POSSIBLE CONTROL OF HYDROGEN PEROXIDE PRODUCTION AND DEGRADATION IN MICROSOMES DURING MIXED FUNCTION OXIDATION REACTION *

Alfred G. Hildebrandt, Manfred Speck, and Ivar Roots
Institut für Klinische Pharmakologie der Freien Universität Berlin,
1 Berlin 45, Hindenburgdamm 30 (Klinikum Steglitz), Germany

Received August 14,1973

SUMMARY

The fate of hydrogen peroxide has been investigated in rat liver microsomes. The net rate of formation of H_2O_2 appears to be independent of concomitant substrate hydroxylation in microsomes from controls and phenobarbital treated animals. If rats are pretreated with Pregnenolone-16α-carbonitrile, H_2O_2 formation increases significantly during N-demethylation of aminopyrine. However, H_2O_2 is consumed in microsomes from 3-Methylcholanthrene treated rats if aminopyrine and NADPH are present. Since the H_2O_2 formation and consumption are dependent on induction by different agents and on presence of substrates, its fate might be linked to the spin state of cytochrome P-450.

H_2O_2 formation has been shown to occur in microsomes, both by an indirect method (1) and directly (2,3). The question has been raised (1,2,3) whether the H_2O_2 production in microsomes is merely the result of NADPH oxidation, or also partly due to cytochrome P-450 autoxidation.

The present paper reports both the formation and the degradation of hydrogen peroxide in microsomes during mixed function oxidation reactions. The appearance of H_2O_2 as an intermediate during hydroxylation reactions, as well as its consumption in the presence of substrate and NADPH seems to be related both to rat pretreatment with different inducing agents and to the type of substrate present. Since induction and substrate addition are known to modify the spin state of cytochrome P-450 (4,5), the hypothesis is proposed that the balance of high and low spin cytochrome determines the fate of H_2O_2 in hydroxylation reactions.

METHODS

As described elsewhere (6,7), microsomes were isolated by differential centrifugation from livers of male rats (150-180 g), both controls and rats subjected to different treatments. Phenobarbital (PB): 80 mg per kg i.p. 72, 60, 48, and 24 hours prior to decapitation. Pregnenolone-16α-carbonitrile (PCN) (3-β-hydroxy-

* Supported in part by Deutsche Forschungsgemeinschaft

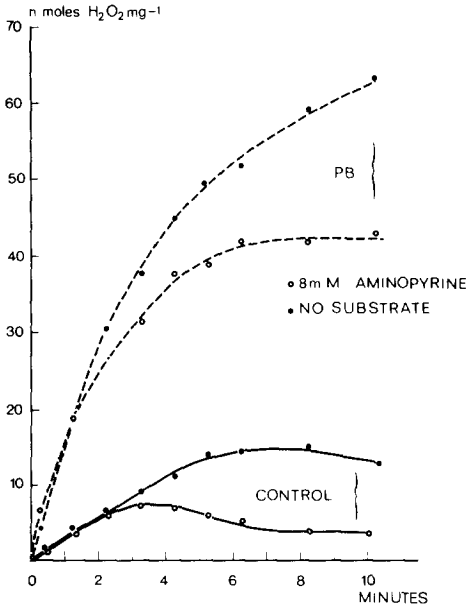

Fig. 1 Determination of H_2O_2 in microsomes from PB treated rats and from controls.
Liver microsomes were diluted to a protein concentration of 1.5 mg per ml in a buffer mixture containing 0.2 mM sodium azide, 50 mM Tris-chloride buffer pH 7.4, with 150 mM KCl, 10 mM $MgCl_2$, 6.4 mM isocitrate, 5 µl of isocitrate dehydrogenase (Boehringer, Mannheim, 50µ = 0.1 unit). When included in the buffer mixture aminopyrine was 8 mM. The reaction was initiated by addition of 300 µM NADPH and incubations were carried out at 30°C with constant shaking in a waterbath. Aliquots (1.5 ml) were transferred into test tubes containing 1.5 ml trichloroacetic acid (5 % w/v) and centrifuged. 0.2 ml 10 mM ferroammoniumsulfate and 0.1 ml 2.5 M potassium thiocyanate were subsequently added to 2.0 ml supernatant. (T = 4°C). The extinction of the developed Fe $(SCN)_3$ was measured at 480 nm and compared against controls (H_2O_2 added to microsomes in absence and presence of aminopyrine).

20-oxo-5-pregnene-16α-carbonitrile): 20 mg per kg was given orally in 0.5 ml of an aqueous suspension containing 1% gum arabic 48 and 24 hours prior to decapitation. 3-Methylcholanthrene (3-MC) in 0.5 ml corn oil was injected i.p. once daily for 3 days at a dose of 4 mg per 150 g body weight. Protein was determined by the biuret method (8). Spectral changes were measured with an Aminco Chance DW 2 spectrophotometer. Hydrogen peroxide was assayed as recently described (9) and as shown under the figures. All incubates were performed in duplicates.

RESULTS

The production of H_2O_2 during oxidation of NADPH has been determined in microsomes from controls and PB treated animals and plotted as a function of time (Fig. 1). Treatment of rats with PB increases the H_2O_2 production corresponding

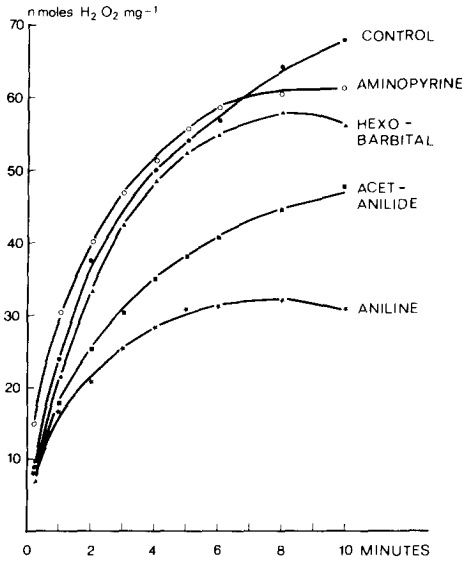

Fig. 2 Effect of substrates on rate and extent of H_2O_2 formation in microsomes. Concentration of substrates when added to buffer mixture was: 8 mM aminopyrine, 4 mM hexobarbital, 4 mM acetanilide, 4 mM aniline. Microsomes from PB treated rats. Conditions as described under Fig. 1.

to the known effect of phenobarbital on NADPH cytochrome c reductase (10). More important is the fact that N-demethylation of aminopyrine has no significant effect upon the initial rate of H_2O_2, suggesting that in microsomes from controls and PB treated animals the two reactions are independent from each other. This might indicate that the observed net H_2O_2 formation occurs solely as a result of NADPH-oxidase activity and not as an autoxidation product of cytochrome P-450 during substrate hydroxylation. On the other hand, this could also be explained by an increased formation and degradation of H_2O_2 in presence of substrate so that the net production of H_2O_2 remains unchanged.

Although the presence of aminopyrine seems to have no effect on the initial rate of net H_2O_2 production, it does effect the extent of production quite considerably. It is interesting to note in Fig. 2 that the addition of type II substrates such as acetanilide or aniline decreases the steady state concentration of H_2O_2 quite significantly, in contrast to type I substrates. Since substrate addition reveals conformational changes at the enzyme (11), the question arises

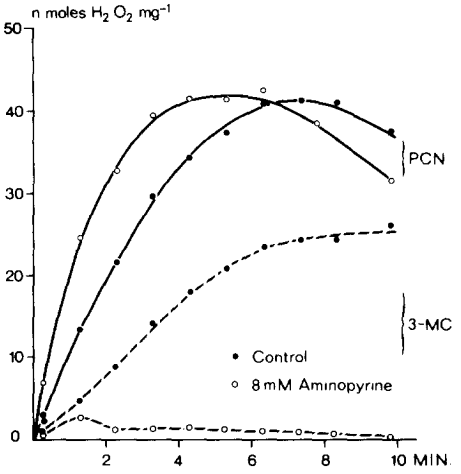

Fig. 3 Determination of H_2O_2 in microsomes from PCN and 3-MC treated rats. Conditions as described under Fig. 1.

whether upon substrate addition a change in the electron density distribution associated with the heme of cytochrome P-450 might coincide with a change of the mechanism of oxygen activation, reflected by respective changes in H_2O_2 formation or degradation. In other words, is it possible that a change from low spin to high spin cytochrome P-450 leads to a form which prevents further reduction of H_2O_2 and which consequently enhances net production of H_2O_2 ? Likewise, the converse should be true if a change from high spin to low spin cytochrome P-450 produces a species of cytochrome P-450 which degrades H_2O_2.

It was previously recognized (5) and later confirmed (12,13) that PB treatment of animals results in an increase in low and high spin hemoprotein, whereas pretreatment of animals with 3-MC stimulates the formation of high spin cytochrome P-450. The enhancement of low spin ferric cytochrome P-450 seems to be significant in animals treated with PCN (14). As a consequence, addition of type I substrates like ethylmorphine to microsomes from PCN treated animals leads to a more pronounced type I spectrum as seen after PB treatment (15).

Such a transition upon substrate addition in microsomes from PCN treated animals might inhibit the further reduction of H_2O_2. The converse should then be true after 3-MC treatment, since this yields an inverted type I spectral change upon

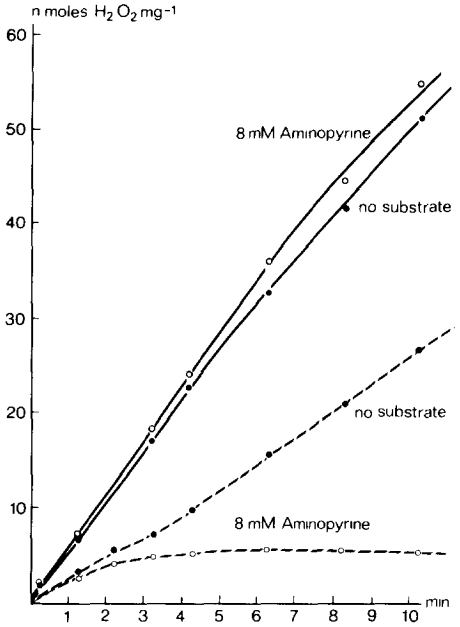

Fig. 4 Effect of aminopyrine on NADPH dependent H_2O_2 generation and consumption as compared to H_2O_2 produced via glucose oxidase reaction. Microsomes from 3-MC treated animals. Conditions as described under Fig. 1 but with additional 120 mM glucose. The reaction was started either by addition of 3.3 µg glucose oxidase, 1.1.3.4, (190 EU = 1 mg; Serva Heidelberg) (solid line curve) to 15 ml buffer or by 300 µM NADPH (dashed line curve).

aminopyrine or ethylmorphine addition. Consequently, an increased degradation of H_2O_2 should be observed if spectral changes and spin states correlate with H_2O_2 production and degradation.

This is obviously the fact as seen in Fig. 3, which compares H_2O_2 formation in microsomes in absence and presence of aminopyrine. Treatment of rats with PCN yields in absence of aminopyrine a higher net formation of H_2O_2 as compared to microsomes from 3-MC treated rats. This is in agreement with the effects of these compounds on NADPH cytochrome c reductase activity (14). More important is the observation that N-demethylation of aminopyrine in microsomes from PCN treated rats yields an increased formation of H_2O_2 which by far exceeds the initial rate of H_2O_2 formation seen in the absence of aminopyrine. On the other hand, with 3-MC microsomes H_2O_2 formation is lower when aminopyrine is present.

TREATMENT	ADDITION TO INCUBATE: 50mM METHANOL	8mM AP	8mM AP + 50 mM METHANOL		
	(1) $HCHO_M$	(2) $HCHO_{AP}$	(3) $HCHO_{(AP+M)}$	(4) $HCHO_{(AP+M)} - HCHO_{(AP)}$	(4) in % of (1)
CONTROL	9.0	10.8	19.8	9.0	100
PB	16.7.	23.4	38.9	15.5	93
3-MC	12.2	6.5	14.8	8.3	66
PCN	18.0	20.5	51.7	31.2	151

Table 1 Indirect determination of H_2O_2 formation in microsomes from rats subjected to different treatment.
The figures represent means of rate of HCHO formation from methanol or aminopyrine in absence of azide. HCHO was determined according to Nash (21). Conditions otherwise as described under Fig. 1.

These determinations have been performed in the presence of 2×10^{-4}M azide to inhibit catalase. To avoid possible artifacts due to the presence of azide, the rates of H_2O_2 production have been determined by the rates of HCHO formation from the metabolism of methanol with catalase in excess (1). One can see from Table 1 that in microsomes from controls and PB treated animals the methanol dependent HCHO formation ($HCHO_M$) occurs independently of the aminopyrine dependent HCHO formation ($HCHO_{AP}$), since the rate of formaldehyde obtained in the presence of methanol and aminopyrine ($HCHO_{AP+M}$) equals the sum of the separate reactions ($HCHO_{AP+M} = HCHO_{AP}+HCHO_M$). However, 3-MC treatment yields lower rates of HCHO formation from methanol, as determined indirectly from the difference of the rates of HCHO formation ($HCHO_{AP+M} - HCHO_{AP} = HCHO_M$). This indicates a decreased rate of H_2O_2 production when aminopyrine is present. The opposite is true for PCN microsomes, since the rate of HCHO formation from both methanol and aminopyrine by far exceeds the sum of the separate reactions, as seen from the direct determinations.

One must show that the observed degradation of H_2O_2 relates to NADPH dependent mixed function oxidation and not to catalase activity. To properly correct for the catalase contribution, although 2 x 10^{-4}M azide was present, H_2O_2 was generated via glucose oxidase in microsomes from 3-MC treated animals, in absence and presence of aminopyrine (Fig. 4). In contrast to the significant decrease of H_2O_2 in presence of NADPH, no such loss of H_2O_2 could be observed in microsomes when H_2O_2 was generated from GOD. Since aminopyrine has no effect in the latter case, this suggests that the decrease is due to the presence of NADPH and aminopyrine and not to catalase activity.

DISCUSSION

The reduction oxidation cycle of cytochrome P-450 during substrate hydroxylation is a two electron process (16,17) which should be linked to production of H_2O_2 (18). The present paper indicates that the fate of H_2O_2 in this reaction might depend on the balance of high to low spin cytochrome P-450. H_2O_2 appears as an intermediate under suitable conditions which might exist if high spin or low spin is favoured in the presence (18), or absence, respectively, of substrates, according to the following proposal (18) :

$$2\ Fe^{3+} + 2O_2^- \xrightarrow{2H^+} 2\ Fe^{3+} + O_2 + H_2O_2$$

A degradation of H_2O_2 takes place especially in microsomes from 3-MC treated rats. However, while high spin is favoured in the absence of substrate (13), low spin seems to be increased in the presence of substrate.

The subtleties of H_2O_2 formation control in microsomes can be followed by reference to the P_1 and P_7 isoenzymes of Turnip peroxidases (19, 20). Analogous to the situation in microsomes, the differences between the different peroxidases can be seen in the absorption spectra of the unliganded proteins. The balance between high spin and low spin Fe (III) states determines whether the respective isoenzyme functions as an oxygenase, or reacts with H_2O_2 as a peroxidase.

The relationship between the H_2O_2 formation and degradation, and the spin state as determined by difference spectrophotometry might be indicative that a similar situation applies in microsomes.

Acknowledgement:

The authors thank Miss H. Rechenberg, Mr. D. Schwald, and Mrs. A. Borstel for excellent technical help.

REFERENCES

1. Gillette, J.R., Brodie, B.B., and La Du, B.N., J. Pharm. Exptl. Therap. 119, 532 (1957)
2. Thurman, R.G., Ley, H.G., and Scholz, R., Europ. J. Biochem. 25, 420 (1972)
3. Hildebrandt, A.G., Roots, I., and Kohlen, W., Naunyn-Schmiedeberg's Arch. Pharmacol. Supp. to Vol. 274, R52 (1972)
4. Hildebrandt, A.G., Remmer, H., and Estabrook, R.W., Biochem. Biophys. Res. Comm. 30, 607 (1968)
5. Cammer, W., Schenkman, J.B., and Estabrook, R.W., Biochem. Biophys. Res. Comm. 23, 264 (1966)
6. Hoogeboom, G.W., in: Methods in Enzymology, Vol. I; (Colowick, S.P. and Kaplan, N.O., eds.) New York: Academic Press 1955, p.16
7. Roots, I., and Hildebrandt, A.G., Naunyn-Schmiedeberg's Arch. Pharmacol. 277, 27 (1973)
8. Gornall, A.G., Bardawill, C.J., and David, M.M., J. Biol. Chem. 177, 751 (1949)
9. Hildebrandt, A.G., Roots, I., and Speck, M., Arch. Biochem. Biophys. (in press)
10. Orrenius, S., and Ernster, L., Biochem. Biophys. Res. Comm. 16, 60 (1964)
11. Estabrook, R.W., in: Microsomes and Drug Oxidations, J.R. Gillette et al., eds., p. 221 (Discussion), New York, London: Academic Press, 1969
12. Jefcoate, C.R.E., and Gaylor, J.L., Biochem. 8, 3464 (1969)
13. Peisach, J. and Blumberg, W.E., Proc. Nat. Acad. Sci. USA 67, 172 (1970)
14. Lu, A.Y.H., Somogyi, A., West, S., Kuntzman, R., and Conney, A.H., Arch. Biochem. Biophys. 152, 457 (1972)
15. Werringloer, J., Abstracts 5. Int. Congr. Pharmacol, San Francisco 1972
16. Mason, H.S., in: Advances in Enzymology. Vol. XIX, F.F. Nord, ed., New York: Chider Science Publ., Inc., 1957
17. Estabrook, R.W., Hildebrandt, A.G., Remmer, H., Schenkman, J.B., Rosenthal, O., Cooper, D.Y., in: The 19 Colloquium der Gesellschaft für Biologische Chemie, B. Hess and Hj. Staudinger, eds., p. 142, Berlin, Heidelberg, New York: Springer, 1968
18. Gunsalus, I.C., Wenner-Gren Symposium on Structure and Function of Oxidation Reduction Enzymes. Stockholm, August 1970
19. Ricard, J., Mazza, G., Williams, R.J.P., Eur. J. Biochem. 28, 566 (1972)
20. Williams, R.J.P., Biochemical Society Transactions 1, 1 (1973)
21. Nash, T., Biochem. J. 55, 416 (1953)