Novel Involvement of a Mitochondrial Steroid Hydroxylase (P450c11) in Xenobiotic Metabolism*

(Received for publication, June 5, 1995, and in revised form, July 17, 1995)

Bert-Ove Lund§ and Johan Lund†
From the †Department of Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Uppsala Biomedical Centre, Box 573, S-75123 Uppsala, and §Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital F60, NOVUM, S-14186 Huddinge, Sweden

Adrenocortical mitochondrial cytochrome P450 isozymes of the Cyp11 family normally synthesize steroids with a very strict substrate specificity. However, for the first time, P450c11 was additionally shown to metabolize and bioactivate the adrenotoxic environmental pollutant 3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichloroethene (MeSO₂-DDE). This conclusion is based on a striking correlation between inductions of MeSO₂-DDE and deoxycorticosterone metabolism by forskolin in the adrenocortical cell lines Y1 and Kin-8, inhibition of P450c11-dependent activities in Y1 cells by MeSO₂-DDE, and metabolism of MeSO₂-DDE by non-steroidogenic COS cells after transfection with a cDNA encoding P450c11. The interaction between xenobiotics and glucocorticoid synthesis should focus more attention to xenobiotox-induced hormonal disturbances.

MeSO₂-DDE (3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichloroethene) is a metabolite of the still extensively used insecticide DDT and is therefore a global environmental pollutant. Because of its lipophilicity and persistence, MeSO₂-DDE is bioaccumulated in the food chain, even leading to pollution of human breast milk (1, 2). Animal experiments have shown MeSO₂-DDE to be adrenotoxic to several species after bioactivation in the adrenal cortex mitochondria (3–7). Furthermore, human adrenal mitochondria have also been demonstrated to bioactivate MeSO₂-DDE in vitro (8). It has been suggested, but never demonstrated, that the bioactivation is mediated by mitochondrial cytochrome P450 (7). The adrenal gland contains several P450 isozymes, but in contrast to hepatic P450s, adrenal P450s are generally not active in xenobiotoxic metabolism. The genes encoding the mitochondrial P450 11β-hydroxylase (P450c11), cholesterol side-chain cleavage (P450sc), and aldosterone synthase (P450aldo) belong to the Cyp11 subfamily of the P450 superfamily of genes (9). These enzymes show a strict substrate specificity and are therefore not believed to metabolize xenobiotics. P450sc is the first and rate-limiting enzyme in steroid hormone synthesis, localized in all steroidogenic tissues, whereas P450c11 and P450aldo are specifically localized in the adrenal cortex. The latter two participate as the last enzymes in the synthesis of glucocorticoids and mineralocorticoids, respectively. Given the specific mitochondrial damage in the adrenal cortex after MeSO₂-DDE exposure, we decided to investigate the possible involvement of steroidogenic mitochondrial P450s in the metabolism of MeSO₂-DDE. For this purpose we studied the bioactivation of MeSO₂-DDE, i.e. the metabolic formation of reactive MeSO₂-DDE intermediates capable of binding irreversibly to macromolecules or cellular antioxidants, in the mouse adrenocortical tumor cell line Y1. We also studied the bioactivation of MeSO₂-DDE in a mutant of the Y1 cell line, Kin-8, that harbors a defect in the cAMP-dependent protein kinase (10), which normally participates in the hormonal regulation of the steroid-metabolizing enzymes. In Kin-8 cells, as opposed to Y1 cells, ACTH, forskolin, or cAMP are inefficient inducers of the P450 isozymes expressed in these cells, i.e. mitochondrial P450sc, P450c11, and P450aldo. To unambiguously demonstrate the relative importance of the adrenal mitochondrial P450s in the bioactivation of MeSO₂-DDE, experiments were finally conducted in monkey kidney COS cells. These cells do not normally metabolize steroids or MeSO₂-DDE, so by investigating the MeSO₂-DDE-metabolizing capacity in COS cells transfected with expression plasmids encoding the three mitochondrial P450s we tried to identify the enzyme responsible for bioactivation, and presumably toxicity, of MeSO₂-DDE.

EXPERIMENTAL PROCEDURES

Culture of the adrenocortical cell lines Y1 and Kin-8, as well as of COS cells, were performed in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Experiments were conducted using subconfluent cultures containing approximately 2 × 10⁶ cells in 30-mm plate. In some experiments Y1 and Kin-8 cells were treated with forskolin (10 μM, in MeSO₂, Sigma) for 18 h before other additions. Steroid substrates (20 μM, Sigma) and MeSO₂-DDE (synthesized according to Ref. 4) were added from 1000-fold stock solutions in Me₂SO. Enzyme assays were performed after growing cells in the presence of steroid substrate (deoxycorticosterone, corticosterone, or 22-hydroxycholesterol). A 500-μl aliquot of the medium was removed and extracted with chloroform/methanol (2:1). After one additional extraction of the medium with chloroform, the chloroform phases were pooled, evaporated, and dissolved in 50% acetonitrile for HPLC analyses of corticosteroids. A500 mg/ml of the medium was removed and extracted with chloroform/methanol (2:1). After one additional extraction of the medium with chloroform, the chloroform phases were pooled, evaporated, and dissolved in 50% acetonitrile for HPLC analyses of corticosteroids. A500 mg/ml of the medium was removed and extracted with chloroform/methanol (2:1). After one additional extraction of the medium with chloroform, the chloroform phases were pooled, evaporated, and dissolved in 50% acetonitrile for HPLC analyses of corticosteroids.

© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.
protein pellet was exhaustively extracted by repeatedly solubilizing the protein in 1% sodium dodecyl sulfate and precipitating with acetone as described by Baker and Van Dyke (13). The protein was finally dissolved in NaOH, and aliquots were assayed for protein (14) and irreversibly bound radioactivity.

Transfection studies were performed on COS cells grown on 30-mm plates until almost confluent, when they received new medium containing 3 μg of DNA (2 μg of P450 +1 μg of adrenodoxin (ADX)) in the transfection reagent DOTAP (Boehringer Mannheim). After a 20-h transfection period, the cells were grown for 24 h in fresh medium and then new medium containing 20 μM steroid substrate or 4 μM [14C]MeSO2-DDE was added. Medium and cells were harvested after 24 h of incubation, and enzyme activities as well as [14C]MeSO2-DDE metabolism assayed as described above.

RESULTS AND DISCUSSION

Upon treatment of adrenocortical Y1 cells with forskolin, which stimulates adenylate cyclase, these cells metabolized MeSO2-DDE to intermediates that reacted with cellular protein (Fig. 1a) and gave rise to hydrophilic MeSO2-DDE metabolites (Fig. 1b). By thin layer chromatography analyses of the hydrophilic MeSO2-DDE metabolites, the presence of ninhydrin-positive MeSO2-DDE metabolites, presumably glutathione conjugates, was indicated (data not shown). The lack of MeSO2-DDE metabolism in unstimulated cells and the minimal MeSO2-DDE metabolism in forskolin-stimulated Kin-8 cells (Fig. 1) suggested the involvement of cAMP-inducible steroidogenic enzymes in the metabolism of MeSO2-DDE. A similar inducibility of MeSO2-DDE metabolism and one steroid hydroxylase, P450c11, by forskolin in Y1 and Kin-8 cells (Fig. 2) further supported the involvement of steroidogenic P450 in MeSO2-DDE metabolism.

We next tested the ability of the three mitochondrial P450s present in adrenocortical Y1 cells to metabolize MeSO2-DDE. This metabolism was assessed in COS cells transfected with expression plasmids encoding mouse P450c11, P450sc, and P450aldo, respectively, either alone or in combination with a plasmid encoding bovine adrenodoxin (ADX) (15, 16). Adrenodoxin is an iron-sulfur protein that participates in the transfer of electrons from NADPH to the mitochondrial P450s, a process previously shown to be rate-limiting for steroid hydroxylations in transfected COS cells (16). As can be seen in Table I, the transfecions were successful, although cotransfection with the ADX plasmid was necessary to obtain high P450 activities with the respective substrates. Only cells transfected with P450c11 metabolized MeSO2-DDE to any significant extent, as determined by the formation of hydrophilic MeSO2-DDE metabolites in the medium (Table I), and irreversible binding of MeSO2-DDE to protein (Fig. 3). Thus, both formation of hydrophilic metabolites and irreversible protein binding of MeSO2-DDE is mediated by the same isozyme, i.e. P450c11.

In vivo exposure of mice to MeSO2-DDE and in vitro experiments with subcellular adrenal homogenates have shown a selective irreversible binding of MeSO2-DDE in the adrenal cortex (4, 5, 7). The present studies provide direct evidence for an involvement of adrenocortical mitochondrial P450s in the bioactivation of MeSO2-DDE, by showing that minimal MeSO2-DDE or deoxycorticosterone metabolism occurred in uninduced adrenocortical cells and that forskolin induction increased the metabolism of both substrates to the same extent. Furthermore, we unambiguously ascribe the metabolism of MeSO2-

![Image](93x265 to 250x370)

**Fig. 1. Irreversible binding of [14C]MeSO2-DDE to protein (a) and formation of hydrophilic [14C]MeSO2-DDE metabolites in the culture medium (b) of normal and forskolin-induced Y1 and Kin-8 cells. Cells were grown in the presence of 3 μM [14C]MeSO2-DDE for 6–24 h, whereafter medium and cells were separated. After extraction with organic solvents, 14C-labeled metabolites irreversibly bound to protein and hydrophilic 14C-metabolites remaining in the medium were measured. Each point represents mean of duplicate samples. Mean variation between duplicates was 11%.

![Image](104x466 to 239x664)

**Fig. 2. P450c11 activity in normal and forskolin-induced Y1 and Kin-8 cells. The mutant Kin-8 has a defect cAMP-dependent protein kinase, strongly decreasing the expression and inducibility of P450sc and P450c11 (8). Cells were grown in the presence of deoxycorticosterone for 6 or 12 h, whereafter an aliquot of the medium was extracted and analyzed for corticosterone by HPLC. Each point represents mean of duplicate samples. Mean variation between duplicates was 11%.

| Transfected enzyme | Enzyme activity | Hydrophilic [14C]MeSO2-DDE metabolites + ADX
|--------------------|----------------|----------------------------------|
| Control (mock/pCMV5) | NDa | 0b |
| P450aldo | 66 ± 79 (aldosterone) | 1035 ± 55 (aldosterone) | 4.3 ± 0.8 |
| P450c11 | 463 ± 67 (corticosterone) | 27,105 ± 2244 (corticosterone) | 117.4 ± 9.5 |
| P450sc | ND | 1764 ± 114 (progesterone) | 5.2 ± 0.2 |

a The values presented have been subtracted with the amount of radioactivity present in medium of mock-transfected cells.

b None of the three enzyme activities was detected.

**Table 1**

Activities of P450aldo, P450c11, and P450sc and the formation of hydrophilic [14C]MeSO2-DDE metabolites in COS cells transfected with mouse P450 cDNA alone and in combination with adrenodoxin (ADX) cDNA

Transfected COS cells were grown in the presence of steroid substrate or [14C]MeSO2-DDE for 24 h, whereafter the medium was assayed for steroid products or [14C]MeSO2-DDE metabolites. Mean ± S.D. (n = 3); ND, not detected.
DDE to P450c11 through transfection studies in COS cells. To our knowledge, this is the first example of xenobiotic-induced toxicity mediated by mitochondrial steroid-metabolizing P450s. In addition, at concentrations only slightly affecting the cell number (assessed as amount of protein per plate), MeSO₂-DDE was a potent inhibitor of the P450c11-activity in the Y1 cells (Fig. 4). In contrast, equal concentrations of the non-adrenotoxic DDE did not affect P450c11 or cell number (data not shown). This finding may provide an explanation to the previously observed reduced capacity of mice exposed to MeSO₂-DDE to synthesize glucocorticoids (6).

This study thus illustrates two mechanisms, bioactivation of xenobiotics and inhibition of steroid hydroxylases, whereby xenobiotics may interfere with glucocorticoid synthesis. Because the adrenal gland generally accumulates high levels of lipophilic xenobiotics (17, 18), and since a normal capacity to produce glucocorticoids is vital for organisms during stressful periods, the ability of certain xenobiotics to interfere with steroid hormone synthesis may have great significance and deserves further attention.

Acknowledgments—We are grateful to Drs. K. Parker for the P450 cDNAs, M. Waterman for the adrenodoxin cDNA, B. Schimmer for the Kin-8 cells, Å. Bergman for synthesis of MeSO₂-DDE, and M. Bakke for technical advice.

REFERENCES

1. Jensen, S., and Jansson, B. (1976) Ambio 5, 257–260
2. Haraguchi, K., Kuroki, H., and Masuda, Y. (1989) Chemosphere 19, 487–492
3. Jönsson, C.-J., Lund, B.-O., Brunström, B., and Brandt, I. (1994) Environ. Toxicol. Chem. 13, 1303–1310
4. Lund, B.-O., Bergman, Å., and Brandt, I. (1988) Chem.-Biol. Interact. 65, 25–40
5. Jönsson, C.-J., Lund, B.-O., Bergman, Å., and Brandt, I. (1992) Reprod. Toxicol. 6, 233–240
6. Jönsson, C.-J. (1994) Pharm. Toxicol. 74, 58–60
7. Jönsson, C.-J., Rodriguez-Martinez, H., Lund, B.-O., Bergman, Å., and Brandt, I. (1991) Fund. Appl. Toxicol. 16, 365–374
8. Jönsson, C.-J., and Lund, B.-O. (1994) Toxicol. Lett. 71, 169–175
9. Miller, W. L. (1988) Endocr. Rev. 9, 295–318
10. Wong, M., Rico, D. A., Parker, K. L., and Schimmer, B. P. (1989) J. Biol. Chem. 264, 12867–12871
11. Lund, B.-O. (1994) Environ. Toxicol. Chem. 13, 911–917
12. Talalay, P., and Dobson, M. M. (1953) J. Biol. Chem. 205, 823–837
13. Baker, M. T., and Van Dyke, R. A. (1984) Biochem. Pharmacol. 33, 255–260
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15. Domalik, L. J., Chaplin, D. C., Kirkman, M. S., Wu, R. C., Liu, W., Howard, T. A., Seldin, M. F., and Parker, K. L. (1991) Mol. Endocrinol. 5, 1833–1861
16. Zuber, M. X., Mason, J. I., Simpson, E. R., and Waterman, M. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 699–703
17. Hornsby, P. J. (1989) Free Radical Biol. Med. 6, 103–115
18. Szabo, S., and Lippe, I. Th. (1989) Toxicol. Pathol. 17, 317–329