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REFERENCES

1. W. B. Jakoby and W. H. Habig, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), Vol. 11, p. 63. Academic Press, New York (1980).
2. L. F. Chasseaud, *Adv. Cancer Res.* **29**, 175 (1979).
3. N. Motoyama and W. C. Dauterman, *Rev. biochem. Toxic.* **2**, 49 (1980).
4. G. J. Smith, V. S. Ohl and G. Litwack, *Cancer Res.* **37**, 8 (1977).
5. U. Rannug, A. Sundvall and C. Ramel, *Chem. Biol. Interact.* **20**, 1 (1978).
6. P. J. VanBladeren, D. D. Breimer, G. M. T. Rottenveel Smijs, P. Devnijiff, G. R. Mohn, B. Van MetterenWalchli, W. Buijs and G. A. Vendergen, *Corcinogenesis* **2**, 499 (1981).
7. H. Mukhtar, C. E. M. Zoetemelk, A. J. Baars, J. T. Wiljnen, M. M. Blankenstein-Wijnen, P. M. Khan and D. D. Breimer, *Pharmacology* **22**, 322 (1979).
8. C. Guthenberg, K. Akerfeldt and B. Mannervik, *Acta chem. scand.* **B33**, 595 (1979).
9. G. Polidoro, C. D. DiIlio, A. Arduini and G. Federici, *Biochem. Med.* **25**, 247 (1981).
10. G. M. Pacifici and A. Rane, *Drug Metab. Dispos.* **9**, 472 (1981).
11. D. K. Manchester and E. H. Jacoby, *Xenobiotica* **12**, 543 (1982).
12. J. R. Bend, M. O. James, T. R. Devereux and J. R. Fouts, in *Basic and Therapeutic Aspects of Perinatal Pharmacology* (Eds. P. L. Morselli, S. Garattini and F. Sereni), p. 229. Raven Press, New York (1975).
13. M. O. James, G. L. Foureman, F. C. Law and J. R. Bend, *Drug Metab. Dispos.* **5**, 19 (1977).
14. G. Polidoro, G. DiIlio, A. Arduini and G. Federici, *Biochem. Pharmac.* **30**, 1959 (1981).
15. K. Asaoka, H. Ito and K. Takahashi, *J. Biochem., Tokyo* **82**, 973 (1977).
16. B. G. Lake, R. Hopkins, J. Chakraborty, J. W. Bridges and D. V. Parke, *Drug Metab. Dispos.* **1**, 342 (1973).
17. M. S. Fahim, D. G. Hall, T. M. Jones, Z. Fahim and F. D. Whitt, *Am. J. Obstet. Gynec.* **107**, 1250 (1970).
18. E. Schlede and R. Borwiski, *Naunyn-Schmiedeberg's Archs Pharmac.* **281**, 341 (1974).
19. G. W. Lucier, B. R. Sonawane, O. S. McDaniel and G. E. R. Hook, *Chem. Biol. Interact.* **11**, 15 (1975).
20. T. M. Guenthner and P. S. Lietman, *Biochem. Pharmac.* **26**, 577 (1977).
21. J. M. Fysh and A. B. Okey, *Can. J. Physiol. Pharmac.* **57**, 112 (1979).
22. J. V. Bell, M. M. Hansell and D. J. Ecobiochon, *Can. J. Physiol. Pharmac.* **53**, 1147 (1975).
23. P. Rouet, K. Alexandrov, P. Markovits, C. Frayssinet and P. M. Dansette, *Carcinogenesis* **2**, 919 (1981).
24. E. Hodgson, A. P. Kulkarni, D. L. Fabacher and K. Robacker, *J. envir. Sci. Hlth* **B15**, 723 (1980).
25. A. P. Kulkarni, D. L. Fabacher and E. Hodgson, *Gen. Pharmac.* **11**, 437 (1980).
26. C. Guthenberg, R. Morgenstern, J. W. DePierre and B. Mannervik, *Biochim. biophys. Acta* **631**, 1 (1980).
27. J. S. Seidegard, J. W. DePierre, R. Morgenstern, A. Pilotti and L. Ernster, *Biochim. biophys. Acta* **672**, 65 (1981).
28. C. H. Kuo, J. B. Hook and J. Bernstein, *Toxicology* **22**, 149 (1981).
29. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. biol. Chem.* **249**, 7130 (1974).
30. B. Combes and G. S. Stakelum, *J. clin. Invest.* **41**, 750 (1962).

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Evidence for generation of leukotriene B_4 in human polymorphonuclear leukocytes treated with linoleylanilide

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In resting human polymorphonuclear leukocytes (PMNs) the free levels of arachidonic acid are low. However, upon stimulation, the levels of arachidonic acid rapidly increase [1–3]. The interest in the study of the mechanisms by which the intracellular levels of arachidonic acid are controlled has grown during the last years (reviewed in [4]). This is due to the finding that arachidonic acid is precursor of the leukotrienes, which are potent mediators of the inflammatory response (reviewed in [5]). We have previously shown that linoleylanilide, a fatty acid anilide supposcly involved in the toxic syndrome in Spain [6, 7], induces the generation of arachidonic acid from human PMNs [3]. Different to other signals, like phagocitic particles or the ionophore A23187 [1, 2], linoleylanilide induces the generation of arachidonic acid without affecting the synthesis of PAF-acether, lysosomal enzymes or O_2^- [3]. This permits us to explore the mechanisms that control arachidonic acid metabolism in human polymorphonuclear leukocytes with relative independence of other processes. In this study we have investigated the following questions: (1) From which phospholipids is arachidonic acid generated in response to linoleylanilide? (2) By which mechanisms is arachidonic acid generated in response to linoleylanilide? (3) Is the arachidonic acid generated in response to linoleylanilide retained intracellularly or is it released to the extracellular medium? and (4) is the arachidonic acid generated in response to linoleylanilide metabolized by the lipoxygenase pathway?

Materials and methods

Cells. Human PMNs were obtained from venous blood of normal volunteers as in [8].

Effect of linoleylanilide on the content in arachidonic acid of phosphatidylcholine and phosphatidylinositol. Human PMNs were labelled with (5,6,8,9,11,12,14,15-[^{3}H])-arachidonic acid (Amersham, 125 Ci/mmole) as previously described [2, 3]. After washing, PMNs (0.6 ml, 1×10^7 cells per ml) were incubated with linoleylanilide (1 mg/ml) for 2 hr at 37°. After extraction, lipids were subjected to TLC in chloroform/methanol/acetic acid/sodium borate 0.1 M

(75:45:12:4.5 v/v) and the content of radioactivity in phosphatidylcholine and phosphatidylinositol determined [2].

Effect of linoleylanilide on the incorporation of arachidonic acid into phospholipids. Human PMNs (0.6 ml, 1×10^7 cells per ml) were incubated at 37°. At zero-time [^{3}H]-arachidonic acid (0.5 μCi/ml) was added in the absence or presence of 1 mg/ml linoleylanilide. After extraction, lipids were subjected to TLC as described above, and the incorporation of radioactivity into phospholipids determined.

Secretion of archidonic acid in response to linoleylanilide. Human PMNs were labelled with [^{3}H]-arachidonic acid as in [2]. After washing, PMNs (0.6 ml, 1×10^7 cells/ml) were incubated with linoleylanilide (1 mg/ml). At several times, cells were centrifuged 15 sec in a Beckman microcentrifuge and the pellet and supernatant extracted. After extraction, lipids were subjected to TLC in ligroin/diethyl ether/acetic acid (50:50:1, v/v) and the content of radioactivity into arachidonic acid determined [2, 3].

High-performance liquid chromatography separation of lipoxygenase products of human PMNs treated with linoleylanilide. Human PMNs (1.5×10^7 cells per ml) were labelled with [^{3}H]-arachidonic acid (1 μCi/ml) and washed four times [2, 3]. After washing, PMNs (1 ml, 3.5×10^7 cells per ml) were incubated with linoleylanilide (1 mg/ml) for 2–5 min at 37° at which time 2 ml acetone at 4° were added. Lipoxygenase products were extracted as described in [9]. Briefly, tubes were shaken for 2 min and centrifuged at 4°. The clear supernatant was extracted with 2 ml petroleum ether which eliminates most of the free arachidonic acid. The ether phase was discarded and 42 μl formic acid 1 M added. Lipoxygenase products were finally extracted with 2 ml chloroform twice. The combined chloroform phase was dried under a stream of N_2 at 30°, dissolved in 200 μl methanol/water (75:25, v/v) 0.02% acetic acid and 100 μl injected on an Ultrasphere C_{18} Beckman column. After injection, LTB_4 and 5-HETE were eluted in methanol/water (75:25 v/v) 0.02% acetic acid at a speed of 1 ml/min [10]. The recovery of LTB_4 and 5-HETE by this procedure was about 70–80%. Authentic lipid standards (Amersham) were used to calculate the retention time of these compounds. Linoleylanilide was prepared as in [11] and sonicated before use as previously described [3].

Results and discussion

Incubation of human PMNs prelabeled with [^{3}H]-arachidonic acid with 1 mg/ml linoleylanilide for 2 hr reduces the content of radioactivity into phosphatidylcholine and phosphatidylinositol by about 40% whereas the content of radioactivity of phosphatidylethanolamine remained unaffected. Most of the arachidonic acid generated in response to linoleylanilide is released to the extracellular medium (Fig. 1). This is accompanied of a transient accumulation of the intracellular levels of arachidonic acid (Fig. 1). The relative proportions of phosphatidylcholine and phosphatidylinositol in human PMNs are respectively about 50 and 5%, which indicates that the major pool for arachidonic acid in these cells is phosphatidylcholine.

To understand the mechanisms by which linoleylanilide generates arachidonic acid from PMNs, we measured the incorporation of radioactivity into phospholipids in cells stimulated simultaneously with linoleylanilide and [^{3}H]-arachidonic acid. As shown in Fig. 2, the rate of incorporation of radioactivity into phosphatidylcholine was slower in cells treated with linoleylanilide than in control cells. This effect was specific for arachidonic acid. Thus, the rate of incorporation of [^{14}C]-oleic acid into phosphatidylcholine remained unaffected by the addition of linoleylanilide up to 1 mg/ml (not shown). The inhibition by linoleylanilide of arachidonic acid acylation into phosphatidylcholine was dose-dependent (Fig. 3). Concentrations between 0.1–0.2 mg/ml linoleylanilide were sufficient to inhibit the incorporation of labelled arachidonic acid into phosphatidylcholine. This same range of concentration of linoleylanilide is sufficient to trigger the generation of arachidonic acid from human PMNs [3]. The rate of acylation of other phospholipids was unaffected by the addition of linoleylanilide. Figure 3 shows that the incorporation of [^{3}H]-arachidonic acid into phosphat-

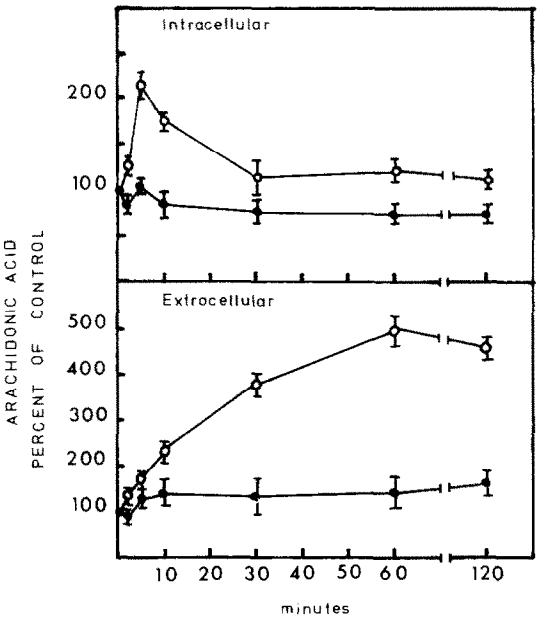

Fig. 1. Effect of linoleylanilide addition on the intracellular and extracellular content of arachidonic acid. Human PMNs were preincubated with ^{3}H-arachidonic acid as described under Materials and Methods. At zero time linoleylanilide (1 mg/ml) was added and the intracellular content of arachidonic acid measured. (○) Activated cells, (●) control cells. Results are the average ± S.E.M. of three independent experiments performed in duplicate.

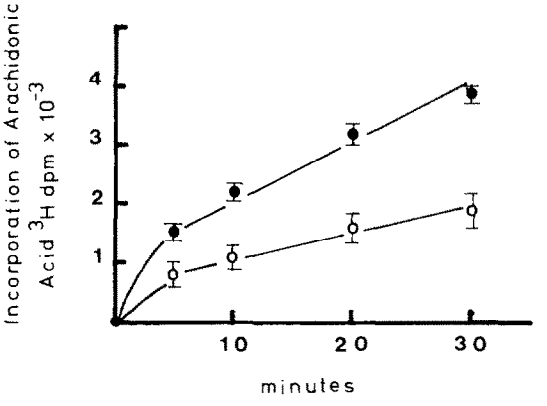

Fig. 2. Effect of linoleylanilide addition on the incorporation of ^{3}H-arachidonic acid into phosphatidylcholine. Human PMNs were incubated at 37° as described under Materials and Methods. At zero time, ^{3}H-arachidonic acid was added in the absence (●) or presence (○) of 1 mg/ml linoleylanilide. After extraction, lipids were subjected to TLC and the incorporation of radioactivity into phosphatidylcholine determined. Results are the average ± S.E.M. of three independent experiments performed in triplicate.

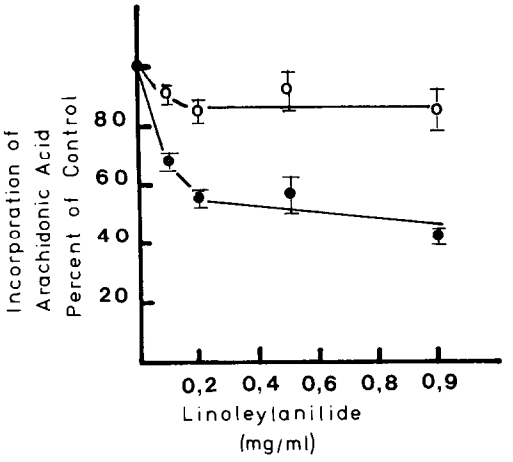

Fig. 3. Effect of different doses of linoleylanilide on the incorporation of ^{3}H-arachidonic acid into phosphatidylcholine and phosphatidylinositol. Conditions were as described for Fig. 2. The incorporation of ^{3}H-arachidonic acid was determined 30 min after the addition of ^{3}H-arachidonic acid. (○) Phosphatidylinositol, (●) phosphatidylcholine. Results are the average ± S.E.M. of three independent experiments performed in triplicate.

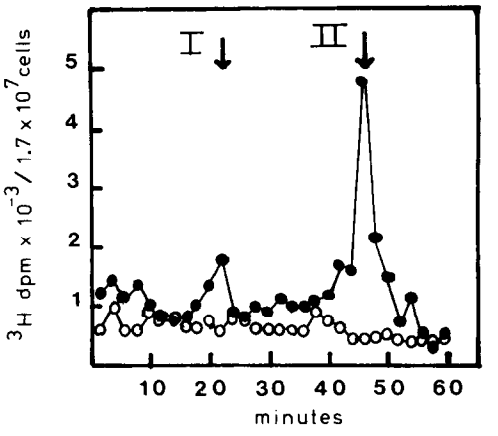

Fig. 4. High performance liquid chromatography separation of lipoxygenase products of human PMNs treated with linoleylanilide. Human PMNs were labelled with ^{3}H-arachidonic acid as described under Materials and Methods. PMNs were then stimulated with 1 mg/ml linoleylanilide for 2 to 5 min at 37° and lipids extracted for HPLC analysis. (●) Cells treated with linoleylanilide, (○) control cells. The retention time of authentic standards is indicated by arrows: I, LTB_4 and II, 5-HETE. This figure shows the results obtained from a single experiment. The same pattern was obtained in more than 5 independent experiments.

idylinositol was unaffected by the addition of up to 1 mg/ml linoleylanilide. These results indicate that linoleylanilide generates arachidonic acid from phosphatidylcholine, at least in part, by inhibiting the acylation of arachidonic acid. Whether this effect is due to decreased esterification or to an effect on the arachidonoyl specific acylCoA synthetase, an enzyme recently described [12], cannot be answered. The observation that the effect of linoleylanilide is confined to phosphatidylcholine suggests, however, an effect on the esterification reaction. Furthermore, our results do not exclude the possibility that part of the arachidonic acid generated in response to linoleylanilide may be also produced by increased phospholipase activity. The mechanism by which linoleylanilide generates arachidonic acid from phosphatidylinositol probably involves the activation of the phosphatidylinositol cycle, since we have previously shown that linoleylanilide enhances the incorporation of [^{3}H]-inositol into phosphatidylinositol [3].

Finally, we have investigated the effect of linoleylanilide addition to human PMNs on the metabolism of arachidonic acid by the lipoxygenase pathway. As shown in Fig. 4, linoleylanilide induces the synthesis of LTB_4 and 5-HETE. These compounds were identified by their mobility under two sets of conditions on HPLC as compared to authentic standards. As a control experiment, cells were stimulated with 5 μM A23187 under conditions known to stimulate the synthesis of LTB_4 and 5-HETE [13]. The behavior on HPLC and quantities of these compounds obtained after A23187 addition coincided with those obtained after linoleylanilide addition and identified as LTB_4 and 5-HETE. The amount of radioactivity recovered in five independent experiments migrating as LTB_4 and 5-HETE in cells incubated 2–5 min with 1 mg/ml linoleylanilide was respectively 1280 ± 310 dpm/3.5 × 10^7 cells and 8700 ± 2450 dpm/3.5 × 10^7 cells. No significant amount of LTB_4 or 5-HETE was detected in control cells treated the same way (Fig. 4). The accumulation of LTB_4 or 5-HETE in response to linoleylanilide was transient with a maximum about 2–5 min after activation to recover prestimulation levels within 30 min (not shown). A similar kinetics has been previously described in human PMNs stimulated with A23187 [13] or zymosan [1]. About 5% of the arachidonic acid released is converted to LTB_4 after linoleylanilide addition. Probably, this indicates that the synthesis of LTB_4 after linoleylanilide addition only reflects the liberation of arachidonic acid from phospholipids and this compound does not stimulate the LTB_4 synthetic system.

In conclusion, the present results show that the addition to human PMNs of linoleylanilide generates arachidonic acid from phosphatidylcholine and phosphatidylinositol. Most of the arachidonic acid is released to the medium whereas a small part is converted to LTB_4. Whether the effect of linoleylanilide on LTB_4 synthesis is related to the inflammatory reactions observed in the patients affected by the oil toxic syndrome remains to be determined.

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REFERENCES

1. C. E. Walsh, M. B. Waite, I. J. Thomas and L. P. DeChatelet, *J. biol. Chem.* **256**, 7228 (1981).
2. M. Garcia Gil, F. Alonso, V. Alvarez Chiva, M. Sanchez Crespo and J. M. Mato, *Biochem. J.* 206. 67 (1982).
3. M. Garcia Gil, M. van Lookeren Campagne, S. Alemany, A. M. Municio and J. M. Mato, *FEBS Lett.* **162**, 151 (1983).
4. R. F. Irvine, *Biochem. J.* **204**, 3 (1982).

5. R. Snyderman and E. J. Goetzl, *Science* **213**, 830 (1981).
6. Programa del CSIC para el Estudio del Sindrome Toxico. Vol 1 and 2. Consejo Superior de Investigaciones Cientificas. Madrid (1983).
7. Simposium Nacional del Sindrome Toxico. Ministerio de Sanidad y Consumo. Madrid (1982).
8. F. Alonso, M. Sanchez Crespo and J. M. Mato, *Immunology* **45**, 493 (1982).
9. J. A. Salmon and R. J. Flower, *Methods in Enzymology* **86**, 477 (1982).
10. W. R. Mathews, J. Rokoch and R. C. Murphy, *Analyt. Biochem.* **118**, 96 (1981).
11. C. Casals, P. Garcia Barreno and A. M. Municio, *Biochem. J.* **212**, 339 (1983).
12. D. B. Wilson, S. M. Prescott and P. W. Majerus, *J. biol. Chem.* **257**, 3510 (1982).
13. P. Borgeat and B. Samuelsson, *J. biol. Chem.* **254**, 2148 (1979).

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Increased microsomal oxidation of ethanol by cytochrome P-450 and hydroxyl radical-dependent pathways after chronic ethanol consumption*

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Isolated rat liver microsomes can oxidize a variety of alcohols to their corresponding aldehydes [1, 2]. Recent experiments have suggested that microsomes have the potential to oxidize alcohols by two pathways [3–5]. One pathway involves an interaction of ethanol with cytochrome P-450 and appears to be independent of a significant role for oxygen radicals such as the hydroxyl radical (·OH)† [3, 5]. In the presence of iron, e.g. iron–EDTA, microsomes generate ·OH [6], and alcohols can be oxidized via interaction with ·OH generated during microsomal electron transfer [3–7]. Chronic consumption of ethanol by rats results in a 2- to 3-fold increase in the activity of the microsomal alcohol-oxidizing system [8, 9]. The content of cytochrome P-450 is increased, and a distinct cytochrome P-450 isozyme is induced which proves to be more active than control cytochrome P-450 in catalyzing the oxidation of ethanol [10]. Similar results were reported recently for the rabbit liver system [11, 12]. Liver microsomes from ethanol-fed rats catalyze the oxidation of two typical ·OH scavengers, KTBA and DMSO, at rates which are 2- to 3-fold greater than rates found with control microsomes [13]. These results suggest that the production of ·OH by microsomes may be increased after chronic ethanol consumption, and that this increase may contribute to the increase in ethanol oxidation by microsomes.

The present studies were carried out to evaluate the relative roles of the cytochrome P-450 pathway and the OH·-dependent pathway in catalyzing the oxidation of ethanol by microsomes isolated from rats chronically fed alcohol, and from their pair-fed controls, and to investigate which of the pathways appears to be responsible for the increase in ethanol oxidation by microsomes from ethanol-fed rats.

Materials and methods

Male, Sprague–Dawley rats were fed for 4 weeks a nutritionally adequate liquid diet in which ethanol provided 36% of the total calories. Pair-fed littermates consumed the same diet except that carbohydrate isocalorically replaced ethanol [14]. Prior to the day of sacrifice, the rats received 2 aliquots of diets, one in the morning and one in the evening. Liver microsomes were prepared as previously described, washed once, and suspended in 125 mM KCl [7]. The oxidation of ethanol and DMSO was assayed as previously described [13]. The final concentration of ethanol was 50 mM while DMSO was present at either 33 or 100 mM. When indicated, 0.1 mM EDTA was added to the reaction system. The DMSO binding spectrum was determined by the method of Peterson *et al.* [15] using a Perkin–Elmer model 554-dual beam spectrophotometer. The concentration of DMSO was 140 mM, and microsomal protein was approximately 0.4 mg. The content of cytochrome P-450 [16] and the activity of NADPH-cytochrome *c* reductase [17] were determined by the indicated references.

All values refer to the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed by either the paired or unpaired Student's *t*-test.

Results and discussion

Microsomal oxidation of ethanol. Microsomal oxidation of ethanol was doubled after chronic ethanol treatment (Table 1). Table 1 shows that, when EDTA was added, the rate of ethanol oxidation was increased 2-fold with both microsomal preparations. This increase probably reflects the chelation of adventitious iron in the microsomes to produce an iron–EDTA chelate, which catalyzes the production of ·OH [6]. However, the ethanol-oxidizing activity by microsomes from the ethanol-fed rats was still 2-fold greater than the rate for the pair-fed controls. Desferrioxamine, which blocked nearly completely the production of ·OH by microsomes from control chow-fed rats [3], produced 50% inhibition of the rate (in the presence of EDTA) of ethanol oxidation by both microsomal preparations (Table 1). The rate of ethanol oxidation in the presence of EDTA plus desferrioxamine was the same as the rate in the absence of any chelating agent (compare lines 1 and 3 of Table 1), suggesting that desferrioxamine blocks the increase of ethanol oxidation produced by EDTA.

With both microsomal preparations a significant rate of ethanol oxidation persisted in the presence of a concentration of desferrioxamine which nearly completely blocks the production of ·OH ([3]; see below). This desferrioxamine-insensitive cytochrome P-450-mediated rate of ethanol oxidation was nearly 2-fold greater by microsomes from ethanol-fed rats (Table 1). By subtracting the rate of ethanol oxidation in the presence of desferrioxamine (or in the absence of any chelating agent) from the total rate of ethanol oxidation in the presence of EDTA, the ·OH-dependent rate of ethanol oxidation could be calculated. This ·OH-dependent rate of ethanol oxidation, which appears to account for about one-half of the total rate

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† Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; DMSO, dimethyl sulfoxide; and KTBA, 2-keto-4-thiomethylbutyric acid.