hormones on hepatic enzymes involved in drug metabolism. The present data also suggest that the three polypeptides although belonging to the same hormone "family", interact via different intracellular mediators to produce the observed effects on the transferases.

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REFERENCES

1. Dutton GJ, *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, FL, 1980.
2. Okolicsanyi L, Frei F, Magnenat P and Naccarato R, Multiplicity and specificity of UDP-glucuronyltransferase. *Enzyme* **12**:658–673, 1973.
3. Bock KW, Josting D, Lilienblum W and Pfeil H, Purification of rat liver microsomal UDP-glucuronyltransferase. Separation of two enzyme forms inducible by 3-methyl-cholanthrene or phenobarbital. *Eur J Biochem* **98**:19–26, 1979.
4. Wishart GJ and Campbell MT, Demonstration of two functionally heterogeneous groups within the activities of UDP-glucuronosyltransferase towards a series of 4-alkyl-substituted phenols. *Biochem J* **178**:443–447, 1979.
5. Onishi S, Kawade N, Itoh S, Isobe K and Sugujama S, Postnatal development of uridine diphosphate glucuronyltransferase activity towards bilirubin and 2-aminophenol in human liver. *Biochem J* **184**:705–707, 1979.
6. Burchell B, Isolation and purification of bilirubin UDP-glucuronyltransferase from rat liver. *FEBS Letters* **111**:131–135, 1980.
7. Falamy CN and Tephly TR, Separation purification and characterization of three isoenzymes of UDP-glucuronyltransferase from rat liver microsomes. *Arch Biochem Biophys* **227**:248–258, 1983.
8. Matern H, Matern S and Gerok W, Isolation and characterization of rat liver microsomal UDP-glucuronyltransferase activity toward chenodeoxycholic acid and testosterone as a single form of enzyme. *J Biol Chem* **257**:7422–7429, 1982.
9. Thulin L, Effects of gastro-intestinal polypeptides on hepatic blood flow and splanchnic circulation. *Acta Chir Scand Suppl.* **441**:8–31, 1973.
10. Ricci GL, Michiels R, Fevery J and De Groote J, Enhancement by secretin of the apparently maximal hepatic transport of bilirubin in the rat. *Hepatology* **4**:651–657, 1984.
11. Ricci GL and Fevery J, The action of VIP on bile secretion and bile acid output in the non-anaesthetized Rat, *Biochem Pharmacol.* **34**:3765–3767, 1985.
12. Østergaard Thomsen O and Lersen JA, The effect of glucagon, dibutyrylic cyclicAMP and insulin on bile production in the intact rat and the perfused rat liver. *Acta Physiol Scand* **111**:23–30, 1981.
13. Constantinopoulos A and Matsaniotis N, Augmentation of uridine diphosphate glucuronyltransferase activity in rat liver by adenosine 3′, 5′-monophosphate. *Gastroenterology* **75**:486–491, 1978.
14. Ricci GL and Fevery J, Stimulation by secretin of bilirubin UDP-glycosyltransferase activities and of cytochrome P-450 concentration in rat liver. *Biochem J* **182**:881–884, 1979.
15. Said SI and Mutt V, Polypeptide with broad biological activity: isolation from small intestine. *Science* **169**:1217–1218, 1970.
16. Cooper B, Eakins MN and Slater TF, The effect of various anaesthetic techniques on the flow rate, constituents and enzymic composition of rat bile. *Biochem Pharmacol* **25**:1711–1718, 1976.
17. Heirwegh KPM, Van de Vijver M and Fevery J, Assay and properties of digitonin-activated bilirubin uridine diphosphate glucuronyltransferase from rat liver. *Biochem J* **129**:605–618, 1972.
18. Zakim D and Vessey DA, Techniques for the characterization of UDP-glucuronosyltransferase, glucose-6-phosphatase, and other tightly-bound microsomal enzymes. *Meth Biochem Anal* **21**:1–37, 1973.
19. Bensadouse and Weinstein D, Assay of proteins in the presence of interfering materials. *Anal. Biochem* **70**:241–250, 1976.
20. Wishart GL, Functional heterogeneity of UDP-glucuronyltransferase as indicated by its differential development and inducibility by glucocorticoids. *Biochem J* **174**:485–489, 1978.
21. Thulin L and Hellgren M, Choleretic effect of vasoactive intestinal peptide. *Acta Chir Scand* **142**:235–237, 1976.

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Ethanol modulation of rat alveolar macrophage superoxide production

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The production of superoxide (O_2^-) by phagocytic cells, such as the alveolar macrophage, is involved in both bacterial killing and inflammation [1]. Mechanisms involved with the limitation of O_2^- production are, therefore, of interest for two reasons: excessive production of extracellular O_2^- is potentially toxic to normal tissue; and factors that reduce the level of O_2^- production could reduce antibacterial effectiveness [2]. Stimulation of O_2^- production has been proposed to involve phosphatidylinositol turnover, intracellular Ca^{2+} mobilization and activation of protein kinase C [3, 4], although there is some debate about which processes are necessary or sufficient [5, 6]. Effects of long chain alcohols on neutrophils have been reported [7]; however, the effects of ethanol on alveolar macrophage O_2^- production have not been examined previously. In this investigation, we explored the observations that ethanol acted both as a weak agonist for O_2^- production and, more strikingly, as an inhibitor of O_2^- production, produced by

the potent agonist, phorbol 12-myristate-13-acetate (PMA).

Methods

Ferricytochrome *c* (type VI), ionomycin, superoxide dismutase, *N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and other buffer salts were obtained from the Sigma Chemical Co., St. Louis, MO. PMA was obtained from Calbiochem, San Diego, CA. Fura-2AM was obtained from Molecular Probes, Eugene, OR. Alveolar macrophages were prepared from virus-free, 200–250 g Sprague–Dawley rats (Charles River, Inc., Wilmington, MA) as previously described [8]. The cells remained viable, as measured by trypan blue staining following ethanol addition (up to 500 mM). Superoxide production was assayed by continually monitoring ferricytochrome *c* reduction [9]. Intracellular calcium was measured following loading of 5×10^6 cells/ml with fura-2AM (2 μM) for 15 min followed by an additional 30-min incubation after a 10-fold dilution with buffer. Following loading, the assay was performed in HEPES buffer containing 135 mM NaCl, 5 mM KCl, 1.0 mM $MgSO_4$, 1.3 mM Ca^{2+}, 5.0 mM glucose and 10 mM HEPES. Fluorescence was followed using an excitation wavelength of 340 nm with emission at 510 nm.

Results and discussion

Addition of ethanol (75–500 mM) to suspensions of alveolar macrophages resulted in a concentration-dependent production of O_2^- (Fig. 1). No stimulation was observed below 75 mM ethanol. The use of specific pathogen, virus-free rats and freshly prepared reagent buffers was essential since the effect of ethanol as an agonist was difficult to observe if O_2^- production by "resting" cells occurred.

A rise in intracellular Ca^{2+} has been associated with O_2^- production following addition of concanavalin A in macrophages [10] and f-Met-Leu-Phe in neutrophils [3]. Addition of 300 mM ethanol to fura-2 loaded macrophages resulted in an immediate and sustained elevation of intracellular Ca^{2+} (Fig. 2) from a normal resting concentration of 124.9 ± 5.2 nM [10] to 260 nM Ca^{2+}. The intracellular Ca^{2+} remained elevated for at least 20–30 min (not shown). The initial increase in Ca^{2+} occurred in the presence of EGTA, indicating that a portion of the Ca^{2+} originated from intracellular stores (Fig. 2). The pattern of increase in intracellular calcium was not typical of agonist-induced changes in intracellular calcium, which show a decline after several minutes. Comparison of the spectra of resting cells and cells in ethanol by setting the spectra equal at 360 nm, the isosbestic point of fura-2, indicated that a true increase in intracellular calcium occurred. No change in fluorescence was observed following ethanol addition in cells that did not contain fura-2.

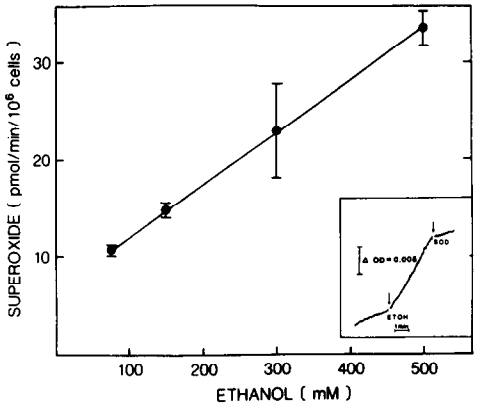

Fig. 1. Ethanol stimulation of superoxide production in alveolar macrophages. Various concentrations of ethanol were added to suspended macrophages (1.0×10^6 cells/ml) after equilibrium for 15 min at 37°. The rate of superoxide production was calculated after subtracting the initial baseline rate of ferricytochrome *c* reduction (not sensitive to superoxide dismutase) before ethanol addition. Data are the means ± SD for four separate preparations. The inset shows an experiment where 165 mM ethanol was added to 1.0×10^6 cells/ml. The reduction of ferricytochrome *c* was blocked with superoxide dismutase (SOD).

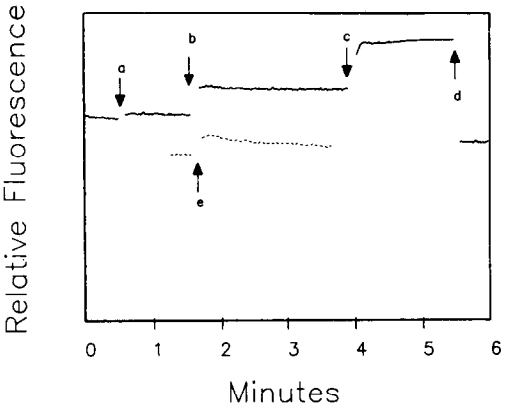

Fig. 2. Effect of ethanol on free intracellular calcium in alveolar macrophages. Solid lines: Fura-2 loaded cells were suspended at 5×10^6 cells/ml without stirring in the presence of 1.3 mM Ca^{2+}. Key: (a) stirring control, (b) 300 mM ethanol, (c) 20 μM ionomycin, and (d) 5 mM EGTA. Dotted lines: Cells stored in calcium-free buffer plus 30 μM EGTA. Key: (e) 300 mM ethanol.

PMA, which directly activates protein kinase C, is a much more potent agonist for O_2^- production than is ethanol (33.9 ± 1.7 pmol/min/10^6 cells for 500 mM ethanol vs 730 ± 38 pmol/min/10^6 cells for a saturating concentration, 10 ng/ml, of PMA). PMA addition alone does not increase intracellular calcium [12]. Preincubation in ethanol inhibited subsequent phorbol ester stimulation of O_2^- production (Fig. 3). After preincubation with 100 mM ethanol for 30 min, PMA-stimulated O_2^- production was inhibited 75% (Fig. 3, inset). Ethanol inhibited PMA-stimulated O_2^- production at all concentrations of PMA tested including saturating concentrations (10 ng/ml). Preincubation in ethanol was essential for inhibition of the response to PMA. No inhibition was observed when PMA and ethanol were added simultaneously (Fig. 3), indicating that ethanol did not interfere with the ferricytochrome *c* reaction with O_2^-.

The continued presence of high concentrations of ethanol was required for inhibition of PMA-induced O_2^- production. When the cells were preincubated with 300 mM ethanol in a small volume and subsequently diluted to a non-inhibiting concentration (25 mM) prior to the addition of PMA, the inhibitory effect of ethanol was not observed. Cells diluted into 300 mM ethanol-containing buffer were inhibited. Cells preincubated in 300 mM ethanol were not irreversibly damaged since these cells were able to generate O_2^- in response to PMA.

Based upon previous investigations of the regulation of alveolar macrophage O_2^- production, we can offer several possible mechanisms for the limitation of PMA-stimulated O_2^- production by ethanol. One possible mechanism is related to the ethanol-associated increase in intracellular Ca^{2+}. We have shown previously that other stimuli (concanavalin A or A23187), which increase intracellular calcium, are associated with regulated O_2^- production in

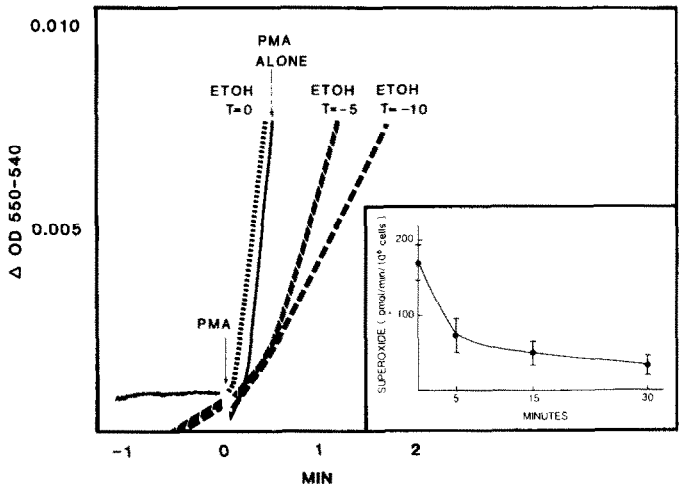

Fig. 3. Effect of preincubation with ethanol on PMA-induced superoxide production. PMA (1 ng/ml) was added to 10^6 cells at time zero. Ethanol (100 mM) was added at time 0, 5 min before PMA addition, or 10 min before PMA addition. Superoxide production was always superoxide dismutase inhibitable. The inset shows experiments where PMA (1 ng/ml) was added to 10^6 cells/ml after preincubation with ethanol (100 mM) for 0, 5, 15, and 30 min. Values are means ± SD for four separate experiments.

alveolar macrophages [11]. For instance, when A23187 or concanavalin A or ethanol is added alone, O_2^- production increases, then gradually slows and ceases after 10 min. Such cells are stimulated to produce O_2^-, but apparently a mechanism is also triggered which limits O_2^- production. In contrast, when phorbol ester is added to macrophages, O_2^- production continues for at least 20 min without sign of abatement [11]. When agonists that increase intracellular Ca^{2+} (A23187 or Con A) are added with phorbol ester, the net result is a diminution of total O_2^- production as compared to that produced by phorbol ester alone, although there is a synergistic increase in the initial rate of O_2^- production [11]. These results imply that, in alveolar macrophages, events related to a rise in intracellular Ca^{2+} may have both a stimulatory and an inhibitory effect on O_2^- production. By analogy, ethanol, through elevation of intracellular calcium, may exert these two effects by the same mechanisms.

The concentrations of ethanol used in our studies were sufficient to increase membrane disorder [12]. Phospholipase C is dependent upon lipids for activity [13]. It is possible that the increase in membrane disorder following ethanol addition to macrophages is sufficient to influence phospholipase C activity and thereby stimulate inositol phosphate metabolism and internal Ca^{2+} release. There is support for this hypothesis in studies by Hoek *et al.* [14] who have shown that ethanol, at concentrations similar to those used in the present study, causes phosphatidylinositol breakdown and intracellular Ca^{2+} mobilization in hepatocytes. Another possible mechanism in which disordering of membranes could have been responsible for the effect of ethanol upon O_2^- production by the alveolar macrophage could have been interference with protein kinase C activation and transformation to the plasma membrane. The rapid reversal of the inhibitory membrane disorder could have affected the NADPH oxidase directly since many of its components are membrane bound.

In summary, ethanol interacted with signal transduction pathways important in O_2^- production in the alveolar macrophage. Ethanol was a weak agonist for O_2^- production and produced an increase in intracellular Ca^{2+}. More strikingly, ethanol strongly inhibited PMA-induced O_2^- production.

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REFERENCES

1. Forman HJ and Thomas, MJ, Oxidant production and bactericidal activity of phagocytes. *Annu Rev Physiol* **46**: 669–680, 1986.
2. Segal AW, Cross AR, Garcia RC, Borregaard NB, Valerius NH, Soothill JF and Jones OTG, Absence of cytochrome b_{245} in chronic granulomatous disease. A multicenter European evaluation of its incidence and relevance. *N Engl J Med* **308**: 245–251, 1982.

3. Snyderman R, Smith CD and Verghese MW, Model for leukocyte regulation by chemoattractant receptors: roles of a guanine nucleotide regulatory protein and polyphosphoinositide metabolism. *J Leukocyte Biol* **40**: 785–800, 1986.
4. McPhail LC, Clayton CC and Snyderman R, The NADPH oxidase of human polymorphonuclear leukocytes. *J Biol Chem* **259**: 5768–5775, 1984.
5. Rossi F, Grzeskowiak M and Della Bianca V, Double stimulation with fMLP and Con A restores the activation of the respiratory burst but not of the phosphoinositide turnover in Ca^{2+}-depleted human neutrophils. A further example of dissociation between stimulation of the NADPH oxidase and phosphoinositide turnover. *Biochem Biophys Res Commun* **140**: 1–11, 1986.
6. Pozzan T, Lew DP, Wollheim CB and Tsien RY, Is cytosolic ionized calcium regulating neutrophil activation? *Science* **244**: 1413–1415, 1986.
7. Yuli Y, Tomonaga A and Snyderman R, Chemoattractant receptor functions in human polymorphonuclear leukocytes are divergently altered by membrane fluidizers. *Proc Natl Acad Sci USA* **79**: 5906–5910, 1982.
8. Forman HJ, Nelson J and Fisher AB, Rat alveolar macrophages require NADPH for superoxide production in the respiratory burst. *J Biol Chem* **255**: 9879–9883, 1980.
9. Forman HJ and Nelson J, Effect of extracellular calcium on superoxide release by alveolar macrophages. *J Appl Physiol* **54**, 1249–1253, 1983.
10. Forman HJ, Nelson J and Harrison G, Hyperoxia alters the effect of Ca^{2+} on rat alveolar macrophage superoxide production. *J Appl Physiol* **60**: 1300–1305, 1986.
11. Dorio RJ, Nelson J and Forman HJ, A dual role for calcium in regulation of superoxide generation by stimulated rat alveolar macrophages. *Biochim Biophys Acta* **928**: 137–143, 1987.
12. Taraschi TF and Rubin E, Effects of ethanol on the chemical and structural properties of biologic membranes. *Lab Invest* **52**: 120–131, 1985.
13. Majerus PW, Connolly TM, Deckmyn H, Ross TS, Bross, Ishii H, Bansal VS and Wilson DB, The metabolism of phosphoinositide-derived messenger molecules. *Science* **234**: 1519–1526, 1986.
14. Hoek JB, Thomas AP, Rubin R and Rubin E, Ethanol-induced mobilization of calcium by activation of phosphoinositide-specific phospholipase C in intact hepatocytes. *J Biol. Chem* **262**: 682–691, 1987.

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Inhibition of folate-dependent enzymes by 7-OH-methotrexate

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Methotrexate (MTX-Glu_1*; 4-NH_2-10-CH_3-pteroyl glutamate), a widely used antineoplastic agent, is thought to exert its antitumor effect by virtue of its tight-binding inhibition of dihydrofolate reductase (EC 1.5.1.3; DHFR) [1]. MTX has been shown to undergo intracellular polyglutamation in a manner similar to the polyglutamation of physiologic folates [2]. With a long intracellular $T_{\frac{1}{2}}$, these MTX polyglutamates (PGs) become the predominant form of intracellular drug in malignant cells and, to a lesser extent, in normal tissues. The MTX-PGs retain the inhibitory potency of the parent compound for dihydrofolate reductase [3]. Polyglutamated forms of MTX are also potent inhibitors of the folate-requiring *de novo* pyrimidine synthetic enzyme thymidylate synthase (EC 2.1.1.45; TS) [4] and aminoimidazole-carboxamide ribonucleotide transformylase (EC 2.1.2.3; AT) [5], one of two folate-requiring enzymes involved in *de novo* purine synthesis.

7-OH-MTX is the major metabolite of MTX, in which the 7-position of the pteridine ring is hydroxylated by aldehyde oxidase. This metabolite was first described by Johns and Loo in rabbits and was found to have intermediate potency as an inhibitor of DHFR, with a K_i of 6.6×10^{-9} M [6], or 2–3 orders of magnitude less than MTX. However, the metabolite is found in high concentration in plasma, particularly in patients treated with high-dose MTX, and becomes the predominant drug form 10–12 hr after MTX treatment [7, 8]. In a study of patients treated with high-dose MTX (140–350 mg/kg), the plasma concentration of 7-OH-MTX exceeded that of MTX 3–10 hr following the infusion of high-dose MTX [9]. At later time points, when plasma levels of MTX were in the range of 10^{-7} M, the level of 7-OH-MTX was found to be 17–140 times higher than that of MTX, and the plasma half-life of 7-OH-MTX was found to be 23.8 hr, or about three times longer than the terminal $T_{\frac{1}{2}}$ for MTX.

7-OH-MTX has been found to be polyglutamated intracellularly *in vitro* in the human acute lymphoblastic leukemia cell line (MOLT 4) at approximately the same rate and extent as MTX [10]. In Ehrlich ascites cells, the rate of polyglutamation of 7-OH-MTX exceeded that for MTX by a factor of 2.7 at equimolar extracellular drug levels, and 7-OH-MTX has been shown to be transported more efficiently than MTX in these cells [11].

Aside from their inhibition of DHFR, 7-OH-MTX and its polyglutamates have not been shown to be inhibitors of other folate-requiring enzymes. In this paper, we report the potencies of 7-OH-MTX and its tetraglutamated form as inhibitors of the *de novo* pyrimidine synthetic enzyme TS and the *de novo* purine synthetic enzyme AT.

Materials and methods

Chemicals. AICAR, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, 2-mercaptoethanol type I, dextran (clinical grade), deoxyuridine monophosphate (dUMP), bovine serum albumin fraction V, acid-washed activated charcoal, folic acid (crystalline), and L-ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). Purified folic acid pentaglutamate ($PteGlu_5$) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). A Bio-Rad Protein Assay kit was purchased from Bio-Rad Laboratories (Richmond, CA). [5-^{3}H]dUMP (20 Ci/mmol) was obtained from the Moravek Biochemical Co. (Brea, CA). 7-OH-MTX-Glu_1 and 7-OH-MTX-Glu_4 were prepared and purified by high-pressure liquid chromatography according to published methods [12]. All other chemicals were of the highest quality obtainable.

* Abbreviations: MTX, methotrexate (the total number of glutamyl groups appended to the pteroyl moiety of MTX is denoted by the suffix -Glu_n); TS, thymidylate synthase; AT, phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase; DHFR, dihydrofolate reductase; and PG, polyglutamate.