Vol. 110, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 27, 1983 Pages 525-529

DETERMINATION OF EPOXIDE HYDROLASE ACTIVITY IN WHOLE CELLS (HUMAN LYMPHOCYTES) AND ACTIVATION BY BENZOFLAVONES

H.R. Glatt, T. Wölfel and F. Oesch

Institute of Pharmacology, Obere Zahlbacher Strasse 67,
D-6500 Mainz, Germany

Received October 7, 1982

SUMMARY: Epoxide hydrolase (epoxide hydratase, epoxide hydrase, E.C. 3.3.2.3) activity so far has only been measured in subcellular preparations. We show here that, with the highly lipophilic substrate (^{3}H)-benzo(a)pyrene 4,5-oxide, the activity can be determined in intact cells. Whole human lymphocytes hydrolyze it at a similar rate to that in lymphocyte homogenate.

We have previously reported that cultivation of lymphocytes in a medium containing 5,6-benzoflavone leads to an increase in epoxide hydrolase activity. We now demonstrate that this stimulation is due to enzyme activation and that enzyme induction does not contribute to this increase to any measurable extent. Moreover, both 5,6-benzoflavone and 7,8-benzoflavone activate epoxide hydrolase. This activation occurs not only in cell homogenate, but also - with a similar concentration-response relationship - in whole lymphocytes. Hence measurement of epoxide hydrolase activity in subcellular preparations reflects the activity in these intact cells. Furthermore, insofar as a concentration of 1 μM of the benzoflavones is sufficient to cause a measurable (10 to 20 %) activation, it appears likely that foreign compounds can activate epoxide hydrolase in man.

INTRODUCTION: Various aromatic and olefinic compounds are metabolized to electrophilically reactive epoxides, which can covalently bind to cellular components and, thereby, elicit carcinogenic, mutagenic and other cytotoxic effects (1,2). Epoxide hydrolases (2-5) inactivate many epoxides and are involved in the formation of highly reactive vicinal dihydrodiol-epoxides (6-7) from polycyclic aromatic hydrocarbons. They probably are a decisive factor in determining the susceptibility of an organism towards such compounds. So far epoxide hydrolase activities have only been determined in subcellular preparations. It is unclear whether the cellular epoxide hydrolase activity is comparable to that observed in subcellular preparations; whether it is affected similarly by added modulators or if endogenous activators and inhibitors or other intracellular factors influence its activity.

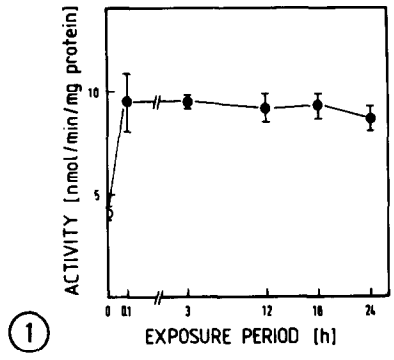
0006-291X/83/020525-05$01.50/0

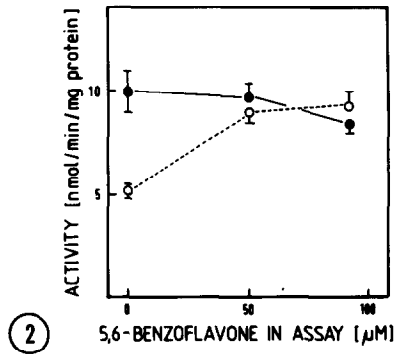
In previous studies we have reported that 5,6-benzoflavone and 7,8-benzoflavone stimulate epoxide hydrolase activity in homogenates of both human lymphocytes and rat hepatoma cells (8,9). These benzoflavones are of particular interest, because they inhibit or potentiate the carcinogenicity and genotoxicity of various compounds (10-13), effects which were attributed to the inhibition or induction of some cytochrome P-450 dependent monooxygenases. We have now investigated the stimulation of epoxide hydrolase in more detail, particulary whether or not it also occurs in whole cells.

MATERIALS AND METHODS: Lymphocytes were isolated and, where indicated, cultured as described (8) with the exception that Hanks' balanced salt solution in the culture medium was replaced by Earle's balanced salt solution. Epoxide hydrolase activity was determined by incubation of cells or cell homogenate with (^{3}H)-benzo(a)pyrene 4,5-oxide and differential extraction of the product followed by quantitation by scintillation counting, as detailed elsewhere (8). Homogenates were prepared by ultrasonication of cell suspensions in a solution which contained 150 mM KCl and 10 mM sodium phosphate buffer pH 7.4. The only modification for determination of the enzyme activity in whole cells was that Hanks' balanced salt solution was used as a resuspension and assay medium. The viability of the used cells was always greater than 95 % as judged by the exclusion of trypan blue. All enzyme activity determinations were performed under conditions where product formation was linear with respect to incubation time and protein concentrations.

RESULTS: Addition of 5,6-benzoflavone to the medium of human lymphocytes for the last 24 hours of the culture increases the epoxide hydrolase activity, determined in the cell homogenate, by about 2.2-fold (8). Fig. 1 shows that the same effect is observed, when 5,6-benzoflavone is present in the culture for much shorter periods. Even addition immediately (5 min) before harvesting leads to maximal increase in activity. Moreover, if 5,6-benzoflavone is only added to the homogenate in the enzyme assay as opposed to the culture medium, a similar increase in enzyme activity occurs. If 5,6-benzoflavone is used in both culture and enzyme assay at optimal concentration, the effect is the same as when either treatment is performed alone (Fig. 2).

When the same assay for determination of epoxide hydrolase activity, as normally used with lymphocyte homogenate, is applied to whole cells with the sole modification that a physiological buffer (Hanks' balanced salt solution) is used, reproducible results are obtained. Product formation is linear with incubation time for at least 1 hour and with the amount of cells (up to at





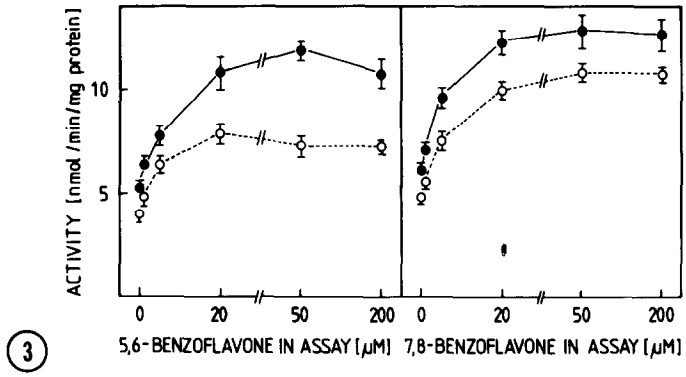


Fig. 1 Epoxide hydrolase activity in homogenized human lymphocytes after various periods of culturing in the presence of 5,6-benzoflavone. 5,6-Benzoflavone was used at a concentration of 27 μM and added in acetone (final concentration 1 °/₀₀). The control (open symbol) received only acetone (0.1 h before the end of the culture). The cells of all treatment groups were seeded and harvested at the same time. Different duration of treatment was obtained by addition of 5,6-benzoflavone at different time points. Values are means with standard deviations for 4 determinations.

Fig. 2 Effect of 5,6-benzoflavone in the assay for determination of epoxide hydrolase in human lymphocytes which have been cultured in the presence (●) or absence (o) of 5,6-benzoflavone. The total culture time was 72 h. 5,6-Benzoflavone (27 μM) or the solvent only (acetone, final concentration 1 °/₀₀) was present for the last 24 h of the culture. Epoxide hydrolase activity was determined in homogenized cells. The substrate, benzo(a)pyrene 4,5-oxide, and 5,6-benzoflavone were added in acetonitrile (final concentration in all incubations 7 %). Values are means with standard deviations for 4 incubations.

Fig. 3 Effect of 5,6-benzoflavone and 7,8-benzoflavone on the epoxide hydrolase activity in whole (●) and homogenized (o) native human lymphocytes. The substrate, benzo(a)pyrene 4,5-oxide, and the benzoflavones were added to the assay in acetonitrile (final concentration in all incubations 7 %). Values are means with standard deviations for 4 incubations.

least 2 mg protein per incubation) (data not shown). The specific activity is slightly higher than if determined in cell homogenate and is stimulated by the addition of 5,6-benzoflavone (Fig. 3, left panel). Compared to cell homogenate, the stimulation may be slightly stronger, but otherwise occurs with a very

similar concentration-response relationship. 7,8-Benzoflavone (Fig. 3, right panel) produces practically the same effects as 5,6-benzoflavone. At a concentration of 1 μM, both isomers cause a 10 to 20 % increase in activity in whole as well as in homogenized cells.

DISCUSSION: 5,6-Benzoflavone and 7,8-benzoflavone activate epoxide hydrolase. A previously reported (8) increase in epoxide hydrolase activity in cultured lymphocytes upon addition of 7,8-benzoflavone to the culture medium can be fully explained by this activation inasmuch as treatment of the culture has no additional effect, when epoxide hydrolase is measured in the presence of maximally activating concentrations of 5,6-benzoflavone.

The observations that 7,8-benzoflavone and 5,6-benzoflavone activate epoxide hydrolase and produce effects at very low (μmolar) concentrations contrast with reports by Alworth et al. (16) who found activation by 7,8-benzoflavone, but not by 5,6-benzoflavone and worked at concentrations of 0.5 and 1 mM. However, these authors used a different substrate (styrene 7,8-oxide) and epoxide hydrolase of a different source (rat liver microsomes) and added a detergent (1 % Tween 80) to the assay. Since epoxide hydrolase is also activated in homogenates of a rat hepatoma cell line (9), species differences are not likely to be the cause of the discrepancy in the results.

Epoxide hydrolase activity was reproducibly measurable in whole cells using a minor modification of the normal assay. This contrasts with studies using Reuber hepatoma cells, where strongly variable results were obtained due to problems at the level of the separation of product and substrate (9). Possibly lymphocytes retain less epoxide substrate during separation than hepatoma cells. However, it should also be noted that in lymphocytes the concentration of substrate was reduced to 10 μM for assaying epoxide hydrolase activity (8), whereas in other cases (17), including in hepatoma cells (9), concentrations of 50 to 150 μM were used. The reason for the use of a low, but still saturating concentration of substrate with lymphocytes was their extremely low specific activity which required the reduction of the blank value to a minimum (8). Possibly this reduction also results in a lower epoxide contamination in the diol extract.

Close similarities between intact and broken cells with regard to epoxide hydrolase activity and its modulation by 5,6- and 7,8-benzoflavone indicate that results obtained in studies on subcellular preparations can provide valid information on the enzyme in its physiological environment. This is of particular importance for the investigated examples with 5,6- and 7,8-benzoflavone, because (i) these compounds activate epoxide hydrolase at very low concentrations, (ii) are active with human epoxide hydrolase and (iii) are frequently used in carcinogenicity and mutagenicity studies as inhibitors or inducers of cytochrome P-450 dependent monooxygenases (11-16). It seems likely that their effects are mediated by epoxide hydrolase as well as via modulation of monooxygenases.

ACKNOWLEDGMENT: We thank C.H. Walker, University of Reading, for critically reading the manuscript. This work was supported by the Bundesministerium für Forschung und Technologie.

REFERENCES

1. Daly, J.W., Jerina, D.M. and Witkop, B. (1972) Experientia 28, 1129-1149
2. Oesch, F. (1973) Xenobiotica 3, 305-340
3. Oesch, F. in J.W. Bridges and L.F. Chasseaud (eds.), Progress in Drug Metabolism, John Wiley, Chichester, England (1979) pp 513-531
4. Lu, A.Y.H. and Miwa, G.T. (1980) Ann. Rev. Pharmacol. Toxicol. 20, 513-531
5. Hammock, B.D., Gill, S.S., Mumby, S.M. and Ota, K. in R.S. Bhatnagar (ed.) Molecular Basis of Environmental Toxicity, Ann Arbor Science, Ann Arbor, Mich., in the press, pp 229-272
6. Sims, P., Grover, P.L., Swaisland, A., Pal, K. and Hewer, A. (1974) Nature 252, 326-328
7. Jerina, D.M., Lehr, R., Schaefer-Ridder, M., Yagi, H., Karle, J.M., Thakker, D.R., Wood, A.W., Lu, A.Y.H., Ryan, D., West, S., Levin, W. and Conney, A.H. in H.H. Hiatt, J.D. Watson and J.A. Winsten (eds.) Origins of Human Cancer, Cold Spring Harbor Laboratory, New York (1977) pp 639-658
8. Glatt, H.R., Kaltenbach, E. and Oesch, F. (1980) Cancer Res. 40, 2552-2556
9. Raphael, D., Glatt, H.R., Protić-Sabljić, M. and Oesch, F. (1982) Chem.-Biol. Interact., in the press.
10. Wattenberg, L.W. and Leong, J.L. (1968) Proc. Soc. exp. Biol. Med. 128, 940-943
11. Gelboin, H.V., Wiebel, F.J. and Diamond, L. (1970) Science 170, 169-171
12. Bowden, G.T., Slaga, T.J., Shapas, B.G. and Boutwell, R.K. (1974) Cancer Res. 34, 2634-2642
13. Wattenberg, L.W., Loub, W.D., Lam, L.K. and Speier, J.L. (1976) Fed. Proc. 35, 1327-1331
14. Slaga, T.J., Berry, D.L., Juchau, M.R., Thompson, S., Buty, S.G. and Viaje, A. in R.I. Freudenthal and P.W. Jones (eds.), Carcinogenesis Vol. 1, Raven Press, New York (1976) pp 127-138
15. Schürer, C.C., Bartram, C.M., Glatt, H.R., Kohl, F.V., Mangels, W., Oesch, F. and Rüdiger, H.W. (1980) Biochim. Biophys. Acta 609, 272-277
16. Alworth, W.L., Dang, C.C., Ching, L.M. and Viswanathan, T. (1980) Xenobiotica 10, 395-409
17. Schmassmann, H.U., Glatt, H.R. and Oesch, F. (1976) Anal. Biochem. 74, 94-104