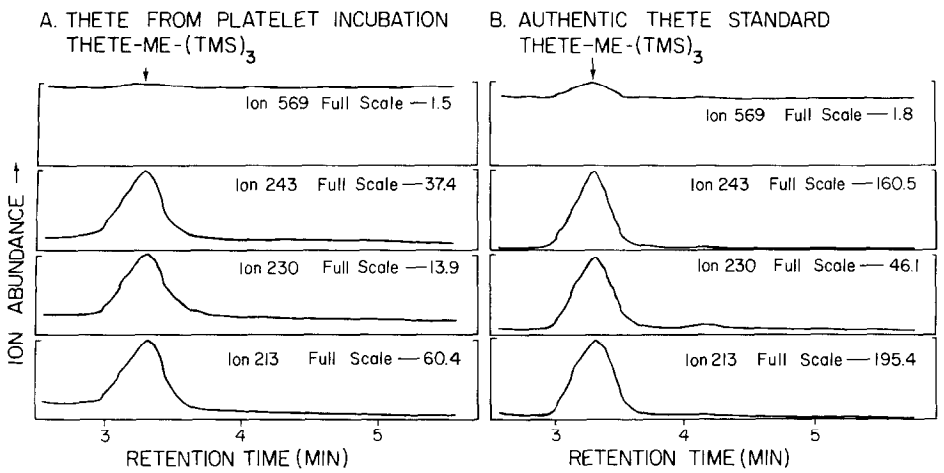

Fig. 2. Selected Ion Monitoring GC/MS of THETE from Platelets of Selenium Deficient Rats. (Panel A) Platelets from selenium deficient rats were incubated with arachidonic acid as described in Fig. 1 and the acidic ethyl acetate extract was derivatized for GC/MS analysis. (Panel B) An authentic sample of THETE methyl ester tris-trimethylsilyl ether (THETE-ME(TMS)$_3$) was also prepared. Both samples were analysed by monitoring mass ions (m/e) 213, 243 and 569 with dwell times of 100, 100 and 700 msec respectively. The GC oven was set at 220°C.

another set of incubations (without aspirin pretreatment) from more selenium deficient and control rats (strain Sprague Dawley, 4 animals per group) maintained 5-6 weeks on the experimental diet. Again there was a marked stimulation of THETE synthesis (3-fold) in the deficient group. In contrast, there was no effect of selenium deficiency on thromboxane B_2 (TXB_2) synthesis.

TABLE 1

Stimulation of THETE Synthesis in Platelets from Selenium Deficient Rats.

	Conversion of [1-^{14}C]-Arachidonic Acid (dpm x 10^{-3})			
METABOLITE	EXPERIMENT #1[a]		EXPERIMENT #2[c]	
	Control	Deficient	Control	Deficient
THETE	1.7±0.3[b]	6.7±1.5 ($p<.05$)	2.6±0.7	6.9±1.2 ($p<.05$)
HETE	63±1	44±5 ($p<.025$)	91±3	74±10 ($p<.2$)
TXB_2	N.D.[d]	N.D.	8.7±0.6	8.6±0.8

[a] Strain Fisher 344, N=3
[b] ± S.E.M.
[c] Strain Sprague Dawley, N=4
[d] not determined since platelets were treated with aspirin

TABLE 2
Loss of Glutathione Peroxidase Activity in Platelets from Selenium Deficient Rats.

Enzyme Source	Activity	
	Control	Deficient
Platelets	5.3±0.2[a,b]	0.7±0.1 ($p<.001$)
Erythrocytes	87±6[c]	11±1
Plasma	2.7±0.4[d]	0.11±0.03 ($p<.001$)

[a] ±1 S.E.M., N=6
[b] umole/min/10^{11} platelets
[c] umole/min/ml packed erythrocytes
[d] umole/min/ml plasma (platelet poor)

The decrease in HETE synthesis in the second group of selenium deficient rats was not statistically significant.

The glutathione peroxidase activity (Table 2) of both the plasma and red cells from the animals on the selenium deficient diet was very low (4% and 13% respectively) in comparison to controls. These results confirm that the animals in the deficient group were indeed selenium deficient (14). The glutathione peroxidase activity of platelets was also low (13% of controls) in the deficient animals. The data shown in Table 2 is based on assays using t-butyl hydroperoxide as a substrate. Very similar losses in activity in plasma and platelets were obtained, using hydrogen peroxide as substrate (unpublished results).

DISCUSSION

The platelet lipoxygenase-derived trihydroxy fatty acids have been proposed to arise from the 12-hydroperoxides produced by platelets based on their lipoxygenase origin, chemical structure and similarity to other trihydroxy fatty acid rearrangement products of fatty acid hydroperoxides (15,4,5). We hypothesized that 8,9,12-THETE and 8,11,12-THETE are formed in platelets when some 12-HPETE escaped glutathione peroxidase-catalyzed reduction to 12-HETE as shown in Figure 3 (16). In the experiments reported here, platelet glutathione peroxidase levels were altered by dietary selenium de-

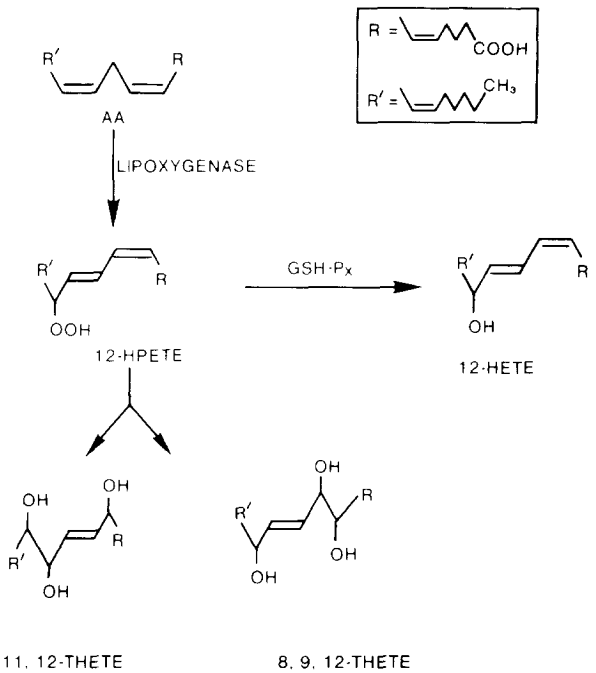

Fig. 3. Proposed Role of Glutathione Peroxidase (GSH-Px) in Determining Product Distribution of the Platelet Lipoxygenase Pathway.

ficiency in the rat and thus provided a model system to further study the role of glutathione peroxidase in lipoxygenase metabolism.

Dietary selenium deficiency (5-6 weeks) in rats markedly decreased platelet glutathione peroxidase activity to only 13% of control levels. These glutathione peroxidase deficient platelets also showed marked alterations in lipoxygenase metabolism of arachidonic acid. Deficient platelets showed a 3- to 4-fold increased formation of 8,9,12-THETE and 8,11,12-THETE in comparison to platelets from control animals. In further support of the interpretation shown in Figure 3, there appeared to be a slight decrease in 12-HETE formation, although the decrease was not always statistically significant (c.f. Table 1). Further work will be necessary to evaluate the possible roles of non-selenium dependent (7) and other peroxidases (17) in the lipoxygenase pathway. In regards to the measurement of 12-HETE formation, Siegel *et al.* (18) have reported that 12-HPETE has a chromatographic mobility in system I similar to 12-HETE and may be present in the chromatographic band counted as

12-HETE. In preliminary experiments, however, we have not found 12-HPETE in extracts of either selenium-deficient or control platelets using a TLC system (1) which separates 12-HPETE from 12-HETE.

In contrast to changes in lipoxygenase metabolism induced by selenium deficiency, there was no measurable change in platelet prostaglandin cyclooxygenase metabolism as monitored by the formation of thromboxane B_2 (TXB_2). These results are in accord with the finding that the peroxidase in bovine seminal vesicles, which reduces 15-hydroperoxy-prostaglandin endoperoxide (PGG) to the 15-hydroxy derivative (PGH), does not use glutathione as a reducing agent (17).

While it is possible that the stimulation of 8,9,12- and 8,11,12-THETE synthesis in platelets from selenium-deficient rats may be unrelated to the decrease in platelet glutathione peroxidase levels, their association seems very reasonable. It thus appears that a selenium-dependent glutathione peroxidase plays a previously unappreciated role in platelet lipoxygenase metabolism. Furthermore, this role, i.e. the reduction of 12-HPETE to 12-HETE, appears to be the first clear example of a specific function for selenium as a required component in the normal metabolism of essential fatty acid.

Acknowledgements: The authors wish to thank Dr. Orville A. Levander, U.S.D.A., Beltsville, Md., for provision of animals and diets, the technical assistance of Amy Wong and Ted Simon and support in part by N.I.H. grants 5S07RR539-17, HL 05062 and CA 15356 and N.S.F. grant PCM 07147.

REFERENCES

1. Hamberg, M. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3400-3404.
2. Nugteren, D.H. (1975) Biochim. et Biophys. Acta 380, 299-307.
3. Bryant, R.W. and Bailey, J.M. (1978) Fed. Proc. 37, 1317.
4. Jones, R.L., Kerry, P.J., Poyser, N.L., Walker, I.C. and Wilson, N.H. (1978) Prostaglandins 16, 583-591.
5. Bryant, R.W. and Bailey, J.M. (1979) Prostaglandins 17, 9-18.
6. Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D. and Hoekstra, W.G. (1973) Science 179, 588-590.
7. Lawrence, R.A. and Burk, R.F. (1976) Biochem. Biophys. Res. Com. 71, 952-958.
8. Levander, O.A., Morris, V.C., Higgs, D.J. and Vorma, R.N. (1973) J. Nutr. 103, 536-542.
9. Chaiken, R., Pagno, P. and Detwiler, T.C. (1975) Biochim. et Biophys. Acta 403, 315-542.

10. Hamberg, M., Svensson, J., Wakabayaski, T. and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. U.S.A. 71, 345-349.
11. Bryant, R.W., Feinmark, S.J., Makheja, A.N. and Bailey, J.M. (1978) J. Biol. Chem. 253, 8134-8142.
12. Karpatkin, S. and Weiss, H.J. (1972) New Eng. J. Med. 287, 1062-1066.
13. Thomson, C.D., Rea, H.M., Doesburg, V.M. and Robinson, M.F. (1977) Br. J. Nutr. 37, 457-460.
14. Chow, C.K. and Tappel, A.L. (1974) J. Nutr. 104, 444-451.
15. Farlardeau, P.M., Hamberg, M. and Samuelsson, B. (1976) Biochim. et Biophys. Acta 441, 193-200.
16. Bryant, R.W. and Bailey, J.M. (1980) Advances in Prostaglandin and Thromboxane Res. 6, Samuelsson, B., Ramwell, P.W. and Paoletti, eds., Raven Press, New York, in press.
17. Ohki, S., Ogino, N., Yamamoto, S. and Hayaiski, O. (1979) J. Biol. Chem. 254, 829-836.
18. Siegel, M.I., McConnell, R.T. and Cuatrecasas, P. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 3774-3778.