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GENERATION OF LOW-LEVEL CHEMILUMINESCENCE DURING THE METABOLISM OF 1-NAPHTHOL BY RAT LIVER MICROSOMES

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Abstract—The metabolism of 1-naphthol in rat liver microsomal fractions supplemented with NADPH is accompanied by low-level chemiluminescence which reflects the formation of molecular excited states. Photoemission consists of two phases which both are dependent on microsomal protein and 1-naphthol concentration. The involvement of cytochrome P-450 in the microsomal metabolism of 1-naphthol was indicated by an inhibition of chemiluminescence by aminopyrine or metyrapone. Oxygen is required for light emission. Whereas phase I is hardly influenced by superoxide dismutase, phase II is suppressed. Chemiluminescence was not associated with malondialdehyde accumulation, in contrast to NADPH-dependent lipid peroxidation in microsomal fractions in the absence of 1-naphthol. Phase I of chemiluminescence appears to directly reflect cytochrome P-450-dependent hydroxylation, and phase II is attributed to redox cycling of products arising from these reactions, e.g. the 1,4- and/or 1,2-naphthoquinones as oxidation products of the corresponding dihydroxynaphthalenes.

It has been reported that 1-naphthol is selectively toxic to short-term organ cultures of human colonic tumour tissue, and a potential role as an anticancer agent has been considered [1]. Recently, d'Arcy Doherty *et al.* [2] studied the effects of 1-naphthol and of the metabolites, 1,2- and 1,4-naphthoquinone, on isolated hepatocytes. Whereas most of the 1-naphthol was metabolized to the glucuronide and the sulphate, there was also evidence for other metabolic routes. The finding of increased toxicity of 1-naphthol and the 1,2 and 1,4-naphthoquinones after inhibition of NAD(P)H:quinone reductase by dicoumarol, i.e. surface blebbing and loss of intracellular glutathione together with a lower rate of glucuronide and sulphate formation, supports a toxic mechanism involving redox cycling of the naphthoquinones. The process of redox cycling (see [3]) produces superoxide anion radicals and subsequently other active oxygen species at the expense of cellular reducing equivalents. The NAD(P)H:quinone reductase provides a protective mechanism by maintaining the quinones in the fully reduced state via 2-electron reduction, thus lowering the one-electron reduction and redox cycling.

Recently, the formation of O_2^- in microsomal fractions supplemented with NADPH and 1-naphthol was demonstrated [4]. In the case of menadione (2-methyl-1,4-naphthoquinone) redox cycling and generation of active oxygen species was accompanied by low-level chemiluminescence, probably involving $^1O_2$ light emission [5]. Dicoumarol, as an inhibitor of the NAD(P)H:quinone reductase led to an increased toxicity of menadione in isolated hepatocytes [6], and to an increase of low-level chemiluminescence [5]. In the present work, it is shown that the metabolism of 1-naphthol in microsomal fractions is accompanied by low-level chemiluminescence consisting of two phases, the second of which is attributed to redox cycling of naphthoquinones and concomitant O_2^--production.

MATERIALS AND METHODS

Preparation of microsomal fractions. Microsomal fractions were prepared as described previously [7]; prior to homogenization, livers from anaesthetized male rats (150–200 g body weight) were perfused through the portal vein with ice-cold 0.25 M sucrose, 10 mM triethanolamine/HCl, pH 7.2, at a rate of 28 ml/min for 2–3 min. The isolated microsomal fraction was washed twice in the same medium and stored at −25° until use.

Chemiluminescence measurements. Chemiluminescence measurements were carried out as described previously [8], using a photon counter equipped with an EMI 9658 AM photomultiplier cooled to −25° by a thermoelectric cooler. Photoemission is expressed in counts per seconds. Microsomal fractions were incubated in 0.1 M potassium phosphate buffer, pH 7.4, at 37° in a final volume of 6.5 ml. Samples were gassed with oxygen under constant stirring throughout the measuring period. Reactions were started by addition of NADPH or, in the case of the NADPH-regenerating system, addition of $NADP^+$ through polyethylene tubing. When a NADPH-regenerating system was employed, 15 mM glucose-6-phosphate and 1.0 U/ml glucose-6-phosphate dehydrogenase were added prior to the addition of $NADP^+$ (0.4 mM). The concentration of microsomal protein, NADPH and 1-naphthol (stock solution in DMSO) are given in the text.

Measurement of malondialdehyde. Malondialdehyde accumulation was measured as thio-

barbituric acid reactive material [9] at 535–570 nm using a $\Delta\varepsilon$ of 156 $mM^{-1}\,cm^{-1}$, in parallel with the chemiluminescence reaction by withdrawing 0.1 ml aliquots of the microsomal suspension at different time points. The spectrophotometric assay was carried out in a dual-wavelength spectrophotometer (Sigma Instruments, model ZWS-11, Biochem Co., München, F.R.G.).

Measurement of oxygen uptake. Oxygen uptake was measured by a pO_2 analyser with a Clark-type electrode inserted into a closed cuvette (1.3 ml volume), which was maintained at 37°. The reaction medium in the cuvette was constantly stirred with a magnetic stirrer throughout the measuring period.

Chemicals. 1-Naphthol was obtained from Sigma Co. (München, F.R.G.). Other chemicals were from Merck (Darmstadt, F.R.G.), biochemicals from Boehringer Mannheim (Mannheim, F.R.G.).

RESULTS

1-Naphthol-induced low-level chemiluminescence in microsomal fractions

In the presence of NADPH, microsomal suspensions supplemented with 1-naphthol exhibit chemiluminescence with a two-phase time course as shown for a typical incubation with an NADPH-generating system in Fig. 1, upper trace. The first maximum of light emission, referred to as phase I of chemiluminescence, was reached within several seconds after starting the reaction, whereas the second one (phase II of chemiluminescence) appeared after a time t_{II}, which was about 2 min under the conditions of Fig. 1. Both phase I and phase II light emission were linearly dependent on microsomal protein and were absent in incubations without NADPH. The involvement of superoxide anion radicals in reactions which are responsible for the second phase is demonstrated by the effect of superoxide dismutase (20 μg/ml) (Fig. 1, lower trace). Whereas phase II is suppressed in the presence of superoxide dismutase, phase I is essentially not affected. Controls with boiled superoxide dismutase did not show an inhibition of phase II. As can be seen in Table 1, catalase (40 μg/ml) did not show a marked effect, only a slight increase (15%) in phase II of chemiluminescence occurred. Mannitol, a $OH^{\cdot}$ scavenger exhibits a 22% inhibition of phase I and a 13% inhibition of phase II of light emission.

Both phases are dependent on the concentration of 1-naphthol; at higher concentrations of 1-naphthol it becomes difficult to distinguish between phase I and phase II chemiluminescence, as t_{II} decreases and phase I is overlapped by phase II. However, the maximal chemiluminescence of each incubation, the bulk of which can be attributed to phase II, exhibits a concentration dependence as shown in Fig. 2, with a saturation level at about 0.5 mM 1-naphthol. The apparent K_M was 36 μM. The decrease of the time interval t_{II} upon increasing concentrations of 1-naphthol is also shown in Fig. 2, with t_{II} reaching a minimum again at 0.5 mM 1-naphthol.

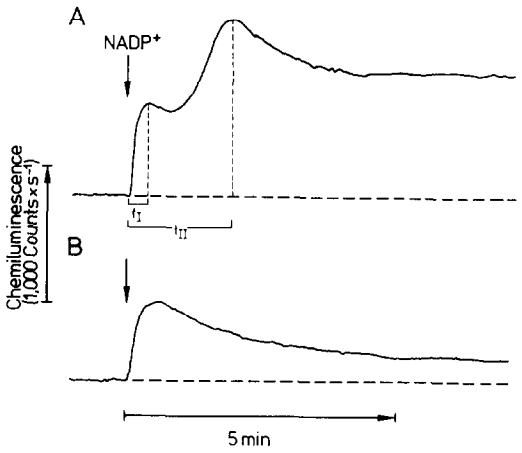

Fig. 1. Time course of 1-naphthol-induced chemiluminescence. Chemiluminescence was recorded from incubations of rat liver microsomal fractions without (A) and with (B) superoxide dismutase (40 μg/ml) present. The microsomal fractions (1.5 mg protein/ml) were incubated in 0.1 M potassium phosphate buffer pH 7.4 at 37°, gassed with oxygen, containing 100 μM 1-naphthol and a NADPH-generating system as described in Materials and Methods.

Comparison of chemiluminescence and malondialdehyde accumulation in the presence and absence of 1-naphthol

Incubations of microsomal fractions with a NADPH-generating system in the absence of 1-naphthol produced chemiluminescence as shown in Fig. 3A. Only very weak light emission occurred in the first 6 min after addition of $NADP^+$, followed by an exponential increase, in agreement with the findings

Table 1. Effects of dicoumarol, catalase and mannitol on 1-naphthol-induced chemiluminescence

Additions	Chemiluminescence		
	Phase I cps (%)	Phase II cps (%)	t_{II} (min) (%)
1-Naphthol (50 μM)	587 ± 12 (100)	878 ± 23 (100)	4.0 ± 0.2 (100)
plus dicoumarol (20 μM) (added after phase I)	—	1133 ± 46 (129)	3.7 ± 0.1 (93)
plus catalase (40 μg/ml)	550 ± 47 (94)	1012 ± 55 (115)	4.0 ± 0.2 (100)
plus mannitol (20 mM)	458 ± 12 (78)	763 ± 7 (87)	4.1 ± 0.1 (103)
plus metyrapone (1 mM)	398 ± 18 (68)	738 ± 30 (84)	8.3 ± 1.6 (208)

Conditions as in Fig. 1. Values are means from N = 3 experiments ± S.E.M.

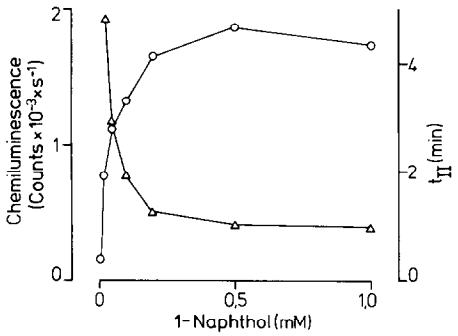

Fig. 2. Dependence of phase II chemiluminescence on the concentration of 1-naphthol. Rat liver microsomal fractions were incubated with a NADPH-generating system under conditions as described in Fig. 1. Circles indicate the height of phase II chemiluminescence; the time between the initiation of the reaction by addition of $NADP^+$ and maximal light emission, t_{II}, is indicated by the triangles.

in [10]. Chemiluminescence was accompanied by the formation of malondialdehyde, measured as thiobarbituric acid-reactive material. In contrast, there was no significant malondialdehyde formation in the presence of 1-naphthol (Fig. 3B), and, besides the above-described 1-naphthol-dependent phases of light emission no further increase of chemiluminescence at later time points was observed. Thus, the observed levels of chemiluminescence at incubation times of 20 min or more after starting the reaction are lower in the presence than in the absence of 1-naphthol; this was found with all concentrations employed (5 μM–1 mM). As malondialdehyde formation and chemiluminescence under conditions as described in Fig. 3A are attributed to lipid peroxidation [10], it is concluded that the metabolism of 1-naphthol inhibits lipid peroxidation or the formation of lipid peroxidation products such as malondialdehyde. The colour development in the TBA assay is not influenced by 1-naphthol.

Oxygen dependence of 1-naphthol-induced chemiluminescence and oxygen uptake during the microsomal metabolism of 1-naphthol

When NADPH was added to a 1-naphthol-containing microsomal suspension, or when a NADPH-generating system was employed as described in Fig. 1, no chemiluminescence was detected when the suspension was gassed with nitrogen instead of oxygen, thus indicating the absolute requirement of oxygen for the formation of light emitting species.

The time courses of chemiluminescence and oxygen uptake of microsomal incubations with 1-naphthol (100 μM) upon addition of NADPH (400 μM) are shown in Fig. 4. The time when maximal oxygen uptake is reached coincides with the maximal light emission of phase II. It appears that the small oxygen uptake in phase I is overlapped by the relatively higher rate of O_2 consumption in phase II.

Effect of aminopyrine and metyrapone on 1-naphthol-induced chemiluminescence

Aminopyrine, a substrate for cytochrome P-450, was used in order to establish the involvement of this microsomal monooxygenase in the mechanism of the formation of light emitting species. Aminopyrine itself did not produce chemiluminescence when metabolized in microsomal fractions. As can be seen in Fig. 5, aminopyrine suppresses both phases of chemiluminescence and shifts t_{II} from 1.5 min to about 6 min when present at a concentration of 0.2 mM or more. The inhibition with 1 mM aminopyrine is 68% for the first and 80% for the second phase. Aminopyrine exhibits a half-maximal effect on phase I and phase II light emission as well as

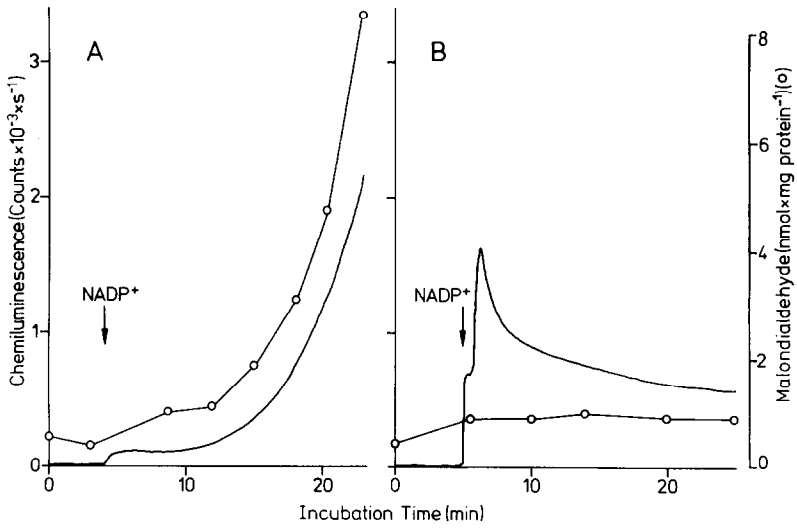

Fig. 3. Chemiluminescence (—) and malondialdehyde accumulation (—○—) without and with 1-naphthol. (A) Control incubation without 1-naphthol; (B) 1-naphthol (200 μM). Malondialdehyde was measured in aliquots removed from the suspension during chemiluminescence measurement. Conditions as described in Fig. 1, also using an NADPH-generating system.

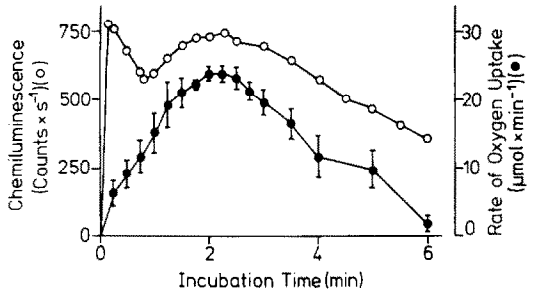

Fig. 4. Relationship between chemiluminescence and the rate of oxygen uptake in the presence of 1-naphthol. Open circles are average chemiluminescence values at the respective time points (2 experiments), filled circles indicate average values (±S.E.M., N = 3) of oxygen uptake determined from the slope of the oxygen uptake time course, rate without 1-naphthol subtracted. Microsomes (1.3 mg protein/ml) were incubated in oxygen-saturated 0.1 M potassium phosphate buffer pH 7.4, 37°, containing 1-naphthol (100 μM). The reactions were started by a single addition of NADPH (0.4 mM), no regenerating system present.

on t_{II} at 40 μM. The capability of aminopyrine to scavenge radicals may contribute to the decrease in phase I and phase II light emission.

To further establish the role of monooxygenase reactions in the metabolism of 1-naphthol, metyrapone, an inhibitor of cytochrome P-450 was employed. As can be seen in Table 1, in the presence of metyrapone phase I chemiluminescence decreases by about 30%. The second phase reaches its maximum much later and is slightly lowered compared to controls without metyrapone.

Inhibition of NAD(P)H:quinone reductase by dicoumarol

Dicoumarol, an effective inhibitor of NAD(P)H:quinone reductase, an enzyme which is also present in microsomes, influenced 1-naphthol-induced chemiluminescence dependent on the time of addition. When it was present at the beginning of the incubation, phase I was lowered by about 25%, phase II was slightly higher and t_{II} was 150% compared to controls. The effects on phase I and t_{II} suggest a competitive metabolism of dicoumarol by cytochrome P-450. As the NAD(P)H:quinone reductase exerts its protective effects by preventing redox cycling of the quinones, as reflected by phase II (see discussion), in further experiments dicoumarol was added after the first phase of light emission, i.e. the interference with monooxygenating reactions was diminished. Under these conditions, a clear increase (29%) of phase II was observed in the presence of dicoumarol (20 μM) (Table 1).

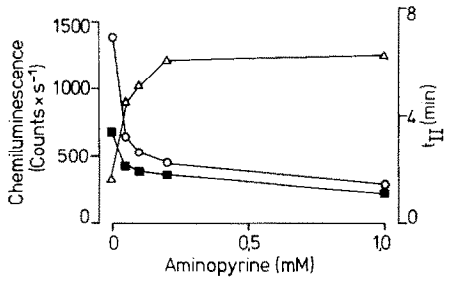

Fig. 5. Effect of aminopyrine on 1-naphthol-induced chemiluminescence. Aminopyrine as a substrate of the microsomal cytochrome P-450 system was added prior to $NADP^+$ to the incubation mixture (conditions see Fig. 1), supplemented with 1-naphthol (75 μM). ■: Phase I chemiluminescence; ○: Phase II chemiluminescence; △: t_{II}, time between addition of $NADP^+$ and maximal light emission of phase II.

DISCUSSION

The present work provides direct evidence for the formation of oxygen-dependent excited species during metabolism of 1-naphthol in microsomal fractions. This can be related to redox cycling, which is supported by the ability of 1-naphthol to stimulate microsomal oxygen consumption and superoxide formation, as reported by Thornalley *et al.* [4]. Two phases of light emission were observed which were different regarding the inhibitory effect of superoxide dismutase, oxygen uptake and time course, depending on the experimental conditions.

Based on the mechanism proposed in [2, 4], 1-naphthol is hydroxylated to the 1,4- and/or 1,2-dihydroxynaphthalenes by monooxygenation. The subsequent oxidation of the hydroquinone to the 1,4- or 1,2-naphthoquinone gives rise to redox cycling of the quinone/semiquinone with formation of superoxide anion radicals. This redox cycling may be catalysed by the NADPH–cytochrome P-450 reductase which has been shown to reduce some quinones to the corresponding semiquinones with subsequent production of O_2^- under aerobic conditions [11]. Thus phase II of chemiluminescence is attributed to superoxide production during redox cycling of the cytochrome P-450-dependent products of 1-naphthol, with excited species such as singlet molecular oxygen as products of O_2^--dependent reactions. This is in agreement with results on other redox cyclers, for example paraquat [10] or menadione [5], where chemiluminescence has been attributed to O_2^--formation and subsequent generation of excited species. The reaction mechanism yielding $^1O_2$ is still under debate. Whereas nonenzymatic dismutation of O_2^- as a source of $^1O_2$ was questioned in biological systems, other cellular components may react with superoxide with subsequent formation of $^1O_2$ (see, for example, [12]).

Involvement of oxygen species such as H_2O_2 or $OH^\cdot$ seems to be of minor importance, as catalase and mannitol did not show marked effects on phase II of light emission (Table 1). The small increase in the presence of catalase may be attributed to a somewhat lowered H_2O_2-dependent enzyme destruction. The involvement of superoxide is supported by the selective effect of superoxide dismutase on phase II (Fig. 1) and the correspondence in the time courses of oxygen uptake and chemiluminescence (Fig. 4). Moreover, the required time to reach maximal light emission, t_{II}, is dependent on the concentration of 1-naphthol (Fig. 2) and is affected by aminopyrine (Fig. 5), a substrate for the cytochrome P-450 system

and metyrapone, an inhibitor of cytochrome P-450 (Table 1). This indicates the cytochrome P-450-dependent formation and accumulation of a substrate not present at zero time of the incubation. This is in agreement with a decreased formation of 1,4-naphthoquinone with 20 μM 1-naphthol in the presence of metyrapone or SKF 525A [13]. However, with higher concentrations of 1-naphthol (200–500 μM) a marked inhibition of covalent binding of 1-naphthol-derived metabolites by SKF 525A was not observed [13, 14].

The described mechanism is further supported by the effect of dicoumarol on the phase II of light emission (Table 1). Dicoumarol is an effective inhibitor of the NAD(P)H:quinone reductase, which is present also in microsomal fractions, though its activity is low compared to cytosolic activity [15]. The NAD(P)H:quinone reductase, which catalyses the 2-electron reduction of naphthoquinones without generating a free semiquinone radical, has been shown to be of protective nature in preventing redox cycling [6, 16] and to decrease low-level chemiluminescence during redox cycling [17].

The lack of malondialdehyde formation in microsomal incubations in the presence of 1-naphthol is in agreement with similar findings with other redox cyclers, i.e. paraquat [10] or menadione (unpublished result). This was interpreted as a shift of the microsomal electron flow from reactions capable of initiating lipid peroxidation to a O_2^--producing mechanism, which in further steps produces $^1O_2$ but cannot initiate lipid peroxidation in a comparable amount.

Phase I of chemiluminescence is related to the monooxygenase-dependent metabolism of 1-naphthol, as it is dependent on oxygen, NADPH, microsomal protein and 1-naphthol and inhibited by aminopyrine and metyrapone. As superoxide dismutase and catalase did not show an effect (Fig. 1, Table 1), the participation of O_2^- and H_2O_2 in phase I is unlikely. The slight inhibition (22%) of phase I by mannitol (20 mM) (Table 1) could point to the involvement of $OH^{\cdot}$ radicals. However, the bulk of light emission seems to be generated by a mechanism not requiring $OH^{\cdot}$. The nature of the excited species responsible for phase I of photoemission remains to be elucidated.

The present work supports microsomal redox cycling of cytochrome P-450-derived products from 1-naphthol, such as 1,2- and 1,4-napthoquinone, with concomitant formation of superoxide anion radicals and provides evidence for the generation of excited species not only as a consequence of microsomal redox cycling (phase II) but also during monooxygenase-dependent reactions (phase I).

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