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GLUTATHIONE DEPLETION IN THE GUINEA PIG AND ITS EFFECT ON THE ACUTE COCHLEAR TOXICITY OF ETHACRYNIC ACID

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Abstract—There is controversy as to whether or not the acute cochlear toxicity of ethacrynic acid (EA) is dependent upon its metabolic conversion to EA-cysteine via conjugation with glutathione. In order to investigate this we examined the acute effects of EA on cochlear potentials in guinea pigs in which glutathione levels were decreased by prior administration of (±)-buthionine sulphoximine (BSO), an inhibitor of glutamylcysteine synthetase. First, we detemined the effects of BSO on hepatic and renal glutathione levels in the guinea pig. Guinea pigs (pigmented animals of both sexes or male albino animals) were killed at intervals up to 72 hr after i.p. administration of 1.6 g kg^{-1} BSO. Livers, and also kidneys in the case of pigmented guinea pigs, were removed and total glutathione (GSH + GSSG) measured. Glutathione levels reached a nadir in the liver at 24–48 hr (11% of control) and in the kidneys at 24 hr (14% of control) after administration of BSO. Hepatic but not renal levels approached control values by 72 hr. There were no sex or strain differences. Pigmented guinea pigs were anaesthetised and their endocochlear potential and a.c. cochlear potential in response to a 4 kHz tone were measured using an intracochlear microelectrode. The depression of these potentials by i.v. administration of 60 mg kg^{-1} EA was not affected by administration of 1.6 g kg^{-1} BSO 24 hr earlier, despite profound depletion of glutathione. Also prior p.o. administration of *N*-acetyl-L-cysteine did not affect hepatic glutathione levels nor modify the toxicity of EA. These results suggest that the acute cochlear toxicity of EA is not altered by glutathione depletion, a finding which argues against a role for the metabolic activation of EA in its ototoxicity.

Administration of large doses of the loop diuretic ethacrynic acid (EA, Fig. 1) causes acute reversible depression of cochlear potentials [1, 2] and the compound action potential in the auditory nerve [3, 4]. It has been suggested that metabolism of EA precedes its toxic action on the ear and the cysteine conjugate of EA (Fig. 1) has been proposed as the species through which EA causes ototoxicity [3]. In this report we present the results of experiments conducted to test the hypothesis that conjugation with glutathione is a prerequisite for the ototoxic action of EA. EA-cysteine is a product of the conjugation of EA with glutatione [5] so that the metabolism of EA to EA-cysteine may be modulated by altering either the level of glutathione or the activity of the transferase enzymes in the liver. Wallin *et al.* [6] have shown that, in the rat, an increase in the conjugation of EA to glutathione followed the induction of glutathione-*S*-transferase by phenobarbital administration. Conversely, conversion of EA to its glutathione conjugate was decreased after pretreatment with diethylmaleate which depletes glutathione stores and inhibits glutathione transferases [6]. In the work reported here the strategy adopted was to deplete stores of glutathione selectivity by pretreatment of animals with (±)-buthionine sulfoximine (BSO). BSO reduces glutathione levels, particularly in the liver [7, 8], because it is a potent inhibitor of α-glutamylcysteine synthetase, a key enzyme in the biosynthesis of glutathione. Thus, we examined the effect of BSO pre-treatment on the acute ototoxic action of EA. The effect of prior supplementation with *N*-acetyl-L-cysteine (NAC) on the ototoxicity of EA was also studied. Guinea pigs were used in these investigations as they are the rodent of choice in ototoxicological studies. However, as little is known about the effect of BSO

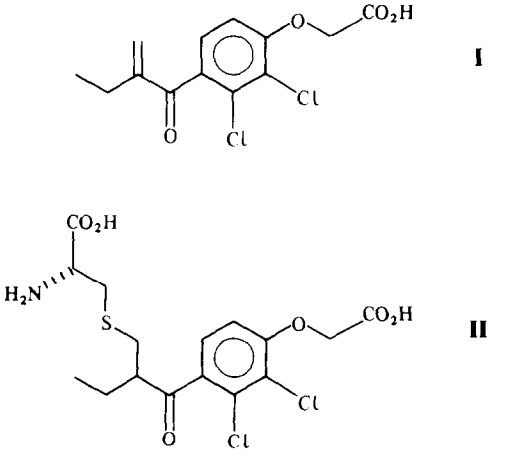

Fig. 1. Structures of ethacrynic acid (I) and its cysteine adduct (II).

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on the guinea pig, the time course of depletion of glutathione in the liver and kidneys of guinea pigs which had received BSO was investigated first.

MATERIALS AND METHODS

EA as sodium ethacrynate (Edecrin) was purchased from Merck, Sharp & Dohme (U.K.), urethane, BSO, NAC and reagents for the glutathione determinations from Sigma (U.K). Male and female pigmented guinea pigs and male albino guinea pigs (480–885 g) were killed by cervical dislocation followed by exsanguination at intervals up to 72 hr after i.p. administration of BSO ($1.6\,g\,kg^{-1}$) in saline. Animals had free access to food and water at all times. The total glutathione (GSH + GSSG) content of liver or kidneys was measured by a method modified from that of Tietze [9]. The tissues were deproteinised prior to assay by homogenisation in 10% metaphosphoric acid and centrifugation followed by pH correction with hot trisodium phosphate. GSH was determined by following at 412 nM (30°) the production of 5-thio-2-nitrobenzoic acid formed during the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) by GSH, the glutathione disulphide (GSSG) subsequently formed being reduced by NADPH in the presence of glutathione reductase. The amount of GSH present in the sample was calculated from a GSH standard curve.

Pigmented guinea pigs, of either sex, were used in studies of the acute effects of EA on cochlear function. Full experimental methods are reported elsewhere [10]. Animals were anaesthetised by i.p. injection of urethane ($1.2\,g\,kg^{-1}$) and prepared by placement of a microelectrode in the cochlear duct for the simultaneous recording of the d.c. endocochlear potential (EP) and the a.c. cochlear potential, or cochlear microphonic (CM), in response to a 4 kHz tone. The intensity of the tone was adjusted to produce an initial CM amplitude of 150 μV rms. EA ($60\,mg\,kg^{-1}$) was administered as a 1-min infusion through a cannula in the external jugular vein 24 hr after i.p. administration of BSO ($1.6\,g\,kg^{-1}$) or saline. The magnitude of the EP and the CM was recorded at 30-sec intervals for 30 min. The animals were then killed for measurement of hepatic glutathione content. In some experiments NAC ($1.2\,g\,kg^{-1}$) was administered p.o. as two equal doses 20 hr and 1 hr before EA. Statistical comparisons between drug-treated and control animals were made using Student's t-test.

RESULTS

The time course of depletion of hepatic and renal glutathione in pigmented animals is shown in Fig. 2A. Hepatic glutathione content was decreased, reaching a nadir, 11% of control glutathione levels, at 24 to 48 hr after administration of BSO. Glutathione levels recovered slowly and by 72 hr hepatic glutathione pools were no longer significantly depleted. Renal glutathione levels reached a nadir after 24 hr at 14% of control kidney levels and recovered more slowly than hepatic glutathione levels, so that after 72 hr renal glutathione pools were still significantly depleted (48% of control levels: $P < 0.001$). Although the numbers were small, the pattern of depletion was identical in both sexes. The time course of depletion of hepatic glutathione levels in male albino guinea pigs treated with BSO (Fig. 2B) was very similar to that observed with pigmented animals. The nadir, 14% of control levels, occurred after 24 hr and recovery was almost complete by 72 hr.

The effect on cochlear potentials of EA ($60\,mg\,kg^{-1}$) administered as a 1-min infusion to pigmented guinea pigs was almost identical in animals pretreated with saline or with BSO (Figs 3 and 4). Although the hepatic glutathione content in the BSO-treated animals was reduced to 8% of the levels in the saline-treated controls, there was no difference between the two groups either in the magnitude of the changes in cochlear potentials induced by EA or in the time to nadir (Fig. 4).

Compared with untreated control animals, hepatic glutathione was found to be depleted by 39% in the animals which received EA after saline. This depletion was probably attributable to the urethane used to induce anaesthesia, and not EA, since urethane alone depleted hepatic glutathione to a similar extent (41% of untreated control value). Pretreatment with *N*-acetyl-cysteine did not prevent the glutathione depletion caused by urethane and did not affect the changes in cochlear potentials (only CM was measured) caused by administration of EA (Fig. 3).

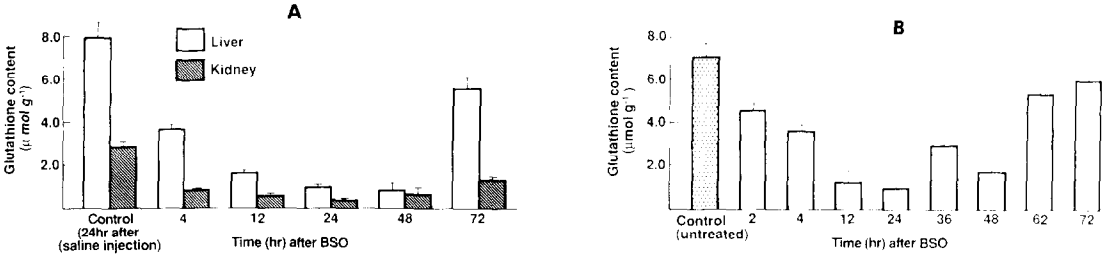

Fig. 2(A). Time course of depletion of hepatic and renal glutathione after administration of (±)-buthionine sulfoximine to pigmented guinea-pigs. Values are the mean ± SE. Four animals (two of each sex) were killed at each time point. (B) Time course of depletion of hepatic glutathione after administration of (±)-buthionine sulphoximine to albino guinea pigs. Values are the mean ± SE. Three male animals were killed at each time point.

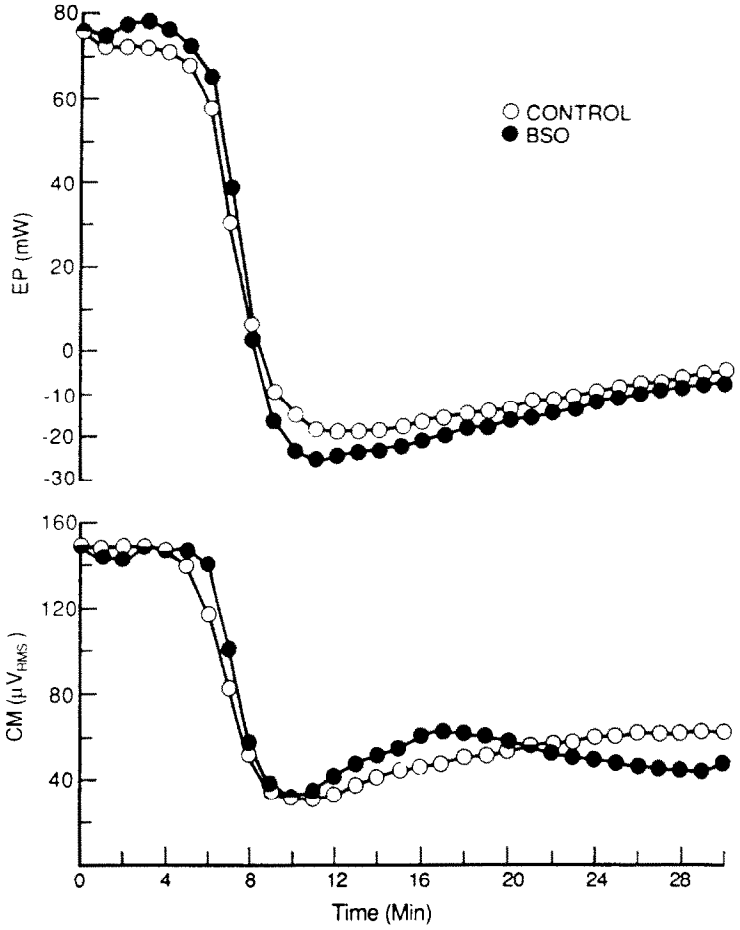

Fig. 3. Changes in endocochlear potential (EP) and a.c. cochlear potential (CM) in pigmented guinea pigs after administration of ethacrynic acid, 60 mg kg^{-1} i.v., at time zero. Each data point is the mean value for four animals pretreated 24 hr earlier either with saline (control) or 1.6 g kg^{-1} (±)-buthionine sulphoximine (BSO). Neither the amplitude of the response nor the time to maximum effect was affected by the pretreatment.

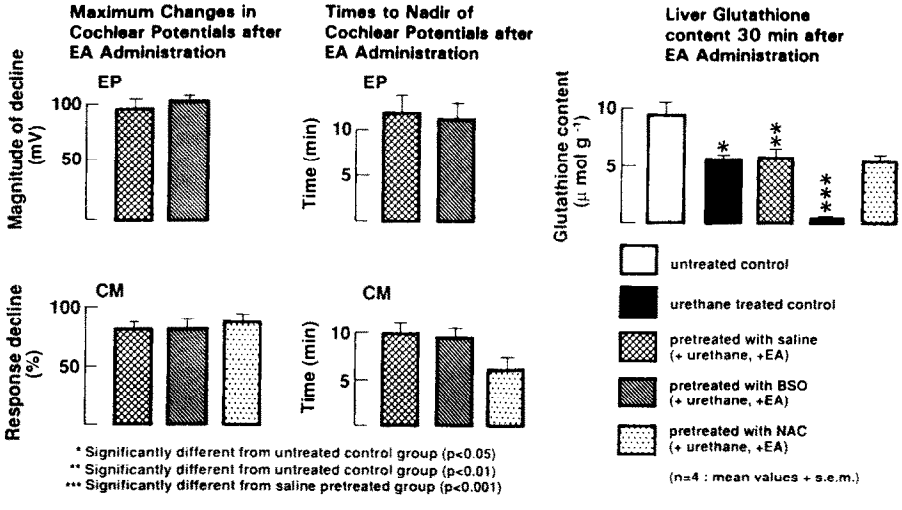

Fig. 4. Influence of various pretreatments on maximum changes and times to nadir of endocochlear potential (EP) and a.c. cochlear potential (CM) after administration of ethacrynic acid (EA). Animals received EA after induction of anaesthesia with urethane. Effects of the different treatment regimens on hepatic glutathione levels are also shown: BSO = (±)-buthionine sulphoximine, NAC = *N*-acetyl-L-cysteine.

DISCUSSION

Although it has been argued that albino animals are inappropriate for use in toxicological studies [11, 12], albino guinea pigs are often used in preference to pigmented guinea pigs and previous studies of glutathione levels in guinea pigs have been conducted using albino animals [13, 14]. Thus, in the present work we have examined both strains.

Hepatic glutathione levels in both albino and pigmented guinea pigs were similar to those reported for other rodent species [14]. Igarashi *et al.* [13] reported levels of hepatic glutathione in albino guinea pigs much lower ($3.8\,\mu mol\,g^{-1}$) than found here. They attributed these lower levels to a lower activity of γ-glutamyl synthetase, a key enzyme in the synthesis of glutathione, and a much greater activity of γ-glutamyl transpeptidase, an enzyme involved in the degradation of glutathione. However, Ecobichon [14], also using albino guinea pigs, found the levels of glutathione in liver to be similar to those reported here. Renal glutathione levels in several rodent species, including the albino guinea pig, were much lower than the values found in liver [14], analogous to our findings in pigmented guinea pigs.

After administration of BSO, hepatic glutathione levels in both strains of guinea pig declined slowly to a nadir at 24–48 hr, recovering slowly over a 72 hr period. This time course of depletion and recovery of hepatic glutathione contrasts markedly with that observed in mice after administration of an identical dose of BSO [8]. In mice the nadir (approximately 30% of control) occurred 4–6 hr after BSO and by 15 hr levels had recovered to 83% of the control value [8]. However, similar to results in the mouse, renal glutathione levels in the guinea pig recover at a much slower rate than hepatic levels after depletion by administration of BSO. A species difference in the rate of recovery of hepatic glutathione levels has been observed previously after depletion of hepatic glutathione pools caused by diethylmaleate [14]. Among four rodent species, albino guinea pigs were conspicuous in their slow ability to replenish depleted hepatic glutathione pools. This finding, confirmed here using a different mechanism of glutathione depletion, has implications for the use of the guinea pig for toxicological studies of compounds which may be either activated or detoxified by conjugation with glutathione. For example, by virtue of their impaired ability to replenish glutathione stores, guinea pigs were more susceptible than rats, mice or hamsters to the toxic effects of diethylmaleate [14], an agent which directly conjugates with glutathione causing rapid depletion of the tripeptide. In contrast, the hepatotoxin, paracetamol, is metabolised to electrophilic species which are detoxified by conjugation with glutathione. Guinea pigs showed much less depletion of hepatic glutathione than mice or hamsters after paracetamol administration, a fact which correlated with the reduced susceptibility of guinea pigs to the hepatotoxic effects of the drug [15].

EA is an example of a compound thought to be toxified by reaction with glutathione, leading to formation of the cysteine conjugate. However, it is contentious whether the EA–cysteine conjugate is the ultimate ototoxic species derived from EA or a proximate toxin which releases EA at the site of toxic action in the cochlea. The hypothesis that EA is ototoxic only after its metabolic conversion to EA–cysteine is supported by three pieces of evidence: (i) the ototoxic potential of EA–cysteine was greater than that of EA [4]; (ii) the time to maximal effect with EA–cysteine could be reduced to 1 min by increasing the dose whereas with EA there was an irreducible latency of 10–15 min [4]; (iii) whereas another ototoxic diuretic, frusemide (which is not conjugated with glutathione [16]), was several times more potent when injected into the arterial blood supply to the cochlear compared with i.v. administration, i.a. administration of EA did not increase its potency in or reduce the latency to maximal effect [17].

There is an alternative explanation for the greater potency of EA-cysteine as compared to EA. *In vitro* studies showed that EA was bound more extensively to plasma proteins than was EA–cysteine [18]. Furthermore, under physiological conditions EA–cysteine spontaneously released a product which resembled EA. Consequently, it was argued that the higher plasma concentration of unbound EA–cysteine would allow more EA–cysteine to reach the cochlear sites where it could liberate EA, possibly the species ultimately responsible for ototoxicity [19]. A similar explanation has been offered for the finding that EA–cysteine is more potent than EA as an inhibitor of active chloride transport in rabbit kidneys [19].

The results presented here show that a profound depletion of tissue stores of glutathione did not alter the early time course of the cochlear toxicity of EA. This finding argues against the hypothesis that the biotransformation of EA to EA–cysteine is a prerequisite for the ototoxicity of EA, but it is not conclusive. It has been shown that conjugation with glutathione is a major metabolic pathway for EA in the rat [5] and that conversion of ethacrynic acid to its glutathione conjugate in this species is decreased if glutathione stores are depleted [6]. However, the metabolic fate of EA in the guinea pig remains to be established. Furthermore, even if conjugation of EA with glutathione is quantitatively important in the guinea pig and if glutathione stores are selectively depleted by BSO administration, it is still conceivable that EA could conjugate directly with cysteine. Further work is indicated to resolve these issues.

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