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Cleavage of folates during ethanol metabolism

Role of acetaldehyde/xanthine oxidase-generated superoxide

Spencer SHAW,* Elizabeth JAYATILLEKE, Victor HERBERT and Neville COLMAN
Departments of Medicine and Pathology, Mount Sinai School of Medicine, New York, NY, and The V.A. Medical Center, Bronx, New York, NY 10468, U.S.A.

Although folate deficiency and increased requirements for folate are observed in most alcoholics, the possibility that acetaldehyde generated from ethanol metabolism may increase folate catabolism has not been previously demonstrated. Folate cleavage was studied *in vitro* during the metabolism of acetaldehyde by xanthine oxidase, measured as the production of *p*-aminobenzoylglutamate from folate using h.p.l.c. Acetaldehyde/xanthine oxidase generated superoxide, which cleaved folates (5-methyltetrahydrofolate > folinic acid > folate) and was inhibited by superoxide dismutase. Cleavage was increased by addition of ferritin and inhibited by desferrioxamine (a tight chelator of iron), suggesting the importance of catalytic iron. Superoxide generated from the metabolism of ethanol to acetaldehyde in the presence of xanthine oxidase *in vivo* may contribute to the severity of folate deficiency in the alcoholic.

INTRODUCTION

The folate deficiency and increased requirement for folate observed in most alcoholics are attributable to multiple mechanisms [1–7]. The possible role of catabolism or cleavage of folic acid in the alteration of requirements for folate caused by ethanol has not been previously demonstrated. *In vivo*, Murphy *et al.* [8] observed that folic acid is metabolized to acetaminobenzoylglutamate, suggesting that catabolism of folates occurs via cleavage at the C^9–N^{10} bond. Innocentini & Duran [9] observed cleavage of folate *in vitro* by red-blood-cell membrane oxidase and horseradish peroxidase and concluded that singlet oxygen rather than superoxide was the predominant free radical in producing cleavage of folate. Recent studies by Haddad *et al.* [10] revealed cleavage of folic acid during the metabolism of a number of drugs (xanthopterin, allopurinol and dihydro-orotic acid), suggesting that the cleavage was due to production of superoxide. The metabolism of ethanol by its major metabolic pathway, via alcohol dehydrogenase, leads to the formation of acetaldehyde, which is metabolized to acetate primarily by the hepatic mitochondrial enzyme acetaldehyde dehydrogenase [11]. The oxidation of acetaldehyde, however, can also occur by reaction with the ubiquitous enzyme xanthine oxidase, during which superoxide radicals are generated [12]. We recently observed that the metabolism of ethanol by alcohol dehydrogenase in the presence of xanthine oxidase promotes peroxidation of lipid membranes [13]. This peroxidation was inhibited by superoxide dismutase and stimulated by addition of ferritin iron, suggesting that peroxidation was initiated by superoxide radicals and potentiated by the presence of catalytic iron mobilized from ferritin during the generation of superoxide.

This prompted us to study whether superoxide generated from the oxidation of acetaldehyde by xanthine oxidase can cleave folates and thus contribute to the altered folate metabolism seen in alcoholics.

MATERIALS AND METHODS

H.p.l.c. measurement of folates and *p*-aminobenzoylglutamate (NH_2Bz-Glu)

Folates and NH_2Bz-Glu were measured by a modification of the h.p.l.c. method of Reingold *et al.* [14]. Samples were chromatographed on an Ultrasil ODS (10 μm particle size) column (4.6 mm × 25 cm) with all buffers run at a flow rate of 1 ml/min. The column was first equilibrated with 5 mM-PIC-A in 0.1 M-sodium phosphate, pH 7.2, for 30 min just before injection of the samples. NH_2Bz-Glu was eluted isocratically with 1 mM-sodium phosphate buffer, pH 7.5, containing 0.5% methanol for 20 min. Folates were then eluted in a stepwise isocratic fashion with 20 min each of the phosphate buffer containing 10, 20 and 40% (v/v) methanol. The column was then washed with 50% methanol in water for 1 h and then re-equilibrated with the PIC-A buffer for 30 min before injection of the next sample. A representative chromatogram is shown in Fig. 1. Concentrations of NH_2Bz-Glu and 5-methyltetrahydrofolate were determined by area of chromatograms determined from standard curves using known standards.

Cleavage of folates

Cleavage of folic acid was determined by the measurement of NH_2Bz-Glu production. This product is produced during the C^9–N^{10} cleavage of the folate molecule and was selected because of ease of quantification. In selected experiments the relationship between the production of NH_2Bz-Glu and the disappearance of

Abbreviations used: NH_2Bz-Glu, *p*-aminobenzoylglutamate; PIC-A, paired-ion chromatography Reagent A (Waters Chromatography Division, Milford, MA, U.S.A.).

* To whom correspondence and reprint requests should be sent, at the following address: General Medical Research, Bronx V.A. Medical Center, 130 W. Kingsbridge Road, Bronx, NY 10468, U.S.A.

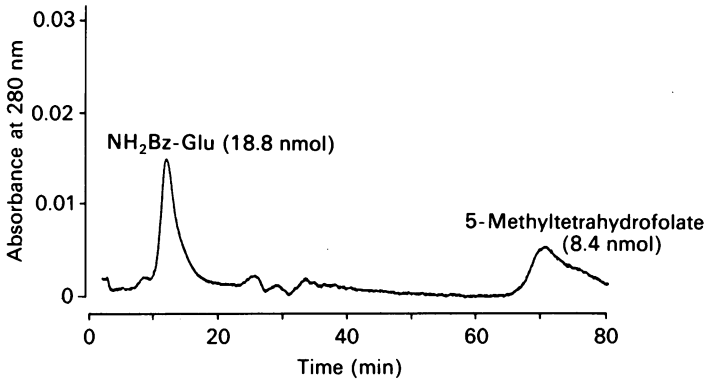

Fig. 1. Representative chromatogram of a standard mixture of 18.8 nmol of NH_2Bz-Glu and 8.4 nmol of 5-methyltetrahydrofolate

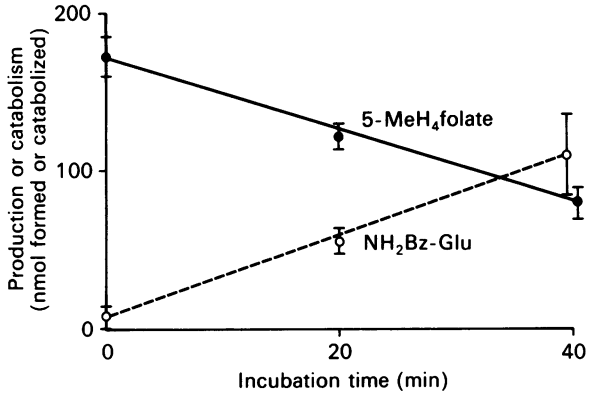

Fig. 2. Relationship between production of NH_2Bz-Glu and catabolism of 5-methyltetrahydrofolate (5-MeH_4folate)

A stoichiometric relationship was observed between the disappearance of 5-methyltetrahydrofolate (——) and the appearance of NH_2Bz-Glu (----) during incubations. Results are means ± S.E.M.

folates was studied as shown in Fig. 2. The production of NH_2Bz-Glu was stoichiometric with the disappearance of 5-methyltetrahydrofolate: 5-methyltetrahydrofolate decreased from 172.1 ± 10.8 nmol to 79.2 ± 10.4 (mean 93.1 nmol) and NH_2Bz-Glu increased from 8.1 ± 6.2 to 109.0 ± 24.6 (mean 101 nmol). In addition to the stoichiometry, production of NH_2Bz-Glu from cleavage was also verified by its co-elution with a known standard of NH_2Bz-Glu by h.p.l.c. and by the correspondence of absorbance spectra of standards of NH_2Bz-Glu run on the column and cleavage products (both with maximum absorbance at 273 nm).

Incubations

Incubations were carried out in sealed 1 ml vials in 0.1 M-phosphate buffer, pH 7.4, at 37 °C. Except where specified, the concentrations of reagents were as follows: folic acid, folinic acid and 5-methyltetrahydrofolate, 333 nmol/ml; xanthine oxidase (from buttermilk), 125 munits/ml; acetaldehyde [0.25 mM; usually added directly to reaction mixtures or generated from the metabolism of ethanol by alcohol dehydrogenase (from equine liver; 100 munits/ml)]; NAD^+, 0.75 mM; ethanol, 20 mM. Acetaldehyde concentrations were verified by head-space gas chromatography on a Perkin–Elmer 2000 head-space gas chromatograph by the method of Korsten *et al.* [15].

Reaction mixtures were incubated for 30 min and stopped by cooling to 0 °C and ultrafiltration in a Centricon-30 (30000 M_r cut-off) for 10 min at 2500 *g*. The filtrate was then immediately analysed by h.p.l.c. All samples were in quadruplicate and incubated in the dark. Results are expressed as means ± S.E.M. of the four determinations, and the significance of differences between groups was determined by Student's *t* test.

RESULTS

The effect of the metabolism of ethanol by alcohol dehydrogenase in the presence of xanthine oxidase on the cleavage of 5-methyltetrahydrofolate is shown in Fig. 3.

As shown in Fig. 4. the ability of acetaldehyde/xanthine oxidase to cleave several forms of folate was compared. Comparable cleavage was obtained with direct addition of acetaldehyde to xanthine oxidase instead of a generating system.

The ability of the thiols cysteine and glutathione to inhibit folate cleavage is shown in Fig. 5.

The importance of superoxide radicals and catalytic iron in producing cleavage were studied as shown in Table 1.

DISCUSSION

The results of the present study demonstrate that the metabolism of acetaldehyde by xanthine oxidase can cleave folates. Cleavage was observed by direct addition of acetaldehyde as well as during the continuous generation of acetaldehyde from alcohol dehydrogenase *in vitro*. Thus the major physiological pathway of ethanol metabolism via alcohol dehydrogenase could play an important role in catabolizing folate.

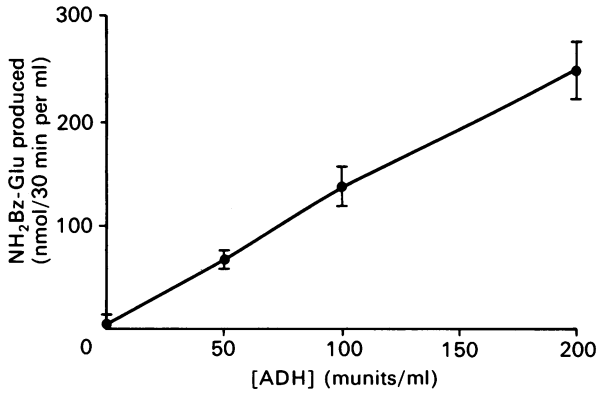

Fig. 3. Cleavage of 5-methyltetrahydrofolate by acetaldehyde/xanthine oxidase

Acetaldehyde generated during the metabolism of ethanol by alcohol dehydrogenase (ADH) in the presence of xanthine oxidase cleaved 5-methyltetrahydrofolate. Suitable blanks lacking either NAD^+, ethanol or xanthine oxidase served as controls. Cleavage was also produced by direct addition of acetaldehyde instead of its generation from ethanol, NAD^+ and alcohol dehydrogenase. Results are means ± S.E.M.

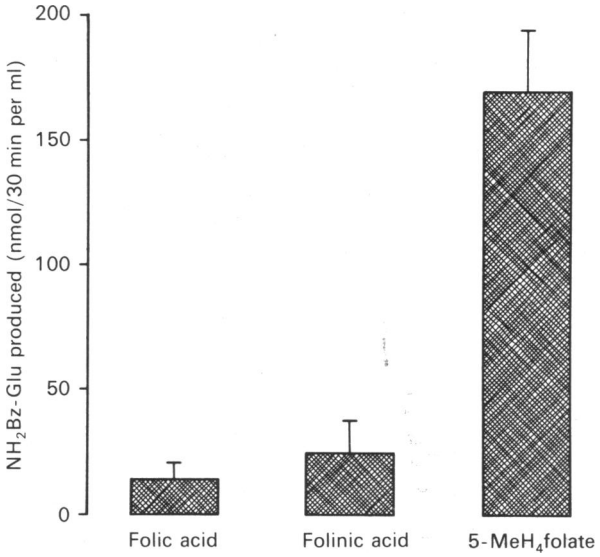

Fig. 4. Comparison of cleavage of different folates by acetaldehyde/xanthine oxidase

5-Methyltetrahydrofolate (5-MeH_4folate) was readily cleaved, whereas folic acid and folinic acid (168 nmol/ml each) were not. The rate of cleavage of 5-methyltetrahydrofolate was significantly increased compared with that observed for either folinic acid or folic acid (both $P < 0.001$). Results are means ± S.E.M.

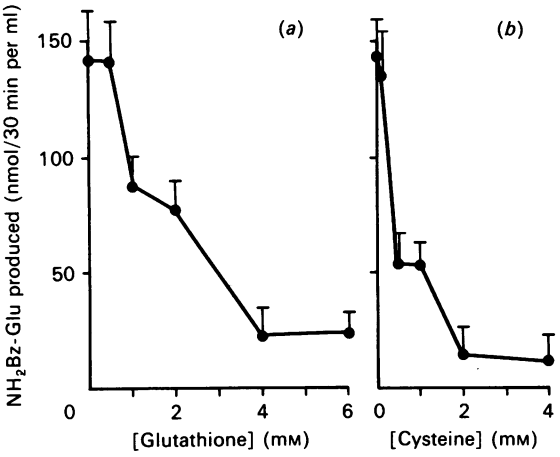

Fig. 5. Inhibition of folate cleavage by thiols

Additions of cysteine (*b*) and glutathione (*a*) to reaction mixtures containing 168 nmol of 5-methyltetrahydrofolate/ml inhibited folate cleavage. Gluthione was inhibitory over the range of concentrations normally present in the liver. Results are means ± S.E.M.

The cleavage of folate was markedly inhibited by the addition of superoxide dismutase, suggesting an important role for superoxide radicals. These results are consistent with those of Haddad *et al.* [10], who observed cleavage of folate *in vivo* due to generation of superoxide by addition of various drugs, but differ from those of Innocentini & Duran [9], who suggested that singlet oxygen might play a more important role in this respect. The reason for these differences is not apparent, but

Table 1. Role of superoxide and iron in ethanol-induced cleavage of 5-methyltetrahydrofolate

Cleavage was markedly inhibited by superoxide dismutase as well as by 4-methylpyrazole (an inhibitor of alcohol dehydrogenase). Addition of ferritin as a source of iron enhanced cleavage, as did direct addition of Fe^{2+}, whereas chelation of iron with desferrioxamine inhibited it. The reaction mixture consisted of 0.75 mM-NAD^+, 20 mM-ethanol, 105 munits of alcohol dehydrogenase/ml, 0.125 unit of xanthine oxidase/ml and 333 nmol of 5-methyltetrahydrofolate/ml. Results are means ± S.E.M. Statistical significance (addition versus no addition): $*P < 0.001$; $**P < 0.01$; $***P < 0.05$.

Addition	NH_2Bz-Glu formed (nmol/30 min per ml)
None (reaction mixture only)	155.1 ± 15.0
Superoxide dismutase (320 units/ml)	18.3 ± 4.2*
4-Methylpyrazole (10 μM)	72.9 ± 13.2**
$FeCl_2$ (100 μM)	280.2 ± 13.3*
Ferritin (1 mg/ml)	206.0 ± 13.8***
Desferrioxamine	
100 μM	121.6 ± 16.8
200 μM	70.1 ± 8.6**

may be a question of methodological interpretation or interconversion of active species.

The addition of ferritin or Fe^{2+} promoted cleavage of 5-methyltetrahydrofolate, and chelation of iron by desferrioxamine, albeit at high concentrations (200 μM), inhibited it, consistent with a possible facilitating role for catalytic iron in folate cleavage. Thomas *et al.* [16] demonstrated that superoxide generated from xanthine/xanthine oxidase could mobilize ferritin iron and promote lipid peroxidation, and we have observed similar mobilization of ferritin iron by acetaldehyde/xanthine oxidase-generated superoxide [17]. Catalytic iron thus generated could promote cleavage through a variety of mechanisms, such as interconversion of free radicals by the Haber–Weiss and Fenton reactions to particular reactive species or through direct reaction with reactive oxygen to form active intermediates capable of promoting cleavage [18].

Although Fe^{2+} may interact with molecular oxygen to produce highly reactive species, tight chelation of iron inhibits such reactions [19,20]. In the presence of iron overload, excess iron may be present as low-molecular-mass complexes that can initiate free-radical reactions [21,22]. Similarly, increased saturation of ferritin may increase its ability to initiate free-radical reactions [23]. Increased hepatic iron stores have been frequently observed in alcoholics [24] as well as in bone marrow during folate deficiency and might thus potentiate folate cleavage by acetaldehyde/xanthine oxidase.

The addition of glutathione to reaction mixtures markedly inhibited cleavage. This inhibition occurred at concentrations of glutathione normally present in the liver. However, after chronic alcohol administration in subhuman primates [25] as well as in man [26], a decrease in hepatic glutathione was observed that, in primate studies, was exaggerated by acute ethanol administration. Thus, after chronic alcohol consumption, this important protective mechanism may be diminished. Furthermore, in the rodent model, chronic alcohol administration

markedly decreased hepatic biliary glutathione [27] measured in the presence of ethanol, and this decrease was accentuated by acute ethanol administration. Although acute ethanol administration has been observed to interfere with the enterohepatic circulation of folates [4], it is unclear to what extent cleavage may contribute to such effects.

In the present study the ability of superoxide generated from ethanol metabolism to cleave three folate compounds (folic acid, folinic acid and 5-methyltetrahydrofolate) was compared. The last-named compound was much more sensitive to cleavage and suggests the possibility that the major folate pools, which are all pools of reduced folates, and in particular the pool of 5-methyltetrahydrofolate, could be selectively depleted by ethanol. Such depletion would further exacerbate the apparent increased methyl-group wastage seen after ethanol administration that may have a significant pathogenetic role in alcohol-mediated liver injury. Methyl-group wastage due to ethanol could occur by a variety of mechanisms, including recently reported inappropriate use of *S*-adenosylmethionine for synthesis of sarcosine via glycine methyltransferase [28]. Interestingly, pterin aldehyde has been shown to be an effective inhibitor of xanthine oxidase [29] and thus may represent a negative feedback cycle to prevent further cleavage.

In conclusion, superoxide-mediated folate cleavage during ethanol metabolism may be a major catabolic mechanism contributing to the increased requirement for folate seen in the alcoholic.

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