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METABOLISM IN RAT LIVER MICROSOMES OF THE NITROXIDE SPIN PROBE TEMPOL

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Abstract—Paramagnetic nitroxide spin labels have been extensively used to probe various biophysical and biochemical properties of the cellular environment. Recently nitroxides have been proposed as contrast enhancing agents in proton magnetic resonance imaging and contrast enhancement has been demonstrated in animal studies. Nitroxides, possessing a stable unpaired electron, increases the relaxation rates of protons, providing an enhancement of contrast. Nitroxides are metabolized intracellularly principally via reversible reduction to hydroxylamines. Rates of reduction depend on the physical characteristics of the nitroxides, in general 5-membered pyrrolidine ring are reduced more slowly than those with a 6-membered piperidine ring. Oxidation back to the nitroxide is relevant for lipid soluble hydroxylamines, while is low for water soluble ones. It is known that nitroxides are metabolized by subcellular fractions (cytosol, mitochondria, microsomes), though the enzymatic and non-enzymatic systems involved are poorly characterized. In the present study, the first of the necessary steps toward a systematic study of the metabolism of nitroxides by subcellular organelles, we have chosen to study the metabolism of 4-hydroxy 2,2,6,6-tetramethylpiperidine-*N*-oxyl in isolated rat liver microsomes. Microsomes were able to reduce Tempol slowly without any substrate addition; when NADPH was added, the reduction rate substantially increased. In phenobarbitone induced rats the reduction rate was significantly higher than in not-induced microsomes. NADPH-dependent reduction rate was inhibited by thallium chloride (an inhibitor of the flavin-centered cytochrome P-450 reductase), superoxide dismutase, and by *N*-ethylmaleimide; menadione increased it. The Tempol reduction rate was not significantly affected by various cytochrome P-450 inhibitors with the sole exception of metyrapone. A solution containing purified cytochrome P-450 reductase and NADPH readily reduced Tempol. Microsomes fortified with NADH were able to reduce Tempol at an appreciable rate. In order to distinguish between reduction of nitroxides to hydroxylamine or destruction of nitroxides following nitroxide reduction, microsomal suspensions were treated with a mild oxidant (ferricyanide 0.5–10 mM). The recovery varied from 40 to 60%, indicating a process of probe destruction leading to as yet unknown metabolites. The present study clearly indicates that, in this model system, cytochrome *c* (P-450) reductase and not cytochrome P-450 is responsible for the observed Tempol metabolism; along with hydroxylamine formation, other Tempol derived metabolites are formed during the process.

Paramagnetic nitroxyl spin labels are relatively stable in a biological environment, rendering them useful as probes of biophysical and biochemical properties of cells [1–3]. Nitroxides have been used extensively to study motion in membranes and macromolecules [2–4], transmembrane potentials [5], surface potential, [6] and pH [7]. Exploiting the interaction between molecular oxygen and broadening of the ESR signal of nitroxide free radicals via Heisenberg spin-exchange, oxygen concentration can be effectively measured intracellularly [8, 9].

Nitroxides are under active investigation as imaging agents for *in vivo* ESR techniques and as contrast agents for *in vivo* nuclear magnetic resonance imaging (MRI) [10, 11]. A potentially important way to obtain additional information is to use agents whose concentrations in the tissues depend on the metabolic states of the tissues [12]. It has been shown that the rate of reduction of some nitroxides is affected by the oxygen concentration [11, 12], with the rate of nitroxide reduction being fastest under hypoxic conditions.

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It has been reported that the major metabolic pathway leads to the formation of the diamagnetic hydroxylamine, which can be observed as spin loss in a ESR spectrometer and loss of the ability to enhance proton relaxation [11]. Although decrease of the ESR signals usually has been considered an undesirable effect, it may be exploited to develop metabolically responsive agents. A thorough understanding of the metabolic pathways of nitroxides, in cells is a key to the use of nitroxides *in vivo* studies. Both enzymatic and non-enzymatic mechanisms for nitroxide metabolism have been postulated [13, 14]. Enzymatic reduction by the electron transport system has been observed in microsomal [15, 16] and mitochondrial fractions [17].

Oxidation of hydroxylamine back to the parent nitroxide has been reported, hence it could interfere with the measurement of the reduction though a relevant re-oxidation has been observed for lipid soluble hydroxylamines and for 5-membered ring hydroxylamines; for 6-membered rings and water soluble ones it is low, negligible in the case of 4-hydroxyl 2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) [18, 19].

The study presented here has been undertaken to gain new insight into the metabolism of the paramagnetic probe Tempol in liver microsomes, within

the frame of a systematic study of the metabolism of nitroxides by subcellular organelles and whole cells.

MATERIALS AND METHODS

Chemicals. 4-Hydroxy 2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) was obtained from Molecular Probes (Junction City, OR); metyrapone, SKF525A, menadione (2-methyl-1,4-naphthoquinone, superoxide dismutase (SOD), potassium ferricyanide $K_3Fe(CN)_6$, ascorbate oxidase, *N*-ethylmaleimide (NEM), NADH, and NADPH were obtained from Sigma Chemical Co. (St Louis, MO), phenobarbitone from Boehringer Biochemia (Mannheim, F.R.G.). Cobaltic protoporphyrin IX (CPP) was purchased from Porphyrin Products (Logan, UT). Inorganic chemicals were of analytical grade. Purified cytochrome P-450 reductase (EC 1.6.2.4) was a generous gift of Dr M. Ingelman-Sundberg, Karolinska Institutet, Stockholm, Sweden.

Microsomal preparation. Adult male albino rats (body wt approx. 200 g) were maintained on a standard laboratory diet. Microsomes were obtained either from livers of male rats or from livers of rats treated with phenobarbitone (PB), an inducer of the mixed function oxidase system (MFOS), administered 1 g/l. in drinking water for 5 days. Microsomes were prepared as described previously [20] and preserved frozen at −80° and used within 3 weeks from preparation. The microsomal pellet was resuspended in an isotonic buffered solution immediately before use at a concentration of 35 mg/ml of protein. Tempol (100 μM), dissolved in saline was added to the microsomal suspension in the presence or absence of NADPH (1 mM). In some experiments various type of metabolic inhibitors were added to the microsomal suspension before the addition of NADPH.

Solutions of CPP were prepared and administered as described by Drummond and Kappas; [21] cobaltous chloride ($CoCl_2$) treatment was carried out as described by De Matteis and Gibbs [22]; the result of the administration was checked by assessing cytochrome P-450 in the microsomal suspension [23].

Measurement of reduction rates of Tempol. Tempol metabolism was studied by following the decay of its concentration in a Bruker 200D ESR spectrometer fitted with a variable temperature cavity. Tempol, along with various inhibitors when indicated, was added to the concentrated microsomal suspension, the mixture was thoroughly mixed in a small vial and sucked in a gas permeable plastic tube (Zeus Industries, Raritan NJ) which was positioned in the ESR cavity, the rate of reduction in the absence of NADPH was recorded; the sample was then recollected in a vial, NADPH was added and the recording procedure was repeated. Initial reduction rates were measured by setting the magnetic field at the peak of the midfield line of the nitroxide and turning the field sweep to zero. The procedure described enables the measurement of the signal decay within 15 sec after NADPH addition, i.e. before the sample reached thermal equilibrium inside the cavity.

Oxygen partial pressure was kept constant by flowing air around the sample tube, the temperature was kept at 37°. Figure 1 shows a typical Tempol spectrum and an example of how the measurements were performed in this study.

Statistical significance was estimated by Student's *t*-test on paired data.

RESULTS

Tempol was reduced by liver microsomes in the absence of any added co-factor; the addition of NADPH significantly increased the reduction rate (Table 1). Microsomes of rats pretreated with PB had a significantly higher reduction rate both in the presence or absence of added NADPH (Table 1). Boiling the microsomal suspension for 15 min almost completely blocked metabolism of nitroxides (Table 1).

An ascorbate-dependent mechanism for reduction of Tempol, reported in various papers [13, 14] was ruled out by experiments performed in the presence of ascorbate oxidase (5 U/ml); such an addition did not result in any variation of the rate of reduction (Table 2).

In order to ascertain the role of the microsomal electron transport chain, centered on cytochrome P-450, the rate of metabolism of Tempol was measured in microsomes supplemented with various inhibitors. The presence in the incubation mixture of cytochrome P-450 inhibitors, listed in Table 1, did not affect significantly the rate of reduction of Tempol in the presence of NADPH with the sole exception of metyrapone (Table 1).

Rats pretreated with $CoCl_2$ show a dramatic decrease in cytochrome P-450 content, while CPP pretreatment causes a decrease in both cytochrome P-450 and cytochrome P-450 reductase [24]. $CoCl_2$ pretreatment did not cause a significant modification in Tempol rate of metabolism, while a statistically significant decrease was observed in microsomes from CPP pretreated rats (Fig. 2).

Thallium chloride ($TlCl_3$), an inhibitor of the electron transport via the flavin present in the cytochrome P-450 reductase [25] caused a moderate, though statistically significant, inhibition of the reduction rate (Table 2). The prominent role played by the reductase was confirmed by experiments performed using NADPH, purified cytochrome P-450 reductase and Tempol. A solution containing 0.1 unit of reductase and Tempol did not result in any observable rate of reduction; after NADPH addition, Tempol was reduced at a rate of 66.5 ± 1.75 pmol/min/mg prot.

The addition of menadione to microsomes results in an increased production of superoxide anion [26]; menadione, added to the microsomal suspension along with Tempol and NADPH, significantly increased the rate of reduction of Tempol. This effect was inhibited by addition of superoxide dismutase (1000 U/ml) added to the suspension (Table 2). Addition of catalase did not elicit any change on the rate of Tempol reduction (Table 2); the combined addition of SOD and catalase, did not give results different from those observed in the presence of SOD only (Table 2).

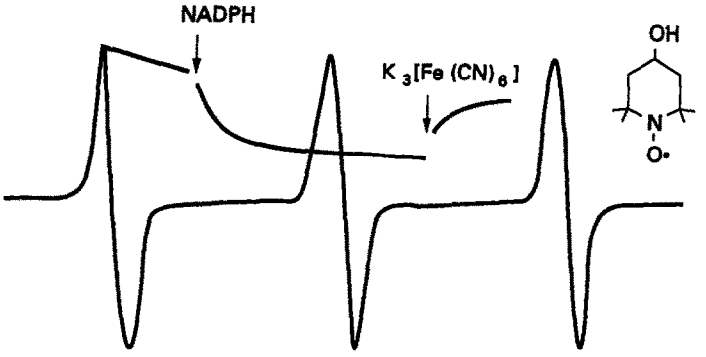

Fig. 1. Typical Tempol spectrum. Immediately after the recording, the magnetic field was set at the peak of the midfield line of the nitroxide, the field sweep was turned to zero and the recording time was set to 1000 sec. Oxygen partial pressure was kept constant (9 mg/l.) by flowing air on to the gas permeable sample tube. Initially the rate of reduction was measured without any further addition for about 5 min, then NADPH was added to the same sample; $K_3Fe(CN)_6$ was added eventually in order to re-oxidize to nitroxide the hydroxylamine formed.

Table 1. Effect of various metabolic inhibitors on the rate of reduction of Tempol in PB induced liver microsomes.

	− NADPH	+ NADPH
PB microsomes	272.5 ± 31.2	1050.7 ± 92.2
Non-induced microsomes	119.7 ± 7.2*	467.8 ± 40.1**
Metyrapone (0.2 mM)	212.8 ± 34.6	615.2 ± 65.7*
+ SKF525A (1 mM)	201.8 ± 35.6	1389.9 ± 67.1
+ Methimazole (10 mM)	248.1 ± 31.1	823.9 ± 63.2
Boiled	73.2 ± 6.3**	42.2 ± 6.4**

Data are in pmol/min/mg protein ± SE where $N \geq 6$.
* $P \leq 0.05$; ** $P \leq 0.001$, when compared to PB microsomes. Student's *t*-test.

Table 2. Effect of various treatments on Tempol rate of reduction in PB induced liver microsome

	− NADPH	+ NADPH
PB microsomes	272.5 ± 31.2	1050.7 ± 92.2
+ Menadione (0.2 mM)	321.3 ± 86.5	2134.1 ± 240.3**
+ $TlCl_3$ (0.8 mM)	138.8 ± 25.3*	713.1 ± 48.4*
+ SOD (100 U)	309.4 ± 75.8	782.7 ± 90.5*
+ Catalase (100 U)	239.7 ± 30.2	992.3 ± 54.2
+ SOD and catalase	215.4 ± 28.6	823.2 ± 45.3
+ GSH (5 mM)	229.1 ± 18.4	1091.7 ± 68.7
+ NEM (5 mM)	134.4 ± 23.8*	359.1 ± 70.2**
+ Ascorbate oxidase (5 U)	156.1 ± 15.3	1064.1 ± 110

Data are in pmol/min/mg protein ± SE where $N \geq 4$.
* $P \leq 0.05$; ** $P \leq 0.001$, when compared to PB microsomes. Student's *t*-test.

Involvement of the microsomal, NADPH-dependent, FAD-containing monoxygenase in the metabolism of Tempol was taken into consideration. The presence in the suspension of methimazole, a potent inhibitor of the microsomal FAD-containing monoxygenase, did not elicit any significant effect.

The involvement of sulfhydryl groups in the reduction of nitroxides has been reported [27]; therefore, we measured the effect of both NEM, which blocks SH-groups and of GSH, source of reactive sulfhydryl groups. NEM decreased the NADPH-dependent rate of reduction; the addition of GSH did not significantly modify the reduction rate (Table 2).

Tempol reduction was observed when NADPH was replaced by NADH in the PB-induced micro-

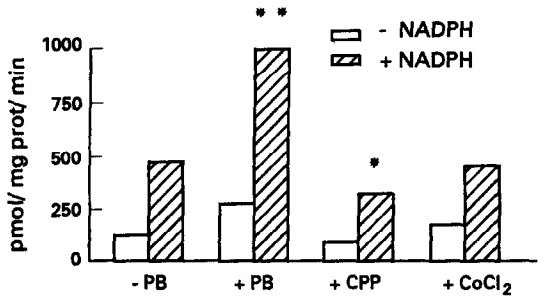

Fig. 2. Initial reduction rate of Tempol reduction observed in microsomes from control rats (−PB), pretreated with PB (+PB), pretreated with cobaltic protoporphyrin (CPP) and $CoCl_2$. Rates were significantly higher in PB microsomes (** $P \leq 0.001$, N=13), and significantly lower in CPP microsomes (* $P \leq 0.05$, N=3) in comparison with microsomes from control animals.

somal suspension, initial reduction rate was 425 ± 96 (N = 5) pmol/min/mg $prot^{-1}$.

Hydroxylamines are the main metabolites of the bioreduction process. The amount of the hydroxylamine in the microsomal suspension can be measured by regenerating the nitroxide adding the weak oxidant $K_3Fe(CN)_6$. Because of the reported effect of ferricyanide at high concentrations on the spectra of nitroxides [28], resulting in broadening of the spectral lines, the effect of ferricyanide was tested in an ample range of concentrations. The results summarized in Fig. 3 indicate that the recovery of the signal, due to re-oxidation of the hydroxylamine, is partial; hence the simple reduction to the hydroxylamine does not explain the loss of the ESR signal.

DISCUSSION

The results reported provide some detailed insight in the metabolism of nitroxide in microsomes. These results are based on a single water soluble nitroxide, extrapolation to other types of nitroxides (e.g. based on a different ring system and/or with different substituting groups) should be made cautiously. With this caveat in mind the picture that emerges is that the metabolism of nitroxide in microsomes is complex, resulting in at least two types of products: the

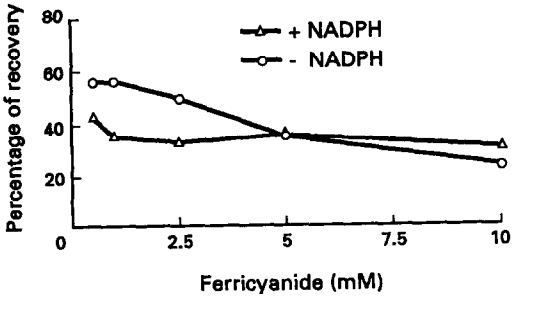

Fig. 3. Effect of increasing concentrations of $K_3Fe(CN)_6$ added to the microsomal suspension, at the end of an incubation performed with or without NADPH. Highest recovery was observed at low $K_3Fe(CN)_6$ concentrations; the lack of complete recovery indicate that the spin loss observed was due both to reduction and to destruction.

hydroxylamine and a product which cannot be oxidized back to the nitroxide by ferricyanide. In experiments performed on cells in culture, appropriate concentrations of ferricyanide can oxidize virtually 100% of hydroxylamine back to nitroxide [9], indicating that the only product of the metabolism, in that model system, is the hydroxylamine; this clearly does not apply in the microsome experiment.

The product, different from the hydroxylamine, has not been characterized in this study, though we suggest that it may arise from the radical–radical reaction between Tempol and carbon or peroxy radicals induced by NADPH addition to microsomes [29].

A significant metabolism of Tempol occurred in the absence of NADPH. The observed rate of reduction was significantly higher in PB induced microsomes both in the absence, or presence of NADPH. The reduction in the absence of NADPH was not due to residual endogenous NADPH, was not inhibited by NEM, $TlCl_3$, SOD, or stimulated by menadione (Table 2). The NADPH-independent reduction can only be partially attributed to a non-enzymatic mechanism (about 25% of reducing activity remained after boiling, Table 1) and does not appear to be related to the presence of ascorbate or sulfhydryl groups (Table 2).

NADPH-dependent metabolism does not relate to cytochrome P-450 presence. Inhibitors of the enzyme(s) had little or no influence on the reduction rate; treatments able to decrease significantly the amount of cytochrome P-450, $CoCl_2$ and CPP, did not affect Tempol reduction rates significantly. A modest effect by CPP, which diminishes both cytochrome and reductase, suggests that cytochrome P-450 reductase may have an important role. The involvement of the flavin centered enzyme is indicated by the inhibitory effect on Tempol reduction rate exerted by thallium chloride and by the stimulatory effect of menadione (Table 2). Support to this view is given by the observed direct interaction between reduced flavin or flavin radicals and nitroxides, described in the literature [30, 31]. The decisive evidence was obtained by the experiment carried out using purified cytochrome P-450 reductase. When Tempol and purified reductase (0.1 U/ml) were mixed, no variation of the Tempol signal was observed; a rapid reduction was induced by the addition of NADPH, giving a clear cut answer to our quest.

Superoxide anion could in part be responsible for Tempol reduction as indicated by some inhibitory effect observed when SOD was added to the microsomal suspension. The lack of an effect of catalase and the lack of a magnification of the combined SOD-catalase addition is consistent with superoxide anion rather than hydroxyl radicals or hydrogen peroxide being the participating species.

The spin loss still observed even in the presence of appropriate concentrations of inhibitors of the reductase or of SOD suggests that electron transfer to Tempol takes place directly at the active site of the flavoenzyme and superoxide anion-mediated reduction is secondary.

Flavin has been shown to be able to reduce nitroxides. In addition to P-450 reductase microsomes

possess a different FAD containing enzyme, the NADPH-dependent monoxygenase [32, 33]. This enzyme does not appear to be involved in the reduction of the nitroxide, because of the lack of any effect related the presence in the suspension of methimazole, a specific inhibitor of the enzyme (Table 1).

A mechanism of Tempol reduction described in cell cultures [27] is dependent on non-protein-bond sulfhydryl groups. In the microsomal suspension GSH is virtually absent and its addition to the suspension did not result in any relevant effect (Table 2). Protein bound sulfhydryl groups are essential to the activity of many enzymes. The inhibitory effect observed when NEM is added to the microsomal suspension is reasonably linked to the non-specific inhibitory effect of NEM on many enzymatic systems, including cytochrome P-450 reductase.

A final comment pertains to the observed NADH dependent reduction of Tempol. Being clearly demonstrated the central role played by the reductase in the microsomal reduction of Tempol, the NADH dependent reduction is plausibly related to cytochrome b_5 reductase and/or to other reductases which can easily transfer electrons from either NADH or NADPH.

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