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Metabolism and Toxicity of *trans,trans*-Muconaldehyde, an Open-Ring Microsomal Metabolite of Benzene

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We have previously hypothesized that ring-opened metabolites may play an important role in benzene toxicity. In this paper we review recent work related to this hypothesis. *trans,trans*-Muconaldehyde (TTM), a six-carbon diene dialdehyde, was shown by our laboratory to be a microsomal metabolite of benzene. This compound is a ring-opened metabolite of benzene that is hematotoxic in mice. The toxicity of TTM may stem in part from its ability to act as a direct-acting alkylating agent involving interaction with cellular sulfhydryls and/or amino groups. On the other hand, metabolism to the diacid *trans,trans*-muconic acid (MA), a known urinary metabolite of benzene, may represent detoxification since this results in loss of electrophilicity of the compound. Preliminary results indicate that TTM can be metabolized to MA *in vitro* and *in vivo*. The interaction of TTM *in vitro* with macrophages and neutrophils, key cells in the bone marrow, results in cell membrane changes, including loss of activity in the plasma membrane-bound NADPH-dependent oxidase and decreases in membrane lipid fluidity. Deoxyguanosine also was found to react with TTM, forming several different products. These findings may be due to TTM acting directly as an alkylating agent.

Introduction

Benzene toxicity has been postulated to involve metabolism to reactive intermediates that bind to cellular constituents, thus interfering with normal cell function. Until recently, the focus has been on ring-hydroxylated metabolites of benzene and their toxic effects *in vivo* and *in vitro* (*1,2*). Our initial suggestion that ring-opening might also be an important metabolic pathway in benzene toxicity (*3*) was based on the identification of *trans,trans*-muconic acid in the urine of rabbits administered ^{14}C-benzene (*4*) and on the potential toxicity of alpha,beta-unsaturated aldehydes (*5*). In order to investigate this hypothesis we have initially studied *trans,trans*-muconaldehyde, a six-carbon diene dialdehyde. In the present paper we review our previous and ongoing studies with this compound in relation to the possible role of ring-opened metabolites in benzene toxicity.

trans,trans-Muconaldehyde (TTM) is an alpha,beta-unsaturated six-carbon diene dialdehyde. Based on its chemical structure (Fig. 1) TTM is expected to exhibit toxicity similar to that of other alpha,beta-unsaturated aldehydes such as inhibition of DNA, RNA, and protein synthesis (*5*), inhibition of respiration (*6*), genotoxicity (*7*), and carcinogenicity (*8*). The idea that TTM might be a hitherto unidentified metabolite of benzene was further supported by reports in the literature indicating the formation of muconaldehyde in aqueous solutions of benzene undergoing pulse radiolysis (*9*) resulting in the formation of hydroxyl radicals.

Formation of TTM from Benzene

We initially confirmed the formation of TTM from benzene in a hydroxyl radical generating system using a Fenton Reagent (*10*). Detection of TTM in a microsomal-metabolizing system required a number of different approaches. TTM was identified as a metabolite of ^{14}C-benzene incubated *in vitro* with mouse liver microsomes in the presence of NADPH (*11*). The pure compound, consisting of yellow needles, melting point to 119 to 121°C (*3*) was synthesized to serve as standard *per se* as well as in derivatized form with 2-thiobarbituric acid (TBA) with which it forms an adduct with a 490-nm absorption max-

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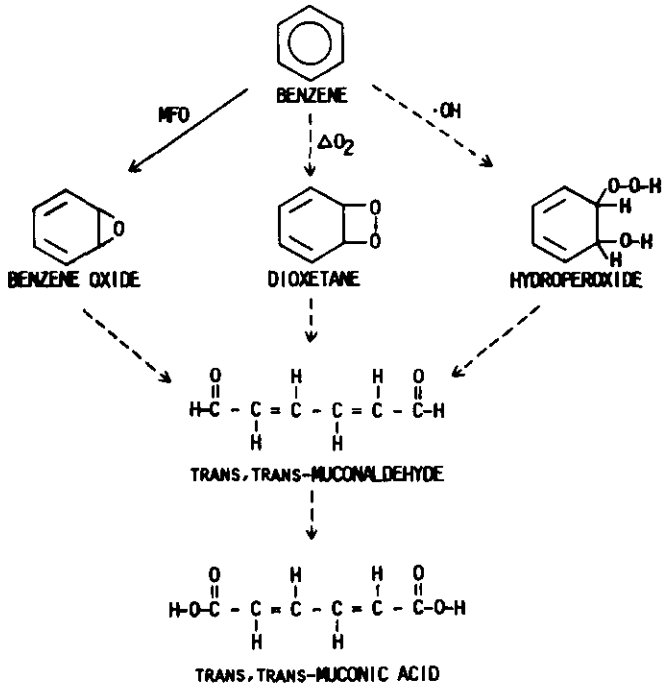

FIGURE 1. Hypothetical pathways for the formation of TTM from benzene. From Latriano et al. (*11*).

imum (*12*). Evidence for TTM formation from benzene included its detection in microsomal-metabolizing mixtures on HPLC chromatograms at retention times of authentic TTM and the TTM-TBA adduct. The radioactivity profile of fractions collected during HPLC analysis contained peaks that eluted with TTM and the TTM-TBA adduct. Collection of the peak presumed to be TTM and subsequent derivatization with 2,4-dinitrophenylhydrazine (DNPH) gave a product with a molecular weight which matched that of the mono-DNPH-TTM derivative as determined by high resolution mass spectrometry. Based on the [^{14}C] label of the TTM HPLC peak, the recoverable TTM formed from 4 mM ^{14}C-benzene is 0.84 nmole/mg protein (0.87 nmole/nmole cytochrome P-450), or 9% of total metabolites, compared with phenol, which constituted 72% of total metabolites and hydroquinone, which accounted for 13% of total metabolites formed during a 10-min incubation period.

Hematotoxicity

Relatively little is known about the toxic effects of TTM and its relation to benzene hematotoxicity. Unlike phenol, which is the precursor of other known ring-hydroxylated metabolites of benzene, TTM is myelotoxic in mice (*13*). Administration of TTM (2 mg/kg, IP) daily for 10 and 16 days resulted in a statistically significant decrease in bone marrow cellularity, red blood cell count, hematocrit, hemoglobin, and increases in white blood cell count and spleen weight at 16 days. Hepatic sulfhydryl concentrations, both total and nonprotein, also decreased markedly while δ-aminolevulinic acid dehydratase, a key enzyme in heme biosynthesis, was not affected.

Reactions of *trans,trans*-Muconaldehyde with Nucleophiles

By virtue of its chemical structure, TTM is electrophilic in nature and therefore can interact with cellular nucleophiles without prior metabolism. Alpha,beta-unsaturated aldehydes in general rapidly react with reduced glutathione (GSH), which adds across the double bond, thus forming the Michael addition product shown in Equation 1.

$$R_2C{=}CH{-}CHO + GSH \rightarrow \underset{\displaystyle GS}{R_2C{-}CH_2{-}CHO} \qquad (1)$$

In our studies the reaction of TTM with GSH was found to proceed rapidly and follow second-order kinetics, first-order with respect to TTM, and to be pH-dependent (*14*). Based on the observed rate constants, TTM is slightly less reactive toward GSH than acrolein, the most reactive alpha,beta-unsaturated aldehyde, and more reactive than *trans*-4-hydroxynonenal, a toxic lipid peroxidation product (*5,15*). The stoichiometry of the reaction and the rate constants suggest that the reaction most likely consists of 1,4 addition, leading to the Michael addition product. Since TTM contains two activated double bonds, it is theoretically capable of reacting with two molecules of thiol, as shown in Equation 2.

$$OHC{-}CH{=}CH{-}CH{=}CH{-}CHO + 2\,GSH \rightarrow \underset{\displaystyle GS\quad SG}{OHC{-}CH_2{-}CH{-}CH{-}CH_2{-}CHO} \qquad (2)$$

The observed stoichiometry of 1.2 (molar ratio of GSH to TTM) at pH 7.4 suggests a second addition of GSH after initial 1:1 adduct formation. TTM therefore has the ability to act as a bifunctional alkylating agent by reaction of the two double bonds with cellular sulfhydryls. It may also act as a bifunctional alkylating agent by Schiff-base formation involving reaction of the aldehyde groups with amino groups as shown in Equation 3:

$$\begin{gathered} R{-}NH_2 + R'{-}NH_2 + \\ OHC{-}CH{=}CH{-}CH{=}CH{-}CHO \rightarrow \\ R{-}N{=}CH{-}CH{=}CH{-}CH{=}CH{-}C{=}N{-}R' \end{gathered} \qquad (3)$$

This bifunctionality suggests that cross-linking reactions may also play a role in the toxic effects of TTM.

In Vitro Metabolism of TTM to *trans,trans*-Muconic Acid

The metabolism of TTM is at present relatively unexplored. Since the aldehyde groups activate alpha,beta-unsaturated double bonds toward nucleophilic attack, metabolism to the alcohol or acid can lead to intermediates in which the double bonds are no longer electrophilic in nature at physiological pH. Both aldehyde groups have to be metabolized in TTM to lead to deactivation of the two double bonds. Metabolism of TTM by cellular aldehyde dehydrogenases, aldehyde reductases, and alcohol dehydrogenases might lead to conjugated diene metabo-

lites containing two carboxylic acid groups or two primary alcohol groups. In addition, compounds containing mixed functional end groups such as an aldehyde group and a carboxyl group or a primary alcohol or a carboxyl group and a primary alcohol group might also be formed. This array of possible metabolites of TTM may also include compounds obtained by oxidation of the double bonds via mixed function oxidases.

Although the metabolism of benzene to *trans,trans*-muconic acid has been demonstrated *in vivo* in rabbits (*4*) and rodents (*16*), the mechanism of MA formation is not known. Recent studies in our laboratory suggest that TTM may be a precursor of MA (*17*). In these studies, TTM was incubated with liver cytosol from male DBA/2 mice and Sprague-Dawley rats in the presence of NAD^+ and pyrazole, an alcohol dehydrogenase inhibitor. Under these conditions, NAD^+ was reduced to NADH, as monitored by an increase in the optical density at 340 nm, suggesting oxidative metabolism. Analysis of the data gave a biphasic Lineweaver-Burke plot for mouse liver cytosol compared with a single unbroken straight line for rat liver cytosol. These results suggest that, depending on the species, one or more aldehyde dehydrogenase(s) may participate in the oxidative metabolism of TTM.

In order to analyze the products formed, ether extracts of the cytosolic metabolism mixtures were analyzed by HPLC using the analytical procedures published by Gad-El Karim et al. (*16*) for the detection of MA. A peak at the retention time of authentic *trans,trans*-muconic acid from the cytosolic extracts incubated with TTM suggests that MA can be formed from TTM. The quantitative aspects of the *in vitro* metabolism of TTM to MA and its relationship to the *in vivo* metabolism of benzene to MA require further exploration. It is of interest to note that *in vitro* metabolism of *trans*-2-hexenal has recently been reported to lead to corresponding alpha,beta-unsaturated carboxylic acid (*18*). On the other hand, *in vitro* metabolism of *trans*-4-hydroxynonenal, a major toxic alpha,beta-unsaturated aldehydic lipid peroxidation product, has been reported to lead to the formation of the corresponding primary alcohol and not the corresponding carboxylic acid (*19*).

In Vivo Metabolism of Benzene and TTM to *trans,trans*-Muconic Acid

trans,trans-muconic acid (MA) is an open-ring metabolite of benzene previously detected in the urine of rabbits (*4*) and mice (*16*) administered benzene. Our laboratory found that MA is also excreted in the urine of male Sprague-Dawley rats injected IP with benzene at doses ranging from 0.5 to 20 mg/kg (*20*). Using an adaptation of the analytical methods reported by Gad-El Karim et al. (*16*) for the measurement of urinary MA, the initial 24-hr total MA excreted in Sprague-Dawley rats ranged from 0.3 to 6.6% of the administered benzene dose.

In preliminary studies, the metabolism of TTM to MA was also examined in mice. For this work, urine was collected from control and TTM-treated mice (two per metabolic cage) into 2 mL 0.2% ascorbic acid. The analytical methods used consisted of deproteinization of samples with methanol-acetic acid, centrifugation and extraction of the supernatants with ether, and dissolving the residue after ether evaporation in methanol followed by gradient HPLC analysis (Maniara et al., unpublished data). In two separate experiments, DBA/2 mice (6–8 per group and 2 per metabolic cage) administered TTM IP at a dose of 2 mg/kg excreted about 40 to 45% of the administered dose in the form of urinary MA during the first 24 hr. Injection of a second dose of TTM at 2 mg/kg 24 hr after the first dose also resulted in excretion of MA at about 40% of the administered dose of TTM. These data indicate that a major metabolite of TTM *in vivo* in mice is MA.

Toxic Effects on Macrophages and Neutrophils

In other studies in our laboratory, we have examined the toxicity of TTM and other alpha,beta-unsaturated aldehydes of toxicological importance with respect to their effects *in vitro* on neutrophils and macrophages. This work is an outgrowth of ongoing work on the effects of oxidants on the lung, effects that may be mediated in part by reactive aldehydes (*21*). Macrophages and neutrophils are also key cells present in bone marrow, and the effects of reactive aldehydes on phagocytic cells *in vitro* may be germane to the mechanism of toxicity of TTM on bone marrow cells *in vivo*.

Incubation of human neutrophils or rat lung macrophages with micromolar concentrations of TTM and other alpha,beta-unsaturated aldehydes leads to a dose-dependent decrease in superoxide anion radical production in stimulated cells (*22,23*). The inhibition of superoxide anion radical production in stimulated phagocytic cells is correlated with decreases in intracellular glutathione, decreases in cell surface sulfhydryls (*23*), and decreases in membrane lipid fluidity (*24*). Concerning these effects in relation to benzene hematoxicity, modification in the structure and function of the plasma membrane of macrophages by TTM may lead to changes in the ability of the macrophage to function as a key cell in the immune system and to communicate with other cells in the bone marrow. Of note are the recent findings by Lewis et al. (*25*) demonstrating inhibitory effects of benzoquinone and other benzene metabolites on the ability of macrophages to be activated for functions important in host defense such as H_2O_2 release, Fc receptor-mediated phagocytosis, interferon gamma priming for tumor cell cytolysis, and bacterial lipopolysaccharide triggering of cytolysis. Thus, TTM and other toxic benzene metabolites have the potential for affecting bone marrow cell function by interaction with marrow macrophages.

Genotoxicity of TTM

TTM has the ability of being genotoxic based on its reaction with deoxyguanosine. Reaction of TTM (10^{-3} M) with deoxyguanosine (2×10^{-3} M) in 0.025 M phosphate

buffer, pH 7.4, for 16 hr at 37°C gave a reaction mixture with a new absorption maximum at 340 nm. HPLC analysis on a C-18 reverse phase bondapack column with 340 nm as the detection wavelength and phospate buffer: methanol (75:25,v:v) as the eluent indicated the presence of three major peaks eluting at 16.1, 18.2, and 19.1 min. Well-resolved minor peaks were detected at retention times of 2.8, 9.9, and 20.2 min, and a group of six peaks eluted between 5.6 and 8.7 min. These results are similar to those obtained in collaborative work with Latriano et al. (*26*) who used radiolabeled deoxyguanosine 5'-phosphate in the reaction with TTM. Alpha,beta-unsaturated aldehydes have been reported to react with deoxyguanosine, forming products with one additional ring involving the N-1 position and the amino group on C-2 (*27,28*).

Conclusions

We have used TTM as the initial compound in studying the potential role of ring-opened metabolites in benzene toxicity. Other potentially toxic ring-opened products, as well as reactive intermediates and conjugates, formed from TTM are also possible. Furthermore, it would not be surprising if benzene toxicity were dependent upon an interaction of both ring-opened and ring-hydroxylated metabolites. These provide future challenging avenues in future benzene research.

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REFERENCES

1. Snyder, R., and Kocsis, J. J. Current concepts of benzene toxicity. CRC Crit. Rev. Toxicol. 3: 265–288 (1975).
2. Kalf, G. F. Recent advances in the metabolism and toxicity of benzene. CRC Crit. Rev. Toxicol. 18: 141–159 (1987).
3. Goldstein, B. D., Witz, G., Javid, J., Amoruso, M., Rossman, T., and Wolder, B. Muconaldehyde, a potential toxic intermediate of benzene metabolism. In: Biological Reactive Intermediates II, Part A (R. Snyder, D. V. Parke, J. J. Kocsis, D. Jallow, G. G. Gibson, and C. M. Witmer, Eds.), Plenum Press, New York, 1982, pp. 331–339.
4. Parke, D. V., and Williams, R. T. Studies in detoxication 49. The metabolism of benzene containing $^{14}C_1$ benzene. Biochem. J. 54: 231–238 (1953).
5. Schauenstein, E., Esterbauer, H., and Zollner, H. Aldehydes in Biological Systems. Methuen, New York, 1977.
6. Khoschsorur, G., Schaur, R. J., Schauenstein, E., Tillian, H. M., and Reiter, M. Intracellulare Wirkung von Hydroxyalkenalen auf tierische Tumoren. Z. Naturforsch. 36c: 572–578 (1981).
7. Marnett, J. L., Hurd, H. K., Hollstein, M. C., Levin, D. E., Esterbauer, H., and Ames, B. N. Naturally occurring carbonyl compounds are mutagens in Salmonella tester strain TA 104. Mutat. Res. 148: 25–34 (1985).
8. Chung, F.-L., Tanaka, T., and Hecht, S. S. Induction of liver tumors in F344 rats by crotonaldehyde. Cancer Res. 46: 1285–1289 (1986).
9. Loeff, I., and Stein, G. Aromatic ring opening in the presence of oxygen in irradiated solutions. Nature 184: 901 (1959).
10. Latriano, L., Zaccaria, A., Goldstein, B. D., and Witz, G. Muconaldehyde formation from ^{14}C-benzene in a hydroxyl radical generating system. J. Free Rad. Med. Biol. 1: 363–371 (1985).
11. Latriano, L., Goldstein, B. D., and Witz, G. Formation of muconaldehyde, an open-ring metabolite of benzene in mouse liver microsomes: a novel pathway for toxic metabolites. Proc. Natl. Acad. Sci. (U.S.) 83: 8356–8360 (1986).
12. Witz, G., Lawrie, N. J., Zaccaria, A., Ferran, H. E., and Goldstein, B. D. The reaction of 2-thiobarbituric acid with biologically active alpha,beta-unsaturated aldehydes. J. Free Rad. Biol. Med. 2: 33–39 (1986).
13. Witz, G., Rao, G. S., and Goldstein, B. D. Short term toxicity of trans,trans-muconaldehyde. Toxicol. Appl. Pharmacol. 80: 511–516 (1985).
14. Rao, G. S., Witz, G., and Goldstein, B. D. The reactivity of trans,trans-muconaldehyde, a possible toxic metabolite of benzene towards glutathione. Toxicologist 2: 122 (1982).
15. Esterbauer, H., Zollner, H., and Scholz, N. Reaction of glutathione with conjugated carbonyls. Z. Naturforsch. 30c: 466–473 (1975).
16. Gad-El Karim, M. M., Sadagopa Ramanujam, V. M., and Legator, M. S. Trans,trans-muconic acid, an open-chain urinary metabolite of benzene in mice. Quantification by high pressue liquid chromatography. Xenobiotica 15: 211–220 (1985).
17. Kirley, T. A., Goldstein, B. D., and Witz, G. Liver cytosolic metabolism of trans,trans-muconaldehyde to trans,trans-muconic acid. Toxicologist 8: 70 (1988).
18. Mitchell, D. Y., and Petersen, D. R. The oxidation of alpha,beta-unsaturated aldehydic products of lipid peroxidation by rat liver aldehyde dehydrogenases. Toxicol. Appl. Pharmacol. 87: 403–410 (1987).
19. Esterbauer, H., Zollner, H., and Lang, J. Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions. Biochem. J. 228: 363–373 (1985).
20. Mitchell, J. M., Goldstein, B. D., and Witz, G. HPLC detection of trans,trans-muconic acid in rats exposed by benzene. Toxicologist 8: 211 (1988).
21. Goldstein, B. D Cellular effects of ozone. Rev. Environ. Health 2: 177–202 (1977).
22. Witz, G., Lawrie, N. J., Amoruso, M. A., and Goldstein, B. D. Inhibition by reactive aldehydes of superoxide anion radical production in stimulated human neutrophils. Chem.-Biol. Interact. 53: 13–23 (1985).
23. Witz, G., Lawrie, N. J., Amoruso, M. A., Goldstein, B. D. Inhibition by reactive aldehydes of superoxide anion radical production from stimulated polymorphonuclear leukocytes and pulmonary alveolar macrophages. Effects on cellular sulfhydryl groups and NADPH oxidase activity. Biochem. Pharmacol. 36: 721–726 (1987).
24. Lawrie, N. J., and Witz, G. Effects of alpha,beta-unsaturated aldehydes on membrane lipid fluidity and phagocytosis of rat alveolar macrophages. Toxicologist 7: 128 (1987).
25. Lewis, J. G., Odom, B., and Adams, D. O. Toxic effects of benzene and benzene metabolites on mononuclear phagocytes. Toxicol. Appl. Pharmacol. 92: 246–254 (1988).
26. Latriano, L., Witz, G., Goldstein, B. D., and Jeffrey A. M. Chromatographic and spectrophotometric characterization of adducts formed during the reaction of *trans,trans*-muconaldehyde with ^{14}C-deoxyguanosine 5'-phosphate. Environ. Health Perspect. 82: 249–251 (1989).
27. Chung, F.-L., Young, R., and Hecht, S. S. Formation of cyclic 1,N^2-*propanodeoxyguanosine adducts in DNA upon reaction with* acrolein or crotonaldehyde. Cancer Res. 44: 990–995 (1984).
28. Winter, C. K., Segall, H., and Haddon, W. F. Formation of cyclic adducts of deoxyguanosine with the aldehydes trans-4-hydroxy-2-hexenal and trans-4-hydroxy-2-nonenal in vitro. Cancer Res. 46: 5682–5686 (1986).