A NEW TYPE OF BIOLOGICAL CHEMILUMINESCENCE: THE MICROSOMAL CHEMILUMINESCENCE OF BENZO[a]PYRENE ARISES FROM THE DIOL EPOXIDE PRODUCT OF THE 7,8-DIHYDRODIOL

J. P. Hamman, D. R. Gorby and H. H. Seliger

McCollum-Pratt Institute and Department of Biology
The Johns Hopkins University, Baltimore, Maryland 21218

Received March 3,1977

Summary: The chemiluminescence, CL, accompanying the metabolism of the carcinogen benzo[a]pyrene, BP, by the aryl hydrocarbon hydroxylase system is a new type of low intensity biological chemiluminescence. It is the result of spontaneous oxygenation of a specific reactive metabolic intermediate; not inhibitable by superoxide dismutase or catalase. The reactive metabolite is the strongly mutagenic 7,8-dihydrodiol-9,10-epoxide, produced enzymatically from the 7,8-dihydrodiol precursor. Hydroxylation of benzo[a]pyrene at the 3 position does not lead to chemiluminescent emission; the CL quantum yields of BP and 3-OH-BP are the same. The CL quantum yields of microsomal metabolism of (-) 7,8-diol-BP and the racemic 7,8-diol-BP are identical. The kinetics of CL of the latter show a much faster initial reaction rate, correlating with the greater reactivity of diol epoxide I formed from (+) 7,8-diol-BP. CL may therefore be used to follow the pathways and the rates of production of the mutagenic diol epoxides of BP.

The adventitious biological chemiluminescence, ABC, observed during aerobic metabolism *in vivo* [1,2] and *in vitro* [3,4,5] can be ascribed to a variety of non-specific radical reactions initiated by $O_2^{\cdot}$ and the decomposition of H_2O_2, released by flavin oxidases and peroxidases. The relation of ABC *in vitro* to metabolism in the natural system is obscured by the structural disruptiveness of enzyme extraction that can lose soluble cofactors; the decoupling of the components of oxygenase systems can result in artifactual release of $O_2^{\cdot}$ and H_2O_2. In the flavin oxidase reaction of bacterial bioluminescence the removal of the aldehyde substrate results in the stoichiometric release of H_2O_2 [6]. This may explain the very high CL

Supported by the U. S. Energy Research and Development Administration under contract no. E4-76-S-02-3277. Contribution no. from the McCollum-Pratt Institute and Department of Biology of The Johns Hopkins University.

Abbreviations: ABC, adventitious biological chemiluminescence; BP, benzo-[a]pyrene; CL, chemiluminescence; diol epoxide I, r-7,t-8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; diol epoxide II, r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; SOD, superoxide dismutase; DMSO, dimethylsulfoxide.

ISSN 0006-291X

of Luminol when this chemiluminescent probe for the radicals $O_2^{\cdot-}$ and $OH^{\cdot}$ is added to the flavoprotein xanthine oxidase [7,8,9,10], to rat liver microsomal extracts in the presence of NADPH [11], and to horseradish peroxidase [11]. $O_2^{\cdot-}$ and H_2O_2 appear to be responsible for the ABC observed for xanthine oxidase [12,13,14,15] and aldehyde oxidase [14] in the absence of Luminol. All of the above referenced ABC are inhibited by superoxide dismutase, SOD, and by catalase. This inhibition by SOD can be used as an operational criterion for non-specific ABC. We have previously proposed that a new type of ABC, the oxygenation of a specific reactive metabolite, should accompany the metabolic hydroxylation of the carcinogenic hydrocarbon benzo[a]pyrene, BP in rat liver microsomes [16,17, 18]. This is different from non-specific microsomal ABC due to the release of $O_2^{\cdot-}$ and H_2O_2.

We now report the experimental evidence for specific ABC. The major chemiluminescence accompanying the microsomal metabolism of BP results from the spontaneous oxygenation of the product of the enzymatic epoxidation of 7,8-dihydrodiol-BP. This ABC is substrate specific, is not inhibited by SOD or by catalase, requires molecular oxygen, and is the result of a metabolic pathway different than that producing the 3-OH-BP metabolite.

MATERIALS AND METHODS

Liver microsomes were isolated from male Long-Evans rats (Charles River Animal Farms) induced with 3-methylcholanthrene [19]. Protein was determined by a modified Lowry procedure [20]. Aryl hydrocarbon hydroxylase activity to produce phenols was determined by a fluorimetric technique [21]. Chemiluminescence intensities and quantum yields were measured as described previously [18]. (-) 7,8-Dihydrodiol-BP was isolated subsequent to microsomal metabolism of BP, by high pressure liquid chromatography [22]. 3-Hydroxy-BP and racemic 7,8-dihydrodiol-BP were obtained from the National Cancer Institute Chemical Repository. The microsomal CL reaction mix contained in 3-ml total volume, 50 mM Tris-HCl, pH 7.6; 3 mM $MgCl_2$, 3 mg microsomal protein plus substrate in 150 µl of methanol. The reaction was initiated by adding NADPH (0.5 mM).

RESULTS AND DISCUSSION

Neither the CL intensity nor the quantum yield of CL of BP in microsome extracts is inhibited by SOD at concentrations up to 60 µg/ml; at this concentration the rate of reduction of cytochrome c by the xanthine oxidase

reaction was inhibited 78%. Catalase (130 units/ml) normally did not inhibit the CL intensity. However in some preparations inhibitions of CL intensities up to 25% were observed. Higher CL intensities were observed upon addition of H_2O_2. Inhibition by catalase from none to 25% is therefore of the H_2O_2 contribution to the CL of the epoxide. H_2O_2 is not an absolute requirement for the microsomal ABC of BP. Synthetic racemic 7,8-dihydrodiol-BP was added to a microsomal reaction mix. The reaction was terminated (by adding 3 ml of acetone at 0°C) after 45 seconds, the approximate time at which a maximum of chemiluminescence was observed. The reactants were extracted into 6 ml of hexane; the hexane extract was evaporated to dryness; the residue was redissolved in 250 µl of DMSO; 50 µl of the DMSO solution were added to 3 ml of 0.05 M Tris-HCl, pH 7.6, and immediately placed in the photon counting geometry. This aqueous solution emitted an oxygen-dependent chemiluminescence that decayed with a first-order rate constant of 0.1 sec^{-1}, confirming the presence, and therefore the production of, a chemiluminescent intermediate. For equimolar quantities of BP and 3-OH-BP metabolized by microsomal extracts, the total light emissions were the same; both BP and 3-OH-BP exhibited the same quantum yield for microsomal chemiluminescence . Therefore the production of 3-OH-BP from BP does not result in chemiluminescence; a different reaction is involved. The quantum yield of CL from 7,8-dihydrodiol-BP is 3 times greater than that from BP (Student t test significant at $P < 0.005$). The initial rate of microsomal CL as a function of 7,8-dihydrodiol-BP concentration shows saturation kinetics. The total microsomal CL emitted during the metabolism of 7,8-dihydrodiol-BP is proportional to the amount added up to 10 µM. The total microsomal CL emitted during the microsomal metabolism of the parent BP is proportional to the initial concentration only below 1 µM, implying competition by pathways not leading to the 7,8-dihydrodiol-BP or to CL, consistent with the higher CL quantum yield of the diol. The relative yields of

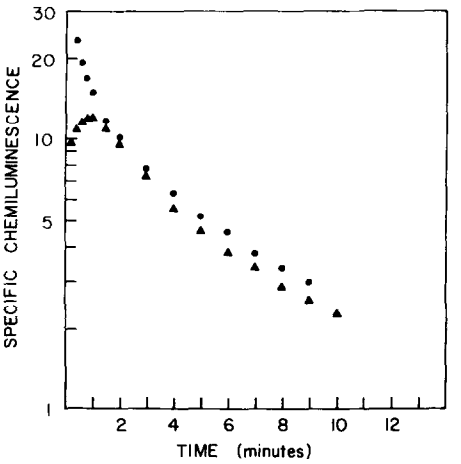

Figure 1. Kinetics of the microsomal chemiluminescence of (-) 7,8-dihydrodiol-BP [▲] and racemic 7,8-dihydrodiol-BP [●]. Reaction conditions were identical to those in Materials and Methods. 3 nmoles of the 7,8-diols were added for each reaction. Injection of NADPH was made with the sample in position in the photon counting geometry. For each individual reaction the chemiluminescent intensity was monitored continuously from t = 0 when the cofactor NADPH was injected. Each point for specific chemiluminescence is the average net chemiluminescence intensity in arbitrary units recorded at that time for three replicate samples divided by the total amount of substrate added to the reaction mix initially.

metabolites formed from BP have been shown to depend on the initial BP concentrations [23] due to the further metabolism of previously formed metabolites. At 2 mM, trichloropropane oxide, an inhibitor of epoxide hydrase [24], reduced the intensities of microsomal CL of both BP and 3-OH-BP by 48-68% while in some cases the CL of the 7,8-diol was slightly enhanced. Inhibition of CL implies that both BP and 3-OH-BP must first be metabolized to diols to produce CL. The ratio of CL to the rate of 3-hydroxy-BP production varies from one preparation to another and is strongly dependent on induction [25].

The time dependences of the microsomal CL from (-) 7,8-dihydrodiol-BP and racemic 7,8-dihydrodiol-BP are quite different and are shown in Fig. 1. Both diols have the same microsomal chemiluminescent quantum yields, but the racemic diol CL shows a much higher initial CL intensity. The slower

rate corresponds to that observed with the (-) diol CL, implying that the more rapid rate seen with the racemic diol CL is due to the (+) diol diasteriomer. The faster rate, resulting in a higher initial CL intensity, indicates a more rapid reaction with molecular oxygen. This is consistent with the much higher chemical reactivity reported for diol epoxide I produced from (+) 7,8-dihydrodiol-BP [26,27] as compared with diol epoxide II produced from the (-) diol. The CL resulting from the oxidation of BP by microsomes is therefore a specific ABC of a mutagenically active diol epoxide metabolite. It appears that, at least for the carcinogen BP, the intensity and the kinetics of microsomal CL may be used to follow the enzyme activities and pathways leading to the production of the highly mutagenic and presumably carcinogenic diol epoxides [28,29,30].

REFERENCES

1. Barenboim, G. M., Domanskii, A. W., and Turoverov, K. K. (1969) Luminescence of Biopolymers and Cells, p. 229, Plenum Press, New York.
2. Quickenden, T. I., and Que Hee, S. S. (1974) Biochem. Biophys. Res. Commun. 60:764-770.
3. Stauff, J., and Ostrowski, J. (1967) Z. Naturforsch. 226:734-740.
4. Howes, R. M., and Steele, R. H. (1971) Res. Commun. Chem. Path. Pharmacol. 2:619-626.
5. Howes, R. M., and Steele, R. H. (1972) Res. Commun. Chem. Path. Pharmacol. 3:349-357.
6. Lee, J. (1972) Biochemistry 11:3350-3359.
7. Totter, J. R., Medina, V. J., and Scoseria, J. L. (1960) J. Biol. Chem. 253:238-241.
8. Totter, J. R., diGros, E. D., and Riveiro, C. (1960) J. Biol. Chem. 253:1839-1842.
9. Greenlee, L., Fridovich, I., and Handler, P. (1962) Biochem. 1:779-783.
10. Fridovich, I., and Handler, P. (1962) J. Biol. Chem. 237:916-921.
11. Nilsson, R. (1969) Biochem. Biophys. Acta 184:237-251.
12. Stauff, J. (1965) Photochem. Photobiol. 4:1199-1205.
13. Stauff, J., and Wolf, H. (1964) Z. Naturforsch. 19B:87-96.
14. Arneson, R. M. (1970) Arch. Biochem. Biophys. 136:352-360.
15. Hodgson, E. K., and Fridovich, I. (1976) Arch. Biochem. Biophys. 172: 202-205.
16. Seliger, H. H. (1975) Photochem. Photobiol. 21:355-361.
17. Hamman, J. P., and Seliger, H. H. (1976) Biochem. Biophys. Res. Commun. 70:675-680.
18. Seliger, H. H., and Hamman, J. P. (1976) J. Phys. Chem. 80:2296-2305.
19. Ernster, L. (1962) J. Cell Biol. 15:541-562.
20. Sutherland, E. W., Cori, C. F., Haynes, R., and Olsen, N. S. (1949) J. Biol. Chem. 180:825-837.
21. Nebert, D. W., and Gelboin, H. V. (1968) J. Biol. Chem. 243:6242-6249.
22. Selkirk, J. K., Croy, R. G., and Gelboin, H. V. (1974) Science 184: 169-171.

23. Holder, G. M., Yagi, H., Jerina, D. M., Levin, W., Lu, A. Y. H., and Conney, A. H. (1975) Arch. Biochem. Biophys. 170:551-566.
24. Oesch, F., Kaubisch, W., Jerina, D. M., and Daly, T. W. (1971) Biochem. 10:4858-4866.
25. Hamman, J. P., and Seliger, H. H., manuscript in preparation.
26. Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., and Jerina, D. M. (1976) Proc. Natl. Acad. Sci. USA 73:3381-3385.
27. Wood, A. W., Wislocki, P. G., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Jerina, D. M., and Conney, A. H. (1976) Cancer Res. 36:3358-3366.
28. Newbold, R. F., and Brookes, P. (1976) Nature 261:52-54.
29. Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H. Hernandes, O., Jerina, D. M., Conney, A. H. (1976) Biochem. Biophys. Res. Commun. 68:1006-1012.
30. Huberman, E., Sachs, L., Yang, S. K., and Gelboin, H. V. (1976) Proc. Natl. Acad. Sci. USA 73:607-611.