Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 186-191

GENERATION OF RESISTANCE TO THE DIPHENYL ETHER HERBICIDE ACIFLUORFEN BY MEL CELLS*

A.R.K. Prasad and Harry A. Dailey+

Department of Microbiology
University of Georgia
Athens, GA 30602-2605

Received July 28, 1995

The diphenyl ether herbicide acifluorfen has been shown to act by inhibition of the terminal enzyme of the protoporphyrin biosynthetic pathway, protoporphyrinogen oxidase (E.C. 1.3.3.4) (PPO), in plant and animal cells. In the present study we show that long term maintenance of murine erythroleukemia (MEL) cells in acifluorfen, which is normally toxic to these cells at 5 μM concentration, results in cells that grow at a near normal rate in 100 μM acifluorfen. Acifluorfen resistant cells do not have increased levels of PPO activity, nor does the PPO made by these cells have increased resistance to acifluorfenin, but these cells accumulate porphyrin and have elevated levels of heme. Data is presented that suggests the resistance of these MEL cells to acifluorfen may be attributable to induction of a cytochrome P450(s).

A variety of diphenyl ether compounds are commonly used as herbicides to control many types of weeds (1-7). The mechanism by which these compounds cause plant death is via light induced peroxide formation and subsequent destruction of cellular substituents, such as lipid membranes. The biochemical basis for this photodestruction has been shown to be the intracellular inhibition of the enzyme protoporphyrinogen oxidase (PPO), the penultimate enzyme of the heme biosynthetic pathway and the last biochemically common step in heme and chlorophyll biosynthesis (8-12). It has been proposed that inhibition of PPO leads to accumulation of the colorless substrate of the enzyme, protoporphyrinogen IX, which apparently diffuses from the chloroplast into the cytosolic compartment where it is oxidized nonspecifically by plant plasma membrane peroxidases (11, 13). The resulting protoporphyrin IX is a highly photosensitive compound which can mediate the formation of reactive oxygen species that, in turn, mediate destruction of cellular components.

*This work supported by grants DK35898 and DK32303 from NIH to H.A. Dailey.

+Corresponding author e-mail:DAILEY@BSCR.UGA.EDU; Fax:706-542-2674.

0006-291X/95 $12.00

As part of our studies on mammalian PPO and regulation of heme synthesis in erythroid cells, we examined the effect of long term culturing of MEL cells in the presence of sublethal concentrations of the diphenyl ether herbicide, acifluorfen. At the start of this project PPO had not been cloned from any source, and so one hope was that decreased sensitivity to acifluorfen might come about in long term culture by an elevation in synthesized PPO. Alternatively, generation of a spontaneous mutation in PPO to acifluorfen resistance would also have been of practical value. The data presented below, however, show that while acifluorfen resistant cell lines were isolated, changes in neither the amount nor biochemical character of PPO were responsible for resistance. The data suggest that, instead, the cells may synthesize a cytochrome P450 for detoxification of acifluorfen.

Materials and Methods

Acifluorfen (97% pure) was purchased from ChemServices, Inc. (PA). Protoporphyrin IX was from Porphyrin Products (Logan, UT), Modified Eagle's medium (αMEM) was from Gibco BRL (Gaithersburg, MD). All other reagents were of the highest quality commercially available.

Murine erythroleukemia (MEL) cells, strain 270, were continuously cultured in the dark at 37°C in a 5% CO_2 atmosphere using αMEM + 7% fetal bovine serum as previously described (14). Viable cell counts were by Trypan Blue exclusion. For cells treated with acifluorfen the desired final concentration of the compound was obtained by addition of the appropriate amount of freshly prepared 100 mM stock solution of acifluorfen in dimethylsulfoxide (DMSO). In all instances the final concentration of DMSO was less than 0.2%, a concentration well below that required to induce differentiation of MEL cells.

Cellular heme was assayed fluorometrically following oxalic acid treatment as previously described (15). Endogenous porphyrins were determined fluorometrically in 1% sodium dodecyl sulfate (SDS) treated cells. Protoporphyrinogen oxidase (PPO) was assayed fluorometrically (16) as modified by Dailey and Karr (17).

For preparation of cellular extracts, cells were harvested by centrifugation (1,300 x g for 5 min) and washed once in Hanks balanced salts. The cell pellet was then suspended to 10^8 cells/ml in lysis buffer (20 mM Tris Cl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1.0% Triton X-100, 0.05% sodium cholate, 0.5 mM dithiothreitol and 1 μg/ml PMSF). Isolated microsomes were prepared by a modification of Omura and Sato (18). For determination of cytochrome levels in the microsomal fraction, 0.1% sodium deoxycholate was added to decrease sample turbidity. Because this detergent shifts the CO spectra of cytochrome P450 to 420 nm (18), P420 levels are reported below.

Results and Discussion

Previously it has been shown that the diphenyl ether herbicide acifluorfen both inhibits PPO activity and causes an intracellular accumulation of protoporphyrin IX in mammalian cells (19). In the current study we have examined the effect of chronic exposure of cultured MEL cells to acifluorfen. We found that cultured MEL cells which have not previously been exposed to acifluorfen experience 35 to 55% mortality when exposed overnight to 5 to 10 μM acifluorfen, respectively. Surviving cells, however, will continue to grow in the presence of the herbicide and

by gradually increasing acifluorfen concentrations over a period of several transfers, it was possible to adapt cells to grow at near normal rates in concentrations of acifluorfen as high as 100 μM (Table I). It should be noted that cells were maintained in the dark at all times so that light induced cellular destruction did not occur. The data show that adaptation to growth in acifluorfen was a slow process at all concentrations and was definitely not an all-or-none process as one might expect if resistance occurred due to a spontaneous mutation of PPO. This hypothesis is further supported by the data in Table II which clearly show that at all acifluorfen concentrations and passage numbers there is no evidence for the presence of an acifluorfen resistant form of PPO.

Although the data presented in Tables II and III demonstrate an increase in PPO activity in cells maintained for long periods in acifluorfen, it would not be expected that this level of increase could account for the high level of resistance to acifluorfen that is found in these cells. Furthermore, the increases in PPO activity do not correlate well with the increases in cellular resistance to acifluorfen in culture.

Cells isolated from cultures maintained in the presence of acifluorfen are pink to brick red in color whereas control cells are a pale tan color. Exposure of these cell pellets to U.V. light elicits a strong red fluorescence in acifluorfen treated cells but not in control cells. Table IV presents data demonstrating that acifluorfen treated cells have increases in both free porphyrins and heme in comparison to control cells. While the increase in free porphyrin would be expected from the inhibition of PPO by acifluorfen, the significant increase in cellular heme levels demonstrates that cellular heme biosynthesis is active and on-going even with 125 μM

Table I
MEL Cell Growth as a Function of Acifluorfen Concentration and Cell Passage Number[1]

Acifluorfen Concentration in Culture (μM)	Relative Growth Rate[2]	
	43 Passages	150 Passages
0	1.0	1.0
50	0.84	0.96
75	0.77	-
100	0.64	0.88
125	0.33	0.45
150	0.20	0.34

[1]Experimental details are in the text.
[2]Relative growth rate expresses the increase in cell number in five days of cultures exposed to acifluorfen relative to the increase in cell number of cells that were not exposed to acifluorfen.

Table II
Inhibition of Protoporphyrinogen Oxidase Activity
by Acifluorfen in Acifluorfen Resistant Cell Lines

Concentration of Acifluorfen in Cell Culture (μM)	Concentration of Acifluorfen in PPO Assay (μM)	Relative PPO Activity[1]		
		Passage Number		
		53	104	150
control	0	18.0	16.0	22.0
control	2	0	0	0
50	0	20.5	17.3	30.2
50	2	0	1.0	2.0
100	0	18.0	16.8	33.1
100	2	0	0	3.0
125	0	10.5	14.0	24.2
125	2	0	2.0	1.2

[1]Cells that had been maintained in the listed concentration of acifluorfen were harvested and washed to eliminate any contaminating extracellular acifluorfen. Sonicated cell extracts were assayed in the presence of 0 or 2 μM acifluorfen. Activity is expressed as relative fluorescence units per 10^7 cell·30 min.

acifluorfen present. What, then, accounts for this increase in cellular heme content? Since it has been shown that in chick embryo hepatocyte cultures exposure to acifluorfen results in induction of cytochrome P450 isozymes (19), an examination of cytochrome P450 levels in high passage

Table III
Effect of Long-term Exposure of Acifluorfen on
Protoporphyrinogen Oxidase Activity in MEL Cells[1]

Concentration of Acifluorfen in Culture (μM)	% Protoporphyrinogen Oxidase Activity				
	Passage Numbers				
	15	30	53	104	150
0	100	100	100	100	100
25	80	94	108	-	-
50	63	67	124	112	108
75	41	44	138	135	-
100	30	35	188	169	128
125	16	13	80	85	51

[1]Experimental details are in the text.

Table IV
Estimation of Heme and Endogenous Porphyrin Levels of Control and Acifluorfen Treated MEL Cells [1]

Concentration of Culture Acifluorfen (μM)	RFU	
	Heme	Endogenous Porphyrins
0	1.3	0.2
50	9.8	6.3
100	44.6	23.2
125	61.5	31.3

[1] $1x10^7$ cells from passage 150 were harvested, washed once with HBSS and after lysis were centrifuged at 1300 x g to remove the cell debris. The supernatant was further centrifuged at 80,000 x g for 1 hr and 1 ml of the supernatant was used for estimation of heme and endogenous porphyrin levels as described in Materials and Methods.

acifluorfen maintained MEL cells was made. The data (Table V) show a marked increase in cytochrome P450 levels in acifluorfen resistant cells. While products resulting from a cytochrome P450 mediated alteration of acifluorfen were not identified in this study, the data presented above is consistent with the induction of a cytochrome P450(s) that is capable of metabolizing acifluorfen to a compound that does not inhibit PPO.

In summary, we have presented data showing that MEL cells in culture can be adapted to growth in high concentrations of the herbicide acifluorfen, but that this adaptation is a relatively slow process. The metabolic basis for this adaptation is not attributable to either the development of an acifluorfen resistant PPO enzyme or an increased synthesis of an unmodified PPO enzyme. The data suggest that cellular resistance is mediated via induction of a cytochrome P450 which is capable of metabolizing acifluorfen to a non-inhibitory compound. Confirmation of this suggestion will require a detailed study of the products of acifluorfen metabolism and the characterization of cellular cytochromes P450 from the resistant cell lines.

Table V
Cytochrome P-420 Levels in Long Term Acifluorfen Exposed MEL Cells[1]

Acifluorfen Concentration in Culture (μM)	Cytochrome P420 (pmoles/mg protein)
0	39
50	60
100	498
125	395

[1] Microsomes from 150 passage number cells were separated according to the method of Omura and Sato (18). Sodium deoxycholate was added to decrease sample turbidity cholate.

References

1. Kunert, K. -J., and Böger, P. (1981) Weed Sci. *29*, 169-173.
2. Kunert, K. -J., Sandmann, G., and Böger, P. (1987) Rev. Weed Sci. *3*, 35-55.
3. Nicolaus, B., Sandmann, G., Watanabe, H., Wakabayashi, K., and Böger, P. (1989) Pestic. Biochem. Physiol. *35*, 192-201.
4. Lydon, J., and Duke, S. O. (1988) Pestic. Biochem. Physiol. *31*, 74-83.
5. Matringe, M., and Scalla, R. (1988) Plant Physiol. *86*, 619-622.
6. Witkowski, D. A., and Halling, B. P. (1988) Plant Physiol. *87*, 632-637.
7. Duke, S. O., Becerril, J. M., Sherman, T. D., Lydon, J., and Matsumoto, H. (1990) Pestic. Sci. *30*, 367-378.
8. Matringe, M., Camadro, J. M., Labbe, P., and Scalla, R. (1989) Biochem. J. *260*, 231-235.
9. Witkowski, D. A., and Halling, B. P. (1989) Plant Physiol. *90*, 1239-1242.
10. Jacobs, J. M., Jacobs, N. J., Borotz, S. E., and Guerinot, M. L. (1990) Arch. Biochem. Biophys. *280*, 369-375.
11. Jacobs, J. M., Jacobs, N. J., Sherman, T. D., and Duke, S. O. (1991) Plant Physiol. *97*, 197-203.
12. Nandihalli, U. B., and Duke, S. O. (1993) in (Duke, S. O., Menn, J. J., and Plimmer, J. R., eds) Pest Control With Enhanced Environmental Safety. ACS Symp. Ser. no. 524, p. 62-78. Amer. Chem. Soc., Washington.
13. Lee, H. J., Duke, M. V., and Duke, S. O. (1993) Plant Physiol. *102*, 881-889.
14. Lake-Bullock, H., and Dailey, H. A. (1993) Mol. Cell. Biol. *13*, 7122-7132.
15. Sassa, S., Granick, J. L., Eisen, H., and Ostertag, W. (1978) in (Murphy, M.J., ed) In vitro aspects of erythropoiesis, p. 268-270. Springer-Verlag, New York.
16. Brenner, D. A., and Bloomer, J. R. (1980) Clin. Chim. Acta. *100*, 259-266.
17. Dailey, H. A., and Karr, S. W. (1987) Biochemistry *26*, 2697-2701.
18. Omura, T., and Sato, R. (1964) J. Biol. Chem. *239*, 2370-2378.
19. Jacobs, J. M., Sinclair, P. R., Gorman, N., Jacobs, N. J., Sinclair, J. F., Bement, W. J., and Walton, H. (1992) J. Biochem. Toxicology *7*, 87-95.