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PHOSPHATE BALANCE IN GLUTATHIONE-DEPLETED STATES: TOURNIQUET TRAUMA AND BROMOBENZENE INJECTION*

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Abstract—Previous studies have shown that, in the intact mouse, tourniquet trauma decreases the concentration of liver glutathione as well as the ability of the liver to synthesize GSH† after cysteine injection. Prior administration of bromobenzene also decreases liver GSH, but does not decrease GSH synthesis by the liver after cysteine injection. Since the literature indicates that trauma induces depletion of tissue ATP required for GSH synthesis, the present work attempted to study the relationship between liver ATP concentration and the liver's potential for GSH synthesis. In the present experiments the trauma procedure used induced significant increases in liver ATP, whereas bromobenzene preadministration resulted in decreases in liver ATP. Hence, over-all liver ATP concentration does not appear to be limiting for GSH synthesis in trauma. Similar ATP/ADP ratios were obtained for livers of control, traumatized, and bromobenzene-treated mice. Inorganic phosphate and total acid-soluble phosphate were elevated after trauma; total acid-soluble phosphate was depressed after bromobenzene administration.

SINCE the finding by Beck and Linkenheimer[1] that tourniquet trauma causes a dramatic loss of liver nonprotein sulfhydryl, of which the tripeptide glutathione (GSH) makes up the major portion, we have been concerned with the reason for this rapid decrease. A study of the rate of incorporation of the radioactively labeled amino acid, cysteine, into glutathione,[2] led us to suspect that the decreased level of GSH seen after trauma was due to a decreased ability of the traumatized liver to synthesize GSH and/or retain it, rather than to an increased degradation of GSH going on in the liver. Further support to this hypothesis was obtained in experiments in which the GSH-depleted liver, given large amounts of cysteine, showed poor net synthesis.[3] In contrast, animals whose liver GSH was even more drastically depleted, through an injection of bromobenzene, were able to resynthesize their GSH stores rapidly and completely under the same conditions.[3] Since ATP was shown by Yanari *et al.*[4] to be involved in both steps of the synthesis of GSH *in vitro*, and since LePage[5] and, more recently, Stoner and Threlfall[6] found decreased ATP in rat liver after trauma, we hypothesized that a decreased ATP and therefore a lowered energy supply might be the critical factor in the inability of the livers of traumatized mice to resynthesize GSH. Because animals

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† Abbreviations: GSH, glutathione; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NPSH, nonprotein sulfhydryl; TASP, total acid-soluble phosphate; P_i, inorganic phosphate.

pretreated with bromobenzene showed remarkably good ability to resynthesize GSH when given adequate cysteine to replace that lost into the urine as mercapturic acid in the detoxication of bromobenzene, we anticipated finding normal or even elevated quantities of ATP in these animals. As will be shown, the results of this set of experiments were the reverse of those anticipated. It is therefore doubtful that lowered GSH synthesis in the traumatized animal is due to depleted ATP levels.

METHODS

Carworth Farms Webster-strain mice (male, 18 to 26 g), maintained on Rockland Rat Diet, were used. Three experimental groups were studied; control (C), trauma (T), and bromobenzene (BB). All groups were injected intramuscularly with 10 ml peanut oil/kg 18 hr prior to sacrifice; food was removed at this time but water allowed *ad libitum*. The BB group received 750 mg bromobenzene/kg in the peanut oil injection (1·0 ml bromobenzene + 19·0 ml peanut oil). Rubber-band tourniquets were applied to both hind legs of the T group for a period of 3 hr, and these mice were sacrificed 2·5 hr after the tourniquets were removed. All mice were lightly anesthetized with ether prior to immersion in liquid nitrogen. The abdomen was broken open, and about 1 g of liver was dissected out while completely frozen, avoiding the large blood vessels as much as possible.

A protein-free acid extract was prepared from each liver by homogenization of 1 g liver with 9·3 ml ice-cold 0·3 M perchloric acid in a cold-room (5°). One aliquot of the extract was used to estimate nonprotein sulfhydryl (NPSH) by the method of Grunert and Phillips.[7] A second aliquot was neutralized to pH 6·5–7·0 with KOH, diluted fivefold and the potassium perchlorate precipitate removed by centrifugation. This neutralized, diluted extract was used for determination of : (a) inorganic phosphate by the Berenblum and Chain[8] method as modified by Ennor and Stocken;[9] (b) total acid-soluble phosphate by wet ashing followed by the Gomori colorimetric technique;[10] (c) ATP by the luciferase assay of Strehler and Totter,[11] as modified by Cheng[12] and using a Beckman DU spectrophometer with an energy recording adapter to record peak flash intensity; and (d) ADP essentially by the method of Cheng[12], but determining ADP as the difference between pre-existing ATP and total ATP after all ADP in the extract has been transformed to ATP by added phosphocreatine and creatine phosphokinase. Aliquots of the neutralized extract were used immediately for the determination of P_i and were wet-ashed overnight for the TASP determination. The remainder of the neutralized extract was frozen and used the following morning for the determination of ATP and ADP.

Since the luciferase preparation decreases in activity with time, even when held on ice, it was found necessary to make a time correction. Aliquots of the same ATP standard solution were read at various times throughout the analysis, and the change in activity, plotted on semilog paper versus time, was used to obtain a correction factor for the tissue samples. The ATP and the ATP + ADP tubes were read as rapidly as rapidly as possible (1·5 to 2 min per sample) and the time of each analysis noted. The order of reading of the samples was selected from a table of random numbers so that no bias due to the correction factor for time of reading would occur.

Originally it was planned also to estimate mouse liver phosphocreatine, but practically none was found. Rat liver has also been found to contain very little phosphocreatine.[13]

RESULTS

As can be seen from Table 1, both trauma and bromobenzene significantly lowered liver nonprotein sulfhydryl; bromobenzene administration lowered it more than did trauma. This finding was not paralleled by similar changes in the ATP, ADP, or ATP + ADP concentrations. Instead, all three of these quantities were elevated in

TABLE 1. PHOSPHATE BALANCE IN GLUTATHIONE DEPLETION

Component	Control	Trauma	Bromobenzene
	(μmole P/100 g wet weight ± S.E.)		
TASP	(38) *2,680 ± 55	(34) 2,947 ± 73†	(34) 2,336 ± 78‡
P_i	(40) 845 ± 21	(35) 1,020 ± 27‡	(36) 841 ± 40
ATP	(16) 112 ± 13	(16) 152 ± 13§	(16) 85 ± 7
ADP	(16) 255 ± 18	(16) 338 ± 25†	(16) 172 ± 15‡
ATP + ADP	(16) 360 ± 29	(16) 499 ± 37†	(16) 258 ± 18†
	(μmole GSH equiv./100 g wet weight ± S.E.)		
NPSH	(40) 474 ± 14	(36) 295 ± 12‡	(36) 185 ± 15‡
ATP/ADP	0·44	0·45	0·49
P_i/ATP	7·54	6·71	9·89
P_i/ADP	3·31	3·02	4·89

* ( ) Number of animals.
† Significantly different from corresponding control means: $P < 0.01$.
‡ $P < 0.001$.
§ $P < 0.05$.

trauma and depressed in bromobenzene administration. The ratio of ATP/ADP did not differ significantly from the control in either group. Increased concentrations of total acid-soluble phosphate were seen after trauma, and much of this increase could be accounted for by the elevation of inorganic phosphate seen at this time. In the bromobenzne group the total acid-soluble phosphate level was depressed; none of this decrease could be attributed to the P_i. Ratios of P_i/ATP and P_i/ADP showed increases above control values for the bromobenzene group but very slight change for the trauma group.

DISCUSSION

The reports by LePage[5] and by Stoner and Threlfall[6] that rats subjected to tumbling or tourniquet trauma exhibit decreases in ATP and ADP of a number of tissues, including liver, have already been referred to. In actuality, Stoner and Threlfall, who used a specific enzymatic method for ATP, reported significant decreases in liver ATP only for moribund rats or rats sacrificed quite a few hours after removal of hind-leg ligatures. Rats whose liver ATP was estimated shortly after ligature removal did not differ significantly from control, and the decrease in ATP seemed to be a terminal event. The methods available to LePage for ATP and ADP estimations were not specific; his liver ATP values for control rats are much lower than those obtained by others[6, 14] using more specific methods, and many of his rats were moribund at time of sacrifice. The ATP and ADP procedures used in the present work are highly specific, and recoveries for ATP and for ADP added to tissue extracts have ranged from 90–110 per cent. Traumatized mice at time of sacrifice, 2·5 hr after removal of hind-leg ligatures, were not moribund. Hence we must conclude on the basis of the evidence obtained that mice subjected to the particular trauma procedure used by us

have *increased* liver ATP and ADP concentrations as a result of that trauma, and that the bromobenzene pretreatment decreases the concentration of liver ATP and ADP. In earlier experiments,[2, 3] using the same trauma and bromobenzene pretreatment procedures as in the present work, we found that synthesis of glutathione *in vivo* was impaired in the livers of traumatized mice but not in the livers of bromobenzene-pretreated mice. It appears unlikely, therefore, that lowered intracellular ATP concentration can be responsible for the trauma-induced impairment of liver glutatione synthesis. High ATP levels in trauma may be due to a lack of utilization rather than to increased synthesis, whereas in bromobenzene poisoning there may be an increased rate of recycling of ATP, which allows low levels of ATP to support a vigorous synthesis. In trauma, as effected in these experiments, it appears that ATP might be a limiting factor for GSH synthesis only if this trauma, while inducing actual increases in total ATP, also induced massive redistribution of intracellular ATP away from the loci of GSH synthesis.

Total acid-soluble phosphates of mouse liver were elevated in trauma as we used it, with most of the increase attributable to the inorganic phosphate fraction. Stoner and Threlfall[6] and LePage[5] also demonstrate this elevation of P_i in livers of traumatized rats, but do not show an elevated TASP. Although the source of this excess P_i is unknown, calculations made by Trémolières and Derache[15] indicate that the amount of P_i lost from ischemic muscle after tourniquet release is insufficient to account for the elevation of P_i seen in many tissues. It is interesting to speculate whether an increased breakdown of liver phosphoprotein may be occurring under these conditions and might be the source of the increased liver inorganic phosphate. Total acid-soluble phosphates of mouse liver were decreased after bromobenzene administration, but inorganic phosphate remained at control levels. Only about a third of this decrease in liver TASP can be accounted for by decrease in liver ATP + ADP occurring in association with bromobenzene pretreatment. Whatever other organic phosphate is lost from the liver as result of bromobenzene pretreatment, it cannot be phosphocreatine, since we found essentially none of this component in mouse liver.

REFERENCES

1. L. V. BECK and W. LINKENHEIMER, *Proc. Soc. exp. Biol. (N.Y.)* **81**, 291 (1952).
2. S. C. KALSER and L. V. BECK, *Biochem. J.* **87**, no. 3 (1963).
3. S. C. KALSER and L. V. BECK, *Proc. Soc. exp. Biol. (N.Y.)* **109**, 638 (1962).
4. S. YANARI, J. E. SNOKE and K. BLOCH, *J. biol. Chem.* **201**, 561 (1953).
5. G. A. LEPAGE, *Amer. J. Physiol.* **146**, 267 (1946).
6. H. B. STONER and C. J. THRELFALL, *Biochem. J.* **58**, 115 (1954).
7. R. R. GRUNERT and P. H. PHILLIPS, *Arch. Biochem.* **30**, 217 (1951).
8. J. BERENBLUM and E. CHAIN, *Biochem. J.* **32**, 295 (1938).
9. A. H. ENNOR and L. A. STOCKEN, *Aust. J. exp. Biol. med. Sci.* **28**, 647 (1950).
10. G. J. GOMORI, *J. Lab. clin. Med.* **27**, 955 (1941).
11. B. L. STREHLER and J. R. TOTTER, *Meth. biochem. Anal.* **1**, 345 (1954).
12. S. C. CHENG, *J. Neurochem.* **7**, 271 (1961).
13. E. N. FAWAZ, G. FAWAZ and K. VON DAHL, *Proc. Soc. exp. Biol. (N.Y.)* **109**, 38 (1962).
14. W. THORN, J. HEIMANN, G. GERCKEN and B. MÜLDENER, *Z. ges. exp. Med.* **130**, 497 (1958).
15. J. TRÉMOLIÈRES and R. DERACHE, *The Biochemical Response to Injury*, p. 38. Blackwell, London (1958).