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 \( \pi \)-ADH and catalase. On the other hand, several inhibitors of cytochrome P450 (carbon monoxide, \( \beta \)-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 \( \mu \)g streptomycin/ml, 100 \( \mu \)g nystatin/ml) containing \( 1.7 \times 10^6 \) 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% \( \text{CO}_2 \)) 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
[1-14C]ethanol (56–61.6 mCi/mmol; Amersham International Ltd., Bucks, UK) to [14C]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 [14C]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 H2SO4 and the amount of [14C]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 [14C]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) (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 tetrahydrofuran (Aldrich Chemical Co., Milwaukee, WI), (e) 20 mM tetrahydrofuran plus 1,500 U/ml SOD, (f) 20 mM 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 tetrahydrofuran, and (k) 20 nM PMA, 20 mM tetrahydrofuran, 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 Tetrahydrofuran 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 tetrahydrofuran at 37°C for 45 min. PMA (20 nM) was then added to some of the cultures with and without tetrahydrofuran and PMA (20 nM) plus SOD (300 U/ml) to some of the cultures without tetrahydrofuran. 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 H2O2-generating System or H2O2. 3-ml volumes of HBSS containing 1 mg ethanol/ml (in-
cluding \([1^4C]\)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 \([1^4C]\)acetate generated was quantitated as described earlier. The additives included \((a)\) 0.2-100 μg/ml glucose oxidase (133,000 U/g, from \textit{Aspergillus niger}; Sigma Chemical Co.), with and without 1,500 U/ml catalase, and \((b)\) 250-1,500 μM H₂O₂ (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. Tetrahydrofuran and tetrahydrofuran 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. Tetrahydrofuran caused a slight but significant reduction in the PMA-induced stimulation of acetate production and tetrahydrofuran 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**

| Additive                        | Rate of production of acetate \(\text{nmol/10^7 cells/h}\) | Change with addition |
|---------------------------------|----------------------------------------------------------|---------------------|
| 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               |
| Tetrahydrofuran                 | 5 813 286                                               | -60.4               |
| Tetrahydrofuran + 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 + tetrahydrofuran           | 5 11,799 2,083                                           | +474.4              |
| PMA + tetrahydrofuran + 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 |  |  |  |
|------------------------------------|--|---|---|
| No additive SOD                    |  | <0.001 |  |
| No additive catalase               |  | <0.01 |  |
| No additive SOD + catalase         |  | <0.001 |  |
| No additive tetrahydrofuran        |  | <0.005 |  |
| No additive tetrahydrofuran + SOD |  | <0.005 |  |
| SOD tetrahydrofuran               |  | <0.05 |  |
| SOD SOD + catalase                 |  | <0.01 |  |
| SOD SOD + tetrahydrofuran         |  | <0.05 |  |
| Tetrahydrofuran tetrahydrofuran + 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 + tetrahydrofuran         |  | <0.05 |  |
| PMA + SOD PMA + SOD + catalase     |  | <0.05 |  |
| PMA + tetrahydrofuran PMA + SOD + catalase |  | <0.005 |  |
| PMA + tetrahydrofuran tetrahydrofuran + 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) tetrahydrofuran, (e) tetrahydrofuran 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 | Rate of production of acetate | Change with addition |
|----------|------------------------------|----------------------|
|          | n   | Mean | SD  | %   |                   |
| 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 |                   |
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 H₂O₂. 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 H₂O₂ to this macrophage-free incubation mixture was very low both in the presence and absence of glucose oxidase.

### 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|

### Table V

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

| Glucose oxidase, µg/ml | Catalase, U/ml | n | Rate of production of acetate, nmol/10⁷ cells/h | Mean   | SD   |
|------------------------|----------------|---|-----------------------------------------------|--------|------|
| 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.
of added catalase (Table VI). Somewhat unexpectedly, both glucose oxidase and H$_2$O$_2$ 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:

$$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

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:

$$\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2$$

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

$$\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2$$

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$$
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\textsubscript{2}O\textsubscript{2} complex (13) in the presence of low steady-state concentrations of H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O and O\textsubscript{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\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2}-generating system (glucose oxidase plus glucose) or concentrations of H\textsubscript{2}O\textsubscript{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 tetrahydrofuran (2-4). In the present investigation, each of the two additives tetrahydrofuran and SOD caused marked inhibition of ethanol oxidation by unstimulated macrophages and tetrahydrofuran caused a greater inhibition than SOD. When both tetrahydrofuran and SOD were present together, the degree of inhibition was greater than that when SOD alone was present and similar to that when tetrahydrofuran 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 tetrahydrofuran on superoxide production by unstimulated macrophages. In the case of PMA-stimulated macrophages, tetrahydrofuran 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, tetrahydrofuran caused a significant decrease in NBT reduction (i.e., superoxide production) by PMA-stimulated macrophages, raising the possibility that some of
the tetrahydrofuran-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 oxidoreductase, EC 1.1.1.1) which catalyzes 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), tetrahydrofuran (20 mM), and PMA (20 nM), either singly or in various combinations. SOD, catalase, SOD plus catalase, tetrahydrofuran, and tetrahydrofuran 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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