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Biochemical Pharmacology, Vol. 27, pp. 355–358. Pergamon Press, 1978. Printed in Great Britain.

Superoxide anion production by liver microsomes from phenobarbital treated rat

(*Received* 9 *February* 1977; *accepted* 20 *June* 1977)

It is generally agreed that drug hydroxylation is mediated by the NADPH-dependent cytochrome P-450 systems in liver microsomes [1]. The flavoprotein NADPH-cytochrome *c* reductase (EC 1.6.2.4) of the NADPH-specific microsomal electron transport chain, reduces various acceptors among which are ferricytochrome *c* [2], nitroblue tetrazolium [3], and cytochrome P-450 [4] its natural electron acceptor. Cytochrome P-450, once reduced and oxygenated, catalyses the xenobiotic hydroxylation reactions [1]. It has been suggested that, in addition to acting as cytochrome P-450 reductase, NADPH cytochrome *c* reductase may generate superoxide anion (O_2^-).The O_2^- produced could then be the "active oxygen form" which reduces cytochrome P-450 thus permitting the fixation of molecular oxygen (O_2) and further drug hydroxylation [5–8]. Interpretation of these findings have been complicated by the use of the oxidation of epinephrine into adrenochrome as a measure of O_2^- production, since O_2^- may be produced by the direct reaction of epinephrine semiquinone with O_2, as reported by Misra and Fridovich [9].

The aim of the present study was to reinvestigate the rate and mechanism of O_2^- production by liver microsomes from control and phenobarbital treated rats. The effect of drugs added in the incubation medium, on this O_2^- production was also studied. The importance of studying the generation of O_2^- in various conditions is obvious since it has been shown that O_2^- can potentially be harmful in a number of tissues [10–13] and also may participate in the hydroxylation reaction [14].

MATERIALS AND METHODS

Adult Sprague–Dawley female rats, weighing 180–250 g, received daily intraperitoneal injections of phenobarbital (40 mg/kg) dissolved into 0.15 M NaCl for 5 days. Control animals received 0.15 M NaCl alone. The animals were decapitated 48 hr after the last injection, and the livers were immediately removed, weighed, washed into cold 0.15 M NaCl, and stored at −80° for 24 hr. After thawing, 5 g of liver were homogenized in cold 0.34 M sucrose containing 0.04 M Tris–HCl pH 7.5 (Suc-Tris Buffer), with a Potter–Elvejhem type homogenizer. The microsomal fraction was prepared by differential centrifugation according to Shenkman *et al.* [15]. The microsomal pellet of the last centrifugation was resuspended in appropriate volumes of Suc-Tris Buffer for addition to the assay cuvettes. Protein was determined by the method of Lowry *et al.* [16] and cytochrome P-450 according to Omura and Sato [17]. Aniline hydroxylase activity was measured according to Imai and Sato [18] in the presence of 1.25 mM of NADPH and 6.25 mM of aniline. Reduction of cytochrome *c* was measured according to Williams and Kamin [19], with minor modifications. Nitroblue tetrazolium (NBT) reduction was measured as in cytochrome *c* reduction but using NBT instead of ferricytochrome *c*. NADPH-dependent oxygen uptake by liver microsomes, in the absence of exogenous electron acceptors, was measured by the polarographic conventional method using a Gilson oxygraph equipped with a Yellow Spring electrode. NADPH oxidation was measured fluorimetrically by the amount of NADP produced, according to Lowry *et al.* [20] as previously described [21]. The experimental conditions were identical to those used for the measurement of the NADPH-dependent oxygen uptake. O_2^- production by microsomes from control and phenobarbital treated rats, was calculated after determination of superoxide dismutase (SOD) inhibitable reduction of ferricytochrome *c* (or NBT) according to McCord and Fridovich [22]. O_2^- was also measured after determination of SOD apparent inhibitable O_2 consumption. This technique relies on the ability of SOD to dismutate O_2^- according to the following reaction: $2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2$ [22] and can be used if this reaction does not occur spontaneously (in the absence of SOD). This latter condition was verified in the incubation medium used: more than 95 per cent of the O_2^- generated by the xanthine–xanthine oxidase system was recovered in the medium containing the microsomes. Oxygen uptake and O_2^- production were also measured in the presence of drugs added to the incubation medium. Details of the different procedures used are indicated under each table of results. All measurements were made in triplicate for each animal studied. Statistical significance was determined by Student's *t*-test [23]. Reagents: SOD and NBT were purchased from Sigma Chemical Co., glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, NADPH and horse heart cytochrome *c*, from Boehringer Mannheim. Other reagents were the best grade commercially available.

RESULTS AND DISCUSSION

Table 1 presents the results (1) of the total and O_2^- dependent reduction rate of cytochrome *c* and NBT by liver microsomes, and (2) O_2 uptake and associated O_2^-

Table 1. Effect of phenobarbital on dye reduction, oxygen consumption and superoxide anion production by rat liver microsomes and on microsomal cytochrome P-450

	Control	Phenobarbital treated	P
	0.96 ± 0.02	1.92 ± 0.04	<0.001
	nmole $^{-1}$min $^{-1}$ mg microsomal protein $^{-1}$		
Total NADPH cytochrome c reduction activity	54.6 ± 4.0	78.0 ± 6.5	<0.001
NADPH cytochrome c reduction	12.6 ± 1.2	13.2 ± 1.7	<0.4
O_2^- dependent	(23.1 ± 1.2)*	(17.0 ± 2.2)*	NS
Total NADPH NBT reduction activity	60.8 ± 4.8	112.0 ± 6.4	<0.001
NADPH NBT reduction O_2^- dependent	13.6 ± 1.3	15.6 ± 2.3	<0.1
	(22.3 ± 2.0)*	(14.0 ± 2.1)*	NS
O_2 uptake	3.3 ± 0.3	10.5 ± 0.3	<0.001
O_2^- produced	0.96 ± 0.08	7.56 ± 0.50	<0.001
	129.2 ± 2.4)*	(72.0 ± 4.8)*	
NADPH oxidation activity	3.4 ± 0.2	11.1 ± 0.2	<0.001
Aniline hydroxylation activity	0.46 ± 0.07	0.79 ± 0.08	<0.001
Cytochrome P-450 in nmole $^{-1}$ mg microsomal protein $^{-1}$	0.96 ± 0.02	1.92 ± 0.04	<0.001

The reduction of ferricytochrome c was measured according to Williams and Kamin. In NADPH NBT reductase, 15 μM NBT was added instead of ferricytochrome c. The assay medium (1.6 ml) contained 80 μg microsomal proteins. Non enzymatic reductase activities were measured with samples of boiled microsomal fraction. For measurement of O_2^- dependent reductase activity, 10 μl of superoxide dismutase (0.6 mg/ml in Krebs phosphate buffer) were added. Reduction rate of cytochrome c was followed at 550 nm using a millimolar extinction coefficient of 18.5 mM^{-1} cm^{-1} (for NBT, at 600 nm, was 21.5 $mM^{-1}cm^{-1}$). Oxygen uptake was measured by polarography in a cylindrical glass chamber: vol. 1.6 ml, temp. 37°. Assay medium was composed of Krebs phosphate buffer pH 7.4 containing NADPH 1.25 mM and microsomal proteins 40 μg. NADPH oxidation and aniline hydroxylation were measured in the same experimental conditions as oxygen uptake. For O_2^- production, 10 μl of superoxide dismutase prepared as described above were added during the reaction. Reaction was initiated by the addition of NADPH and all oxygen uptake were linear with respect to the incubation time. O_2^- produced was calculated from the differences between O_2 uptake in the presence or absence of SOD × 2 (see Methods). It is to be noted that an NADPH generating system was used for measurements of O_2^- dependent cytochrome c and NBT reduction explaining the high levels of O_2^- measured. All data are the mean, ± 1 standard deviation, of six independent experiments involving different animals. P values represent comparison by unpaired t-test.

production from control and phenobarbital treated rats. It was found that SOD inhibited approximately one fourth of the cytochrome c (or NBT) reduced and of the oxygen uptake in the liver microsomal fraction of control rats. The inhibition of NBT reduction is in agreement with the results reported by Mishin *et al.* [24]. However, these authors did not observe an inhibition of cytochrome c reduction. This discrepancy may be due to the larger amount of cytochrome c that these authors used in their incubation medium. Decreasing cytochrome c from 15 to 10 μM in the incubation medium increased its O_2^- dependent reduction by 10 per cent while increasing cytochrome c (30 μM) decreased by 90 per cent its O_2^- dependent reduction. Thus, the results reported do not strictly correspond to O_2^- produced, since what is actually measured is O_2^- dependent cytochrome c reduction (or NBT reduction), and it is likely that some portion of the O_2^- generated in the reaction mixture is not being detected. SOD inactivated by boiling (or albumin instead of SOD) had no inhibiting effect. Partial blocking by SOD of cytochrome c and NBT reduction indicates that both these electron acceptors are reduced by liver microsomal fractions in two ways: (a) a reductase O_2^--independent pathway (SOD exercises no effect) and (b) and O_2^--dependent pathway (inhibited by SOD). Partial apparent inhibition of oxygen uptake indicates that a fraction of the O_2 uptaken was transformed into O_2^-.

As expected, phenobarbital treatment increased cytochrome P-450, NADPH oxidation activity, aniline hydroxylase activity, and the ability of liver microsomes to reduce cytochrome c (or NBT). However, the O_2^- dependent reduction of cytochrome c (or NBT) was not enhanced, and only the O_2^- independent activity was. This indicates that phenobarbital induces an increase of cytochrome c (or NBT) reductase (O_2^- independent) activity and not of the O_2^- production *when one of these exogenous electron acceptors is present in the medium.* This suggests that the protein with cytochrome c reductase O_2^- independent activity, does not catalyse the production of O_2^- directly from NADPH and O_2.

On the contrary, when O_2^- production was measured in the absence of exogenous electron acceptors (polarographic method), it was observed that phenobarbital treatment enhanced O_2^- production nearly twice as much as O_2 consumption: approximately all the increase of O_2 taken up was reduced into O_2^-. Neither aniline hydroxylase activity, nor NADPH oxidation activity were modified in control and treated rats when 6, 10, 20 or 50 μg of SOD were added to the incubation medium. This showed that SOD does not modify by itself the reactions of these microsomal enzymes. Sasame *et al.* reported, in a recent abstract, that after the cytochrome b5 antiserum inhibits the introduction of the second electron, the oxygenated cytochrome P-450 complex dissociates to form oxidized cytochrome P-450 and O_2^- [25]. It is also well known that O_2 consumption is an index of the reaction rate between P-450 ferrous form and oxygen [26]. Thus, the latter results suggest that the extra electron flux produced by the increase of cytochrome c reductase O_2^--independent activity reduces the cytochrome P-450 with further involvement of the oxygenated form of cytochrome P-450 in the generation of O_2^-. This extra electron flux towards cytochrome P-450 was probably scavenged by cytochrome c (or NBT) when it was in the incubation medium. No increase in

Table 2. *In vitro* effect of coumarin and anilin on oxygen consumption and superoxide anion production by rat liver microsomes

Oxygen uptake and superoxide Anion production measured	Oxygen uptake: Control nmole^{-1} min^{-1} mg protein^{-1}	Oxygen uptake: Phenobarbital treated nmole^{-1} min^{-1} mg protein^{-1}	Ratio	Superoxide anion production: Control nmole^{-1} min^{-1} mg protein^{-1}	Superoxide anion production: Phenobarbital treated nmole^{-1} min^{-1} mg protein^{-1}	Ratio
Without drug	3.3 ± 0.3	10.5 ± 0.3	3.2	0.96 ± 0.08 (29.2 ± 2.4)*	7.6 ± 0.5 (72.0 ± 4.8)*	7.8
†Coumarin	†SS 4.5 ± 0.4	13.9 ± 0.6	3.1	0.81 ± 0.09 (18.0 ± 2.0)*	3.5 ± 0.3 (25.0 ± 1.8)*	4.4
†Aniline	2.2 ± 0.2	7.3 ± 0.5	3.3	0.39 ± 0.05 (17.9 ± 2.2)*	4.7 ± 0.3 (64.0 ± 3.5)*	11.8

* The percentages of the O_2^- dependent activity are within brackets.

Oxygen consumption and superoxide production were measured as indicated in Table 1. The assay medium made up as described in Table 1 contained coumarin 6.25 mM or aniline 6.25 mM dissolved into absolute ethanol. Quantities of oxygen consumption are corrected for microsomal ethanol oxidizing system and all results are corrected for non enzymatic activities. All data are the mean of six different experiments.

* The percentages of the O_2^- produced from O_2 are within brackets.

† S represents a P value <0.01, SS a P value <0.001, and NS a P value <0.4 of the comparison by unpaired *t*-test of the data designated by the square brackets.

the reduction of cytochrome P-450 could therefore occur, explaining why no increase in O_2^- production was observed. It should be noted that O_2^- could be formed either by decomposition of the oxy-complex: $Fe(II)O_2 \rightarrow Fe(III) + O_2^-$ or of the oxy-complex reduced by a further electron: $Fe(II)O_2^- \rightarrow Fe(II) + O_2^-$. The results reported by Sasame *et al.* [25] are however in favour of the first of these two possibilities.

The results reported in Table 2 show that the addition of exogenous drugs in the assay medium, modifies O_2 uptake and O_2^- production. When coumarin (a type I compound) was added to the incubation medium, oxygen consumption was increased by one third in both the control and the treated rats, while the percentage of O_2 reduced into O_2^- was decreased (29 per cent to 18 per cent and 72 per cent to 25 per cent respectively). When aniline (a type II compound) was added, O_2 uptake was decreased by one third in both the control and the treated rats, while the percentage of O_2 reduced into O_2^- decreased (29 per cent to 18 per cent and 72 per cent to 64 per cent respectively). The decrease in the relative O_2^- produced was, thus, very slight in the treated rats. Ratio of O_2^- formation in treated rats on O_2^- formation in control rats as reported in Table 2 showed clearly that coumarin decreased this ratio while aniline increased it. Ratio of O_2 uptake in treated rats on O_2 uptake in control rats was not significantly modified by either drug.

As reported in the first part of this paper, O_2^- was, probably, produced by the dissociation of the oxygenated cytochrome P-450 complex. This permitted to appreciate, indirectly, the steady state level of this complex during drug hydroxylation. It is well established that the initial reaction of the process of drug hydroxylation consists in the rapid binding of the drug to the oxidized form of cytochrome P-450. This binding is associated with spectral changes of the latter which defined type I and type II compounds [15]. Binding of cytochrome P-450 to type I compound (coumarin) is also associated with its transition from the low spin to the high spin form [27]. Cytochrome P-450-substrate complex, then, undergoes rapid reduction by way of cytochrome P-450 reductase. The next step is considered to consist in an interaction of the reduced cytochrome P-450-substrate complex with molecular oxygen giving rise to a ternary oxygenated reduced-cytochrome P-450-substrate complex [28, 29]. This latter complex has been seen to accumulate during the hydroxylation process [29] which would indicate that its formation preceeds the rate-limiting step of the whole process of hydroxylation. The ternary oxygenated reduced-cytochrome P-450 substrate complex is reduced again by a further electron and the drug is hydroxylated. Increase in O_2 uptake associated with a decrease in O_2^- formation, obviously observed in treated rats, suggested that, while the whole process of oxygen utilization was accelerated by coumarin, the ternary oxygenated reduced-cytochrome P-450-substrate complex accumulated to a lesser extent than in the absence of coumarin. It suggested further that an increased activity of the rate-limiting step of drug hydroxylation, which is considered to be that of the second reduction of the oxygenated ternary P-450-substrate complex or, as recently suggested, that of the breaking of the C—H bond of the drug [30, 31] diminished the breakdown of the ternary oxygenated reduced cytochrome P-450-substrate complex. This was more obvious in treated rats. The induction, however, of cytochrome *c* reductase and of cytochrome P-450 seemed slightly more important than that of the rate-limiting step of coumarin-hydroxylation since the percentage of O_2^- production from O_2 uptake was slightly higher (25 per cent) in the phenobarbital treated rats than in the control rats (18 per cent) in the presence of coumarin. Nevertheless, in the presence of coumarin the coupling between electron transfer and hydroxylation occurred favorably.

On the contrary, binding of cytochrome P-450 to type II compound (aniline) has been shown to be caused by the direct interaction of the heme iron with a basic nitrogen of the added compound [15] and cytochrome P-450 remains in a low spin state [27]. This interaction is competitive with carbon monoxide and is thus probably, also, competitive with oxygen. The observation, in the presence of aniline, of a decreased oxygen uptake by microsomes of control and phenobarbital treated rats is in agreement with this suggestion. Aniline, however, decreased O_2^- formation by microsomes of control rats to the same extent as coumarin while it decreased it to a lesser extent in treated rats. The latter observation suggests that, while the whole process of oxygen utilization was decreased, the ternary reduced cytochrome P-450 accumulated, in the presence of aniline, more than in the presence of coumarin. The higher ratio of O_2^- formed by control rats, in the presence of aniline (11.8), than in its absence (7.8) suggested

that the activity of the rate limiting step of aniline hydroxylation was slower, in treated rats, than the formation and breakdown of the ternary reduced cytochrome P-450-substrate complex. Phenobarbital, thus, induced the formation of cytochrome P-450 and cytochrome P-450 reductase much more than that of the rate limiting step of aniline-hydroxylation. In the presence of aniline, uncoupling between electron transfer and hydroxylation probably occurred in phenobarbital treated rats. Steady state concentrations of the ternary complexes of coumarin and aniline have not been directly measured, and our results are only indirect evidence that the O_2^- formation is under the control of the steady state concentration of this complex i.e. under the rate of its formation and the rate of its utilization for drug hydroxylation (further reduction and breaking of the C—H bond of the drug).

In conclusion, the present study while confirming that the O_2^- is produced by liver microsomes also showed that this O_2^- is formed probably by the reduced cytochrome P-450 oxygenated complex or perhaps by this complex further reduced by the second electron transfer, but not directly by cytochrome *c* reductase. It also suggested that the amount of superoxide generated is under the dependence both of the formation of reduced cytochrome P-450 oxygenated form (cytochrome *c* reductase and cytochrome P-450) and of its rate of utilization (further reduction of the complex or C-OH producing steps). We provided, thus, indirect evidence that the steady state concentration of reduced cytochrome P-450 oxygenated complex or its reduced form, linked or not to a drug, is responsible of the amount of O_2^- released in the medium. Moreover, it appeared that, while phenobarbital induced synthesis of cytochrome P-450 and of cytochrome P-450 reductase it induced, in parallel an increased hydroxylating activity of coumarin and not of aniline. Further studies will have to be performed in view of showing direct correlation between concentration of reduced cytochrome P-450 oxygenated form and O_2^- production and in order to appreciate whether drugs other than phenobarbital, produce an increase in O_2^- production and whether this radical is implied, *in vivo*, in the hepatic toxicity of certain drugs administrated alone or in an associated form.

Acknowledgements—We are grateful to H. Troube for her excellent technical assistance.

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