ROLE OF SUPEROXIDE ANION RADICALS IN ETHANOL METABOLISM BY BLOOD MONOCYTE-DERIVED HUMAN MACROPHAGES

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The discovery that macrophages metabolize ethanol to acetate in vitro may be of importance in understanding the pathogenesis of ethanol-induced tissue damage (1). When expressed per unit wet weight, the rate of metabolism of ethanol by these cells is comparable to that by hepatocytes (2). Studies of human and murine macrophages derived from various tissues have revealed that ethanol oxidation by these cells differs markedly from that by hepatocytes in being only slightly inhibited by pyrazole, and therefore, in being largely independent of alcohol dehydrogenase (2–4). Ethanol oxidation by macrophages was also only slightly impaired by 4-iodopyrazole and 3-amino-1,2,4,-triazole, indicating that it was independent of π-ADH and catalase. On the other hand, several inhibitors of cytochrome P450 (carbon monoxide, β-diethylaminoethyl diphenylpropyl acetate, metyrapone, and tetrahydrofurane) markedly suppressed ethanol oxidation, suggesting that macrophages metabolize this alcohol via a cytochrome P450-dependent pathway (2–4). Since it is known that oxygen-derived free radicals mediate the oxidation of ethanol in certain cell-free in vitro systems (5) and that macrophages are capable of generating large quantities of superoxide radicals (6), the possibility arises that superoxide radicals are involved in ethanol oxidation by macrophages. The present paper describes the results of an investigation into this possibility and demonstrates that a substantial proportion of ethanol oxidation by intact macrophages is superoxide dependent.

Materials and Methods

Preparation of Macrophage Cultures. Buffy coats prepared from units of blood obtained from healthy adult blood donors of either sex were supplied by the North London Blood Transfusion Centre and mononuclear cells separated from them using Histopaque-1077 (Sigma Chemical Co., Poole, UK) as described by Nakagawara et al. (7). 10 ml of macrophage growth medium (RPMI 1640, 25% vol/vol newborn calf serum, 100 μg streptomycin/ml, 100 μg nystatin/ml) containing 1.7×10^6 mononuclear cells/ml were placed within each of several 25-cm^2 tissue culture flasks (Sterilin Ltd., Teddington, UK). After incubation at 37°C for 2 h, the growth medium plus the nonadherent cells were decanted. The adherent cells were washed thrice with HBSS at room temperature and cultured at 37°C (100% humidity, 5% CO_2) with 10 ml fresh macrophage growth medium for 4–8 d.

Determination of Rates of Ethanol Metabolism. The monolayers of macrophages were washed twice with HBSS at room temperature and used to determine the rates of oxidation of

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[1-^{14}C]ethanol (56–61.6 mCi/mmol; Amersham International Ltd., Bucks, UK) to [^{14}C]acetate. The basic technique consisted of placing over each macrophage monolayer 3 ml of HBSS containing the appropriate quantities of nonradioactive ethanol (absolute ethanol, BP grade; James Burrough PLC, London, UK) and [^{14}C]ethanol to obtain a total ethanol concentration of 1 mg/ml (pH 7.45) and incubating the cultures at 37°C for 90 min. The incubations were terminated by adding 50 μl 1 M H_2SO_4 and the amount of [^{14}C]acetate formed quantitated by Dowex 1-X8 anion exchange chromatography as described previously (8). The rates of production of acetate were related to the number of macrophages present at the beginning of the incubation with [^{14}C]ethanol. The latter was determined using parallel cultures which were set up at the same time and in the same way as the test cultures and with the same suspension of mononuclear cells. The adherent cells were washed twice with HBSS (37°C) and a measured volume of Isoton containing three drops Zapoglobin per 10 ml (both from Coulter Electronics Ltd., Luton, UK) was added. This lysed the cells and released their nuclei, which were counted using a Coulter Counter (Model ZF; Coulter Electronics, Hialeah, FL) (3).

Effects of Additives on Rates of Ethanol Metabolism by Macrophages. The effects of various substances on the oxidation of ethanol to acetate were determined by including them in the incubation mixture (3 ml of HBSS containing radioactive and non-radioactive ethanol) which was placed over the washed macrophage monolayers. In each experiment, incubation mixtures without additives and with the following additives were placed over monolayers which had been seeded simultaneously with cells from a single donor: (*a*) 1,500 U/ml superoxide dismutase (SOD)[1] (3,050 U/mg protein, from bovine erythrocytes; Sigma Chemical Co.), (*b*) 1,500 U/ml catalase (14,100 U/mg protein, from bovine liver; Sigma Chemical Co.), (*c*) 1,500 U/ml SOD plus 1,500 U/ml catalase, (*d*) 20 mM tetrahydrofurane (Aldrich Chemical Co., Milwaukee, WI), (*e*) 20 mM tetrahydrofurane plus 1,500 U/ml SOD, (*f*) 20 nM PMA (Sigma Chemical Co.), (*g*) 20 nM PMA plus 1,500 U/ml SOD, (*h*) 20 nM PMA plus 1,500 U/ml catalase, (*i*) 20 nM PMA, 1,500 U/ml SOD, plus 1,500 U/ml catalase, (*j*) 20 nM PMA plus 20 mM tetrahydrofurane, and (*k*) 20 nM PMA, 20 mM tetrahydrofurane, plus 1,500 U/ml SOD.

In some experiments, the effects on ethanol metabolism of adding 1,500 U/ml SOD with and without 20 nM PMA were compared with those of adding 150 U/ml SOD with and without 20 nM PMA and of adding 1,500 U/ml heat-treated SOD (60°C for 10 min) with and without 20 nM PMA.

Effects of Additives on Cell Adhesion. To assess the toxicity of the various additives and combinations of additives studied, monolayers of macrophages were incubated for 90 min with HBSS containing ethanol only or ethanol and additives as described above, and the number of residual adherent cells determined. Since adherence is an active, energy-dependent process, the extent of reduction of the number of adherent cells after exposure to an additive was considered to be an index of toxicity.

Effects of Tetrahydrofurane on Superoxide Production. Macrophage monolayers were washed twice with phenol red-free HBSS (PRF-HBSS) and incubated with 3 ml PRF-HBSS or 3 ml PRF-HBSS containing 10 mM tetrahydrofurane at 37°C for 45 min. PMA (20 nM) was then added to some of the cultures with and without tetrahydrofurane and PMA (20 nM) plus SOD (300 U/ml) to some of the cultures without tetrahydrofurane. Nitroblue tetrazolium (NBT, Sigma Chemical Co.) was then added to all the cultures, to a final concentration of 1 mg/ml, and the cultures were incubated for a further 45 min. The NBT-containing solutions were then decanted and the adherent macrophages were washed with methanol (Analar grade: Fisons Scientific Equipment, Loughborough, UK) and left to dry. The insoluble formazan dye was then solubilized by the addition of 3.6 ml 2M KOH followed by 4.2 ml DMSO (Sigma Chemical Co.) to each flask. The optical density of the resulting solution was read at 630 nm against a blank of 3.6 ml 2M KOH and 4.2 ml DMSO using a CE 599 Universal Automatic Scanning Spectrophotometer (Cecil, Cambridge, UK).

Capacity of Catalase to Mediate Macrophage-independent Oxidation of Ethanol in the Presence of an H_2O_2-generating System or H_2O_2. 3-ml volumes of HBSS containing 1 mg ethanol/ml (in-

[1] *Abbreviations used in this paper:* NBT, nitroblue tetrazolium; PRF, phenol red-free; SOD, superoxide dismutase.

cluding [^{14}C]ethanol and nonradioactive ethanol) were incubated in the absence of macrophages with and without various additives at 37°C for 90 min and the amount of [^{14}C]acetate generated was quantitated as described earlier. The additives included (*a*) 0.2–100 μg/ml glucose oxidase (133,000 U/g, from *Aspergillus niger*; Sigma Chemical Co.), with and without 1,500 U/ml catalase, and (*b*) 250–1,500 μM H_2O_2 (Sigma Chemical Co.), with and without 1,500 U/ml catalase.

Results

Effects of Additives on Ethanol Metabolism by Macrophages. The rates of ethanol oxidation under different experimental conditions are summarized in Table I and a statistical analysis of the data given in Table II. It is evident that SOD (1,500 U/ml) caused a substantial inhibition of the conversion of ethanol to acetate, that catalase caused a slight inhibition and that SOD plus catalase caused more inhibition than SOD alone. Tetrahydrofurane and tetrahydrofurane plus SOD caused similar degrees of inhibition. A striking finding was that PMA caused a marked increase in the rate of oxidation of ethanol. This increase was significantly reduced when catalase was also present and completely suppressed when SOD rather than catalase was present. In flasks containing PMA, SOD, and catalase, the rate of ethanol metabolism was less than that in flasks without any additives. Tetrahydrofurane caused a slight but significant reduction in the PMA-induced stimulation of acetate production and tetrahydrofurane plus SOD caused a marked reduction.

Qualitatively similar effects were seen when 150 U SOD/ml were used instead of 1,500 U SOD/ml (Table III). Thus, 150 U SOD/ml caused a statistically significant inhibition of the rate of oxidation of ethanol to acetate by both unstimulated macrophages ($p < 0.001$, paired *t*-test) and PMA-stimulated macrophages ($p < 0.01$, paired *t*-test). In addition, heat-treated SOD (1,500 U/ml) caused a much smaller degree of inhibition of acetate production than unheated SOD (1,500 U/ml) when added to cultures containing both unstimulated macrophages ($p < 0.05$, paired *t*-test) and

TABLE I

Effects of Additives on the Metabolism of Ethanol (1 mg/ml) to Acetate by Human Blood Monocyte-derived Macrophages

Additive	Rate of production of acetate			Change with addition
	n	Mean	SD	
		nmol/10^7 cells/h		%
Nil	8	2,054	201	
SOD*	7	1,044	279	- 49.2
Catalase	6	1,806	180	- 12.1
SOD* + catalase	6	968	171	- 52.9
Tetrahydrofurane	5	813	286	- 60.4
Tetrahydrofurane + SOD*	5	682	360	- 66.8
PMA	7	15,062	2,766	+ 633.3
PMA + SOD*	4	2,054	286	0
PMA + catalase	3	8,145	511	+ 314.3
PMA + SOD* + catalase	3	1,334	120	- 32.1
PMA + tetrahydrofurane	5	11,799	2,083	+ 474.4
PMA + tetrahydrofurane + SOD*	3	1,742	41	- 15.2

* 1,500 U/ml.

TABLE II

Statistical Significance (p) of the Difference between Various Rates of Conversion of Ethanol to Acetate shown in Table I*

Rates of ethanol oxidation compared		
a	*b*	*p*
No additive	SOD	<0.001
No additive	catalase	<0.01‡
No additive	SOD + catalase	<0.001
No additive	tetrahydrofurane	<0.005
No additive	tetrahydrofurane + SOD	<0.005
SOD	tetrahydrofurane	<0.05‡
SOD	SOD + catalase	<0.01‡
SOD	SOD + tetrahydrofurane	<0.05‡
Tetrahydrofurane	tetrahydrofurane + SOD	>0.1‡
No additive	PMA	<0.001
PMA	PMA + SOD	<0.005
PMA	PMA + catalase	<0.05
PMA	PMA + SOD + catalase	<0.01
PMA	PMA + tetrahydrofurane	<0.05‡
PMA + SOD	PMA + SOD + catalase	<0.05
PMA + tetrahydrofurane	PMA + SOD	<0.005
PMA + tetrahydrofurane	PMA + tetrahydrofurane + SOD	<0.01

* Using *t*-test.
‡ Paired *t*-test.

PMA-stimulated macrophages ($p < 0.02$, paired *t*-test), indicating that the effects of 1,500 U SOD/ml were dependent on the presence of undenatured enzyme.

Effect of Additives on Cell Adhesion. The number of residual adherent cells after incubation of macrophage monolayers in the presence of (*a*) SOD (1,500 U/ml), (*b*) catalase, (*c*) SOD (1,500 U/ml) plus catalase, (*d*) tetrahydrofurane, (*e*) tetrahydrofurane plus SOD (1,500 U/ml), (*f*) PMA, (*g*) PMA plus SOD (1,500 U/ml), (*h*) PMA plus catalase, (*i*) PMA, SOD (1,500 U/ml), plus catalase, (*j*) PMA plus tetrahydrofu-

TABLE III

Comparison of the Effects of Two Concentrations of SOD and of Heat-treated SOD on the Metabolism of Ethanol (1 mg/ml) by Unstimulated and PMA-stimulated Blood Monocyte-derived Macrophages

Additive	*n*	Rate of production of acetate: Mean	Rate of production of acetate: SD	Change with addition
		nmol/10^7 cells/h		%
Nil	7	1,936	105	
SOD (150 U/ml)	5	1,559	88	- 19.5
SOD (1,500 U/ml)	5	1,267	268	- 34.5
Heat-treated SOD (1,500 U/ml)	4	1,853	125	- 4.3
PMA	6	9,601	1,683	+ 395.9
PMA + SOD (150 U/ml)	6	3,542	969	+ 83.0
PMA + SOD (1,500 U/ml)	6	2,047	708	+ 5.7
PMA + heat-treated SOD (1,500 U/ml)	4	8,010	2,004	+ 313.7

TABLE IV

Nitroblue Tetrazolium Reduction by Human Blood Monocyte-derived Macrophages with and without Various Additives

Additive	Optical density (630 nm)		
	n	Mean	SD
Nil	5	0.089	0.003
Tetrahydrofurane	5	0.082	0.010
PMA	4	0.470	0.154
PMA + tetrahydrofurane	4	0.234	0.051
PMA + SOD (300 U/ml)	3	0.109	0.061

rane, and (*k*) PMA, tetrahydrofurane, plus SOD (1,500 U/ml) were, respectively, 89.9, 102.9, 100.4, 89.2, 92.4, 96.7, 97.1, 98.9, 105.1, 94.6, and 98.6% of that in the absence of these additives (average values from four experiments). Thus, these additives did not cause major changes in the number of residual adherent cells and appeared to have little or no toxic effect on macrophages, as judged by their ability to remain adherent.

Effects of Tetrahydrofurane on Superoxide Production. As shown in Table IV, PMA-stimulated macrophages reduced much larger amounts of NBT to formazan dye (optical density at 630 nm) than unstimulated macrophages. Furthermore, this PMA-induced stimulation of superoxide production was markedly reduced in the presence of 300 U/ml SOD. Tetrahydrofurane had no effect on NBT reduction by unstimulated macrophages but caused a significant decrease ($p < 0.05$, paired *t*-test) in NBT reduction by PMA-stimulated macrophages.

Capacity of Catalase to Mediate Macrophage-independent Oxidation of Ethanol in the Presence of Glucose Oxidase or H_2O_2. When glucose oxidase was added with and without catalase to a macrophage-free incubation mixture consisting of ethanol and HBSS (which includes 1 g/liter glucose), very little acetate production was observed (Table V). Similarly, the rate of oxidation of ethanol after the addition of H_2O_2 to this macrophage-free incubation mixture was very low both in the presence and absence

TABLE V

Effects of Various Concentrations of Glucose Oxidase with and without Catalase on the Production of Acetate in [^{14}C]Ethanol-containing Macrophage-free Incubation Mixtures

Additive			Rate of production of acetate	
Glucose oxidase	Catalase	*n*	Mean	SD
μg/ml	*U/ml*		*nmol/10^7 cells/h*	
0	0	5	5.06	0.32
0.2	0	5	7.22	1.18
20	0	5	10.50	0.99
20	1,500	5	5.88*	0.85
100	0	5	13.86	1.98
100	1,500	5	6.74‡	0.70

Significance of difference from mean value in incubation mixtures containing the same concentration of glucose oxidase but no catalase (using paired *t*-test).

* $p < 0.001$.

‡ $p < 0.005$.

TABLE VI

Effects of Various Concentrations of H_2O_2 with and without Catalase on the Production of Acetate in [^{14}C]Ethanol-containing Macrophage-free Incubation Mixtures

Additive			Rate of production of acetate		
H_2O_2	Catalase	n	Mean	SD	p*
μM	*U/ml*		*nmol/10^7 cells/h*		
0	0	7	5.59	2.05	
0	1,500	5	4.92	1.14	
250	0	6	11.32	1.59	
250	1,500	6	5.08	1.94	<0.005
500	0	6	14.02	4.14	
500	1,500	6	6.23	1.99	<0.01
1,000	0	6	17.63	2.75	
1,000	1,500	6	5.17	0.41	<0.001
1,500	0	6	20.05	3.31	
1,500	1,500	6	7.97	2.20	<0.001

* For difference from mean value in incubation mixtures containing the same concentration of H_2O_2 but no catalase (using t-test).

of added catalase (Table VI). Somewhat unexpectedly, both glucose oxidase and H_2O_2 caused significantly less acetate production in the presence than in the absence of catalase.

Discussion

The enzyme SOD catalyses the dismutation of superoxide anion radicals and the resultant generation of hydrogen peroxide (9) according to the equation:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

The rate of oxidation of ethanol to acetate by human blood monocyte-derived macrophages was reduced by 13–75% in the presence of 1,500 U/ml of SOD, indicating that some of the ethanol oxidation by these cells was dependent on superoxide anion radicals. This conclusion is supported by the finding that PMA, which stimulates superoxide production by macrophages (7, 10), caused a 4.0–8.3-fold increase in the rate of ethanol oxidation, and that this increase was prevented in the presence of 1,500 U/ml of SOD.

The superoxide-dependent oxidation of ethanol may have been partly mediated via hydroxyl radicals (·OH) generated by the Haber-Weiss reaction:

$$O_2^{\cdot-} + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

or the kinetically much faster iron-catalyzed Haber-Weiss reaction (11, 12):

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}$$

This possibility is consistent with two observations reported here. These are: (*a*) that there was a greater reduction in the rate of oxidation of ethanol by both unstimulated and PMA-stimulated macrophages in the presence of SOD plus catalase than in the presence of SOD alone, and (*b*) that there was a reduction of ethanol oxidation by both unstimulated and PMA-stimulated macrophages in the presence of catalase alone. Superoxide-dependent mechanisms other than those referred to above may also be involved in ethanol metabolism by macrophages since, on its own, catalase caused only a slight inhibition of ethanol oxidation by unstimulated cells. Since catalase may form a catalase-H_2O_2 complex (13) in the presence of low steady-state concentrations of H_2O_2 (which would be generated by macrophages) and thereby promote ethanol oxidation, the addition of catalase could in theory have two conflicting effects: (*a*) inhibition of ethanol oxidation because of the catalase-dependent decomposition of H_2O_2 to H_2O and O_2 and the consequent reduction in the generation of hydroxyl radicals and (*b*) stimulation of ethanol oxidation because of the reaction of the catalase-H_2O_2 complex with ethanol. If this were the case, the extent of inhibition of ethanol oxidation after the addition of catalase would underestimate the importance of hydroxyl radicals in the oxidation of ethanol in the absence of added catalase. However, in the presence of catalase plus either an H_2O_2-generating system (glucose oxidase plus glucose) or concentrations of H_2O_2 known to be produced by macrophages (6) the rate of oxidation of ethanol to acetate in the absence of macrophages was very low. Thus, it appeared that the unexpectedly small reduction of ethanol metabolism observed when catalase was added to macrophage cultures could not be attributed to the confusing effect of catalase-mediated ethanol oxidation. Since spin-trapping techniques have demonstrated that stimulated macrophages do not generate substantial quantities of hydroxyl radicals in the absence of added ferric iron (14) and HBSS does not contain any iron, it seems likely that hydroxyl radicals played only a minor role in mediating the superoxide-dependent oxidation of ethanol observed in the present study.

Previous studies have clearly shown that ethanol metabolism by human and murine macrophages is suppressed by various inhibitors of cytochrome P450, including tetrahydrofurane (2–4). In the present investigation, each of the two additives tetrahydrofurane and SOD caused marked inhibition of ethanol oxidation by unstimulated macrophages and tetrahydrofurane caused a greater inhibition than SOD. When both tetrahydrofurane and SOD were present together, the degree of inhibition was greater than that when SOD alone was present and similar to than when tetrahydrofurane alone was present. If it is assumed that adequate quantities of SOD entered the cells so as to cause the dismutation of virtually all of the superoxide radicals generated intracellularly, these data suggest that inhibitors of cytochrome P450 influence ethanol metabolism at least partly via a superoxide-independent mechanism. This possibility is supported by studies using the quantitative NBT dye reduction test that failed to reveal any effect of tetrahydrofurane on superoxide production by unstimulated macrophages. In the case of PMA-stimulated macrophages, tetrahydrofurane caused a much smaller reduction of ethanol metabolism than SOD, indicating that large quantities of superoxide radicals can be generated over 90 min by stimulated macrophages despite inhibition of cytochrome P450. Nevertheless, tetrahydrofurane caused a significant decrease in NBT reduction (i.e., superoxide production) by PMA-stimulated macrophages, raising the possibility that some of

the tetrahydrofurane-induced reduction of ethanol metabolism by such cells may have resulted from an impairment of the generation of superoxide anion radicals. The mechanisms by which inhibitors of cytochrome P450 interfere with ethanol oxidation by unstimulated and stimulated macrophages require further study.

The major pathway of ethanol metabolism in hepatocytes is based on the cytosolic enzyme alcohol dehydrogenase (alcohol-NAD oxido-reductase, EC 1.1.1.1) which catalyses the NAD-dependent oxidation of ethanol to acetaldehyde (15). Hepatocytes also have minor pathways of ethanol metabolism that are associated with the microsomes and may be more important at high rather than low blood ethanol concentrations (16, 17). Studies using a cell-free system containing purified NADPH-cytochrome P450 reductase and cytochrome P450 derived from hepatic microsomes of phenobarbital-treated rats have suggested that two independent pathways can support NADPH-dependent ethanol oxidation in the liver. One pathway involves hydroxyl radicals which can be generated by the reductase and the other requires the presence of both the reductase and cytochrome P450 and appears to be independent of oxygen-derived free radicals (18). The experiments reported here indicate that ethanol metabolism in intact blood monocyte-derived human macrophages occurs largely via two alcohol-dehydrogenase-independent pathways, one of which is dependent on the generation of superoxide radicals and the other on the activity of cytochrome P450. The subcellular localization of the superoxide-dependent ethanol-metabolizing activity in macrophages is uncertain. However, since SOD has a molecular weight of 32,600 (19), its inhibitory effect on ethanol oxidation is unlikely to have depended on its penetration into the cytosol through the cell membrane. As the main route of entry of SOD into macrophages is likely to be via pinocytosis and during phagocytosis, it seems possible that at least some of the ethanol metabolism by macrophages occurs either at the cell surface or within pinocytotic vesicles or both.

It has been recently suggested (1) that the generation of high concentrations of acetaldehyde around alcohol-metabolizing tissue macrophages may represent an important mechanism of ethanol-related tissue damage. If this hypothesis is correct, the present finding that PMA caused a 4.0–8.3-fold increase in the rate of oxidation of ethanol by macrophages in vitro raises the possibility that organs containing macrophages that have been activated by various stimuli may be particularly prone to ethanol-induced injury in vivo.

Summary

The effects of a number of additives on the rate of conversion of ethanol (1 mg/ml; 21.7 mM) to acetate by monolayers of blood monocyte-derived human macrophages were investigated. The additives studied were superoxide dismutase (SOD; 1,500 U/ml), catalase (1,500 U/ml), tetrahydrofurane (20 mM), and PMA (20 nM), either singly or in various combinations. SOD, catalase, SOD plus catalase, tetrahydrofurane, and tetrahydrofurane plus SOD inhibited ethanol oxidation by 49.2, 12.1, 52.9, 60.4, and 66.8%, respectively. PMA caused a 4.0–8.3-fold increase in the rate of ethanol metabolism and this increase was completely suppressed in the presence of SOD. The data indicate that a substantial proportion of the ethanol metabolism by both unstimulated and PMA-stimulated blood monocyte-derived macrophages was dependent on the generation of superoxide anion radicals.

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