Requirement for Metabolic Activation of Acetylaminofluorene to Induce Multidrug Gene Expression

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Previously we have demonstrated that several xenobiotics can induce multidrug (mdr) gene expression in cultures of primary isolated hepatocytes. One of the best of these xenobiotic inducers in rat hepatocytes is 2-acetylaminofluorene (2-AAF), which induces mdr expression by an enhancement of mdr gene transcription. In all species studied to date, AAF is extensively and variously metabolized. In this study we have sought to determine if AAF per se or a metabolite is responsible for mediating the increase in mdr gene transcription and expression. This study demonstrates that AAF per se is not active, but that the effect of AAF we have observed on mdr gene transcription and expression in the rat is due to the formation of a reactive metabolite(s). Our data indicate that this reactive metabolite is probably N-acetoxy-2-aminofluorene or the sulfate ester of N-hydroxy-AAF. The requirement for the formation of one of these metabolites may explain the differences in species response to AAF, in terms of mdr gene expression, that we have observed. We hypothesize that the mechanism by which mdr gene transcription is increased in response to AAF involves a covalent interaction between a reactive metabolite and an mdr gene regulatory protein. Our current work is concerned with the exploration of this hypothesis. — Environ Health Perspect 102(Suppl 6):209–212 (1994)

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Introduction

The multidrug (mdr) resistance phenotype occurs commonly in tumors prior to and after exposure to chemotherapeutic agents and can arise by several different mechanisms (1–3). One mechanism by which an mdr phenotype is developed is via overexpression of a membrane transport protein, P-glycoprotein (P-gp) (4–7). This protein operates as a plasma membrane pump to transfer xenobiotic molecules across the plasma membrane and out of the cell against a concentration gradient (8–11). Energy for this process is derived from the hydrolysis of ATP (9,10,12). P-gps are encoded by a family of genes consisting of two members in humans, primates, rabbits, and fish; generally three in rodents; four in the dog; and five in the pig (13). The genes generally divide into two classes: mdr1 and mdr2. The mdr1 genes encode a P-gp that confers drug resistance while the mdr2 genes code for a P-gp that does not.

Humans and primates have a mdr1 and mdr2 gene. Rodents also have only one mdr2 gene but have two genes in the mdr1 class. These genes are variously named in the literature, but we refer to them as mdr1a and mdr1b in accordance with the nomenclature proposed by Hsu et al. (14). The P-gps are part of a much larger protein family known as the ABC proteins or ATP-binding cassette proteins (15–17). Members of this family include the yeast STE6 factor transporter to which P-gp is closely related and the cystic fibrosis transmembrane conductor (18,19).

The expression levels of P-gp are variable in tumors both at the time of diagnosis and after treatment with chemotherapeutic agents (20). Normal expression of P-gp is highest in the intestine, kidney (proximal convoluted tubule), on the bile canalicular surface of the hepatocyte, the blood brain barrier, in the adrenal gland, and in the uterus of a pregnant animal (mouse) (21–25). Tumors derived from these tissues normally have the highest pretreatment levels of P-gp (20,26,27). In contrast, tumors of the hemopoietic system appear most likely to develop expression following exposure to chemotherapeutic agents (20,27–29). The normal distribution of P-gp expression suggests that its physiological role may be to expel naturally occurring toxins from the cell (2). This hypothesis is consistent with the substrate specificity of P-gp that is highest against naturally occurring chemotherapeutic agents (30).

Expression of members of the mdr gene family is induced in vivo following partial hepatectomy (31–33) and during chemical carcinogenesis (33). Hepatocytes derived from livers undergoing regeneration following partial hepatectomy demonstrate an increased resistance to hepatotoxins (34). Hepatocytes isolated from rats previously exposed to acetylaminofluorene (AAF) display an increased resistance to several cytotoxic drugs including actinomycin D (35). Expression of the mdr gene family can be induced in rat liver following acute exposure to several xenobiotics (36,37).

Results and Discussion

We are evaluating the hypothesis that P-gp is one of a family of proteins whose function is to pump xenobiotics and their metabolites from cells. We have called this process a phase III system, as it would form the third major response (phase I and II being metabolic transformations) that the cell can make to a xenobiotic insult (Figure 1). By this hypothesis it would be reasonable for the regulatory regions of the P-gp genes, and other related excretory pump genes, to have common regulatory elements so their expression can be coordinately regulated by common protein in response to
toxic stress. Thus, we might postulate that the cell has a family of receptors that have affinities for various classes of substrates and, upon binding a particular xenobiotic, are able to induce expression of those metabolic and excretory genes whose proteins are best able to alter and excrete the xenobiotic (Figure 1). The best clarified such receptor that may form part of this superfamily is the aryl hydrocarbon (Ah) receptor (38). Other possible candidates are the recently identified peroxisome proliferator receptor (39) and proteins that bind to the antioxidant and electrophilic responsive elements (40,41). The existence of different receptors for the different classes of xenobiotics that the cell may encounter is in concordance with the various substrate specificities of the cytochrome P450 isozymes. It would not be in the economic interests of the cell to activate the whole family of cytochrome P450 enzymes to metabolize a xenobiotic that was a substrate for only one.

Previously we and others have demonstrated that several xenobiotics are able to induce the mdr gene expression (33,36,37,42). One of the most potent mdr inducers in the rat is 2-acetylamino fluorene (2-AAF). In rat liver and isolated hepatocytes, AAF is able to induce transcription of the mdr1 genes and thus elevate their expression measured at both the mRNA and protein levels (37). In monkeys AAF is also able to induce mdr expression though the effect is less and more variable than that seen in the rat (Gant et al., unpublished data).

AAF is extensively and variably metabolized in all species (43,44). For this reason, we are conducting a study to determine if AAF per se or a metabolite is responsible for inducing mdr gene family transcription and expression. Differential excretion of AAF also occurs between species (44), so we also have been trying to determine if differences in the route of excretion of AAF metabolites can explain the differential ability of AAF to induce mdr gene expression between species (unpublished data).

All of the work described here has been carried out in the rat primary cultured hepatocyte model as previously described (37). We first tested the effect of both the ring hydroxylated and N-hydroxy metabolites of AAF, to induce mdr expression (Figures 2A and 2B). As we had anticipated, none of the ring hydroxylated forms had any effect on mdr expression in this system. In contrast, both the N-hydroxy and its derivative the N-acetoxy-2-AAF (2-AAAF) were able to induce mdr expression and were 10- to 20-fold more potent as mdr inducers than AAF. This suggested that metabolism through hydroxylation to either the 2-AAAF or further metabolites was necessary for AAF to elicit increased expression of the mdr gene. Additionally fluorene has no ability to induce mdr gene expression, but 2-amino fluorene which can be acetylated to 2-AAF or activated pet se to electrophiles, is equipotent with 2-AAF as an inducer of mdr gene expression (data not shown).

To further establish the role N-hydroxy metabolites in the induction of mdr gene expression by AAF, we attempted to block the induction of mdr gene expression by AAF using α-naphthoflavone (α-NF). α-NF is an inhibitor of the cytochrome P4501A isoforms that are mainly responsible for the catalysis of AAF N-hydroxylation. At concentrations of 1 to 5 μM, α-NF was able to block the ability of AAF to induce mdr gene expression in isolated rat hepatocyte cultures (Figure 3). Interestingly, α-NF was also able to reduce the basal expression of the mdr gene in these cells, suggesting that there may be an endogenous inducer present that requires metabolism also through the cytochrome P4501A enzymes (Figure 3, lanes 3 and 4). To evaluate the role of deacetylases in the formation of the reactive metabolites, we inhibited cellular deacetylases using paraaxon at 1 and 5 μM and tested the ability of AAF and 2-AAAF to induce mdr gene family expression under these circumstances. In microsomes, paraaxon completely inhibits deacetylase in the rat at 0.1 to 1.0 μM (46). Paraaxon was able to inhibit the ability of 2-AAF to induce mdr gene expression but not that of AAF (Figure 4). This demonstrated that the formation of an ultimate electrophilic metabolite was required for the induction of mdr gene expression. The lack of effect of paraaxon on the ability of AAF to induce mdr gene expression can be explained by consideration of the other possible routes by which AAF can be metabolized to electrophilic metabolites. Two other routes of
AAF metabolism to relative metabolites are via formation of the highly reactive \( N \)-hydroxy-sulfate and through formation of the \( N \)-acetoxy-2-aminofluorene from the \( N \)-hydroxy-AAF by \( N,O \)-acetyltransferase. The \( N,O \)-acetyltransferase is unaffected by paraxoan. In rats, further metabolism of the 2-hydroxy-AAF by \( N,O \)-acetyltransferase appears to be more important as a route of carcinogenic activation than sulfation (43). Induction of \( mdr \) gene family expression by 2-AAAF is inhibited by paraxoan, which indicates that the 2-AAAF does not induce per se or decompose to the \( N \)-acetyl electrophile to form covalent adducts to proteins. Rather, it undergoes deacetylation to the \( N \)-acetoxy-2-aminofluorene and then subsequent decomposition to the electrophile.

The requirement for metabolism of AAF to the \( N \)-hydroxy-2-AAAF and the potency of 2-AAAF as an inducer of the \( mdr \) gene family demonstrates that generation of reactive electrophiles and subsequent protein covalent binding may be the route by which AAF is able to induce \( mdr \) expression in the rat hepatocyte. 2-AAAF appears to require a deacetylation to \( N \)-acetoxy-2-aminofluorene to induce \( mdr \) expression. After the electrophile is formed, gene activation could occur either through a direct interaction with DNA, on a gene regulatory element, or via a protein interaction. Our working hypothesis is that induction of gene expression occurs through a specific protein/electrophile interaction. We know that elevation \( mdr \) gene expression occurs through a transcriptional mechanism in response to AAF and other xenobiotics (37) and therefore we postulate that this protein forming an adduct with an AAF metabolite is then, either alone or a heterodimer with another protein, able to activate \( mdr \) gene transcription. Our current work is aimed at identifying these proteins and the mechanism by which they are able to induce \( mdr \) and other gene family expressions.

**REFERENCES**

1. Chabner BA. Karmofsky memorial lecture. The oncologic end game. J Clin Oncol 4: 626–638 (1986).
2. Moscow SA, Cowan KM. Multidrug resistance. J Natl Cancer Inst 80:14 (1988).
3. Hayes JD, Wolf CR. Molecular mechanisms of drug resistance. Biochem J 272:281–295 (1990).
4. Kartner N, Riordan JR, Ling V. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221:1285–1288 (1983).
5. Ueda K, Cardarelli C, Gottesman MM, Pastan I. Expression of a full length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin and vinblastine. Proc Natl Acad Sci USA 84:3004–3008 (1987).
6. Gros P, Neriah YB, Crop JM, Houseman DE. Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 323:728–731 (1986).
7. Riordan JR, Deuchars K, Kartner N, Alon N, Trent J, Ling V. Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature 316:817–819 (1985).
8. Gerlach JH, Endicott JA, Juranka PF, Henderson G, Sarangi F, Deuchars KL, Ling V. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature 324:485–489 (1986).
9. Kamimoto Y, Gattmaisan 2, Hsu J, Arias IM. The function of GP170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. J Biol Chem 264:11693–11698 (1989).
10. Horio M, Gottesman MM, Pastan I. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc Natl Acad Sci USA 85:3580–3584 (1988).
11. Karrant N, Shales M, Riordan JR, Ling V. Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and cell surface P-glycoprotein. Cancer Res 43: 4413–4419 (1983).
12. Ambudkar SV, Leloong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I. Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci USA 89:8472–8476 (1992).
13. Ling V, Bradley G, Veinot LM, Hiruki T, George E. Expression of P-glycoprotein isoforms. In: Drug Resistance as a Biochemical Target in Cancer Chemotherapy (Tsuruo T, Ogawa M, eds). San Diego, California:Academic Press, 1992: 118–127.
14. Hsu SI, Lothenstein L, Horowitz SB. Differential overexpression of three MDR gene family members in multidrug resistant J774A.2 mouse cells. J Biol Chem 264:12053–12062 (1989).
15. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi
U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 346:362–365 (1990).

16. Ames F-LG, Lecar H. ATP-dependent bacterial transporters and cystic fibrosis: analogy between channels and transporters. FASEB J 6:2660–2666 (1992).

17. Juranka PF, Zastawny RL, Ling V. P-glycoprotein: Multidrug-resistance and a superfamily of membrane-associated transport proteins. FASEB J 3:2583–2592 (1989).

18. Raymond M, Gros P, Whiteway M, Thomas DY. Functional complementation of a yeast ste6 by a mammalian multidrug resistance mdr gene. Science 256:232–234 (1992).

19. Riordan JR, Rommens JM, Kerem B-S, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou J-L, Drum ML, Iannuzzi MC, Collins FS, Tsui L-C. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066–1073 (1989).

20. Goldstein LJ, Galski H, Fojo AT, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cosman J, Gottesman MM, Pastan I. Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst 81:116–124 (1989).

21. Croop JM, Raymond M, Habes D, Devault A, Arceti RJ, Gros P, Houseman DE. The three mouse multidrug resistance (MDR) genes are expressed in a tissue specific manner in normal mouse tissues. Mol Cell Biol 9:1346–1350 (1989).

22. Thiebaut F, Tsuru T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular location of the multidrug resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 84:7735–7738 (1987).

23. Cardon-Cardo C, O’Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR, Bertino JR. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proc Natl Acad Sci USA 86:695–698 (1989).

24. Buschman E, Arceti RJ, Croop JM, Che M, Arias IM, Houseman DE, Gros P. mdr2 Encodes a P-glycoprotein expressed in the bile cannula membrane as determined by isofrom specific antibodies. J Biol Chem 267:18093–18099 (1992).

25. Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 84:265–269 (1987).

26. Park J-G, Kramer BS, Lai S-L, Goldstein L, Gazdar AF. Chemosensitivity patterns and expression of human multidrug resistance-associated MDR1 gene by human gastric and colorectal cell lines. J Natl Cancer Inst 82:193–198 (1990).

27. Goldstein LJ, Gottesman MM, Pastan I. Expression of the MDR1 gene in human cancers. In: Molecular and Clinical Advances in Anticancer Drug Resistance (Ozols RF, ed). Norwell, Massachusetts:Kluwer, 1991;101–120.

28. Marie J-P, Zittoun R, Sicik BI. Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and in vitro drug sensitivity. Blood 78:586–592 (1991).

29. Olton WS, Grogan TM, Miller TP. The role of P-glycoprotein in drug-resistant hematologic malignancies. In: Molecular and Clinical Advances in Anticancer Drug Resistance (Ozols RF, ed). Massachusetts:Kluwer, 1991;187–208.

30. Micksch GH, Merlino GT, Galski H, Gottesman MM, Pastan I. Transgenic mice that express the human multidrug-resistance gene in bone marrow enable a rapid identification of agents that reverse drug resistance. Proc Natl Acad Sci USA 88:547–551 (1991).

31. Thorgeirsson SS, Huber BE, Sorrell S, Fojo AT, Pastan I, Gottesman MM. Expression of the multidrug-resistance gene in hepatocarcinogenesis and regenerating rat liver. Science 236:1120–1122 (1987).

32. Marino PA, Gottesman MM, Pastan I. Regulation of the multidrug resistance gene in regenerating rat liver. Cell Growth Differ 1:57–62 (1989).

33. Fairchild CR, Ivy SP, Rushmore T, Lee G, Koo P, Goldsmith ME, Myers C, Farber E, Cowan KH. Carcinogen-induced mdr overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinomas. Proc Natl Acad Sci USA 87:7701–7705 (1987).

34. Roberts E, Ahulwalia MB, Lee G, Chan C, Sarma DSR, Farber E. Resistance to hepatotoxins by hepatocytes during liver regeneration. Cancer Res 43:28–34 (1983).

35. Carr BI. Pleiotropic drug resistance in hepatocytes induced by administration of carcinogens to rats. Cancer Res 47:5577–5583 (1987).

36. Burt RK, Thorgeirsson SS. Coinduction of MDR-1 multidrug resistance and cytochrome P-450 genes in rat liver by xenobiotics. J Natl Cancer Inst 80:1381–1386 (1988).

37. Gant TW, Silverman JA, Bisgaard HC, Burt RK, Marino PA, Thorgeirsson SS. Regulation of 2-acetylaminofluorene-and 3-methylcholanthrene-mediated induction of multidrug resistance and cytochrome P450A1 gene family expression in primary hepatocyte cultures and rat liver. Mol Carcinog 9:499–509 (1991).

38. Poland A, Knutson JC. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol 22:517–554 (1982).

39. Green S. Receptor-mediated mechanisms of peroxisome proliferators. Biochem Pharmacol 43:393–401 (1992).

40. Nguyen T, Pickett CB. Regulation of rat glutathione-S-transferase Ya subunit gene expression. DNA protein interaction at the antioxidant responsive element. J Biol Chem 267:13535–13539 (1992).

41. Friling RS, Bensimon A, Tichauer Y, Daniel V. Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit is controlled by an electrophile-responsive element. Proc Natl Acad Sci USA 87:6258–6262 (1990).

42. Chin K-V, Tanaka S, Darlington G, Pastan I, Gottesman MM. Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. J Biol Chem 265:221–226 (1990).

43. Schur HA, Castonguay A. Metabolism of carcinogenic amino derivatives in various species and DNA alkylation by their metabolites. Drug Metab Rev 15:753–839 (1984).

44. Weisburger JH, Weisburger EK. Biochemical formation pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66 (1973).

45. Silverman JA, Raunio H, Gant TW, Thorgeirsson SS. Cloning and characterization of the rat multidrug resistance (mdr) gene family. Gene 106:229–236 (1991).

46. Yamada H, Lee M-S, Wang CY. N- and O-Deacetylation of N-acetoxy-N-arylamines by mammalian hepatic microsomes. Carcinogenesis 9:1995–2002 (1988).