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Relation of superoxide generation and lipid peroxidation to the inhibition of NADH-Q oxidoreductase by rotenone, piericidin A, and MPP^+

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SUMMARY. The addition of NADH to submitochondrial particles inhibited by agents which interrupt electron transport from NADH-Q oxidoreductase (Complex I) to Q_{10} (rotenone, piericidin A, and MPP^+) results in superoxide formation and lipid peroxidation. A study of the quantitative relations now shows that oxyradical formation does not appear to be the direct result of the inhibition. Although tetraphenyl boron (TPB^-) greatly enhances the inhibition by MPP^+, it has no effect on $O_2^{\bullet-}$ formation or lipid peroxidation. When submitochondrial particles completely inhibited by rotenone or piericidin A are treated with bovine serum albumin to remove spuriously bound inhibitor molecules without affecting those bound at the specific inhibition site, NADH-Q activity remains inhibited and lipid peroxidation occurs but superoxide formation ceases. Thus oxyradical formation may be the result of the binding of inhibitors at sites in the membrane other than those related to the inhibition of electron transport.

Studies in several laboratories established that in submitochondrial particles treated with amytal or rotenone to inhibit the passage of electrons from NADH-Q oxidoreductase (NADH dehydrogenase, Complex I) to Q, the addition of NADH or NADPH leads to superoxide generation and lipid peroxidation (1-3). Hasegawa *et al.* (4) reported that MPP^+ (N-methyl-4-phenylpyridinium), the neurotoxic metabolite of MPTP, likewise causes $O_2^{\bullet-}$ formation and lipid peroxidation in the presence of NADH or NADPH. Their findings were confirmed and extended by Cleeter *et al.* (5) and Singer *et al.* (6).

That treatment of membrane preparations with MPP^+ yields oxyradicals is of considerable interest since there have been recurring speculations in the literature that oxidative stress elicited by superoxide and lipid peroxidation may be a primary cause of the destruction of nigrostriatal cells initiated by MPTP intake. Although redox cycling of MPP^+, proposed (e.g. (7), (8)) as the mechanism of $O_2^{\bullet-}$ generation, has been shown to lack chemical basis under *in vivo* conditions (9), the observation that the inhibition of NADH oxidation by MPP^+ in submitochondrial particles generates $O_2^{\bullet-}$ provides a plausible mechanism for oxidative stress. Thus, reducing equivalents from NADH or NADPH, lodged in the flavin or Fe-S clusters of NADH-Q oxidoreductase, could reduce O_2 directly, although slowly, if electron transport to Q is interrupted. This would accentuate the primary damage to mitochondrial integrity caused by cessation of oxidative phosphorylation and ATP depletion.

Since the data of Hasegawa *et al.* (4) suggested that the inhibition of NADH oxidase activity by MPP^+ may not parallel the rate of $O_2^{\bullet-}$ synthesis, we decided to investigate the quantitative aspects of the relation of oxyradical generation and lipid peroxidation to the interruption of electron transport from NADH to Q. Our findings were briefly summarized recently (10) and are presented in detail and documented in this paper.

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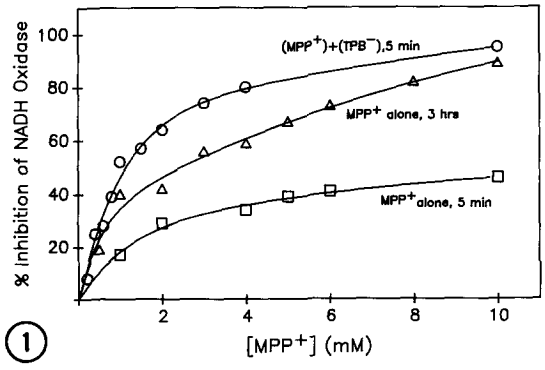
MATERIALS AND METHODS

Submitochondrial particles (ETP) from beef heart mitochondria were isolated (11) and NADH oxidase activity measured spectrophotometrically (12) as described. Superoxide dismutase was from Sigma. MPP^+, rotenone and piericidin A were obtained as in previous studies (13,14). The ETP particles were preincubated with inhibitor at 3 mg/ml protein concentration, in 0.25 M sucrose-0.05 M K phosphate, pH 7.5, for 5 min at 25° C, unless otherwise stated, prior to assay. Unspecifically bound rotenone and piericidin A were removed by washing the inhibited particles with buffered bovine serum albumin (BSA)-sucrose, as in previous work (14). $O_2^{\bullet-}$ generation was followed by oxidation of adrenaline to adrenochrome (2,4) but at 485 nm, in a computer-driven Hitachi U2000 spectrophotometer at 0.3 mg/ml ETP protein concentration against a blank without NADH. The method gave the same results as differential spectrophotometry at 485-585 nm. Lipid peroxidaton was followed by analyzing the malondialdehyde formed during a 5 min incubation in the presence of 0.2 mM ferric chloride, 2 mM ADP, inhibitor, and 0.5 mM NADH (3).

RESULTS

Superoxide production and lipid peroxidation initiated by MPP^+. The tetraphenylboron anion (TBP^-), an ion pairing reagent, greatly enhances the inhibition of mitochondrial oxidation of NAD-linked substrates by MPP^+ and its analogs (15-17). The effect has been erroneously ascribed (15, 18) to be entirely due to the increase in the uptake of MPP^+ by the mitochondria, although it has been shown that this does not account for most of potentiation of the inhibition (16). A major part of the effect is due to facilitating the penetration of MPP^+ into a hydrophobic pocket in NADH-Q oxidoreductase, constituting the inhibition site for interruption of electron transport to Q (16,17). TPB^- does not affect the oxidation of NAD^+-linked substrates in mitochondria, nor the inhibition of NADH oxidation in submitochondrial particles by neutral MPP^+ analogs, such as 4-phenylpyridine. Fig. 1. illustrates the inhibition of NADH-oxidase by $MPP^+ \pm TPB^-$. If the inhibitor is incubated with the ETP particles for 5 min at 25° C and immediately assayed, the resulting inhibition never exceeds 50% in the entire concentration range tested. If incubation with the inhibitor at 25° C is continued for 3 hours prior to assay, the inhibition by 10 mM MPP^+ reaches 80%. The same effect is seen if the preincubation with MPP^+ is at 4° C. In contrast, if a catalytic amount of TPB^- (10 μM) is present, over 90% inhibition is reached in 5 min or less (Fig. 1).



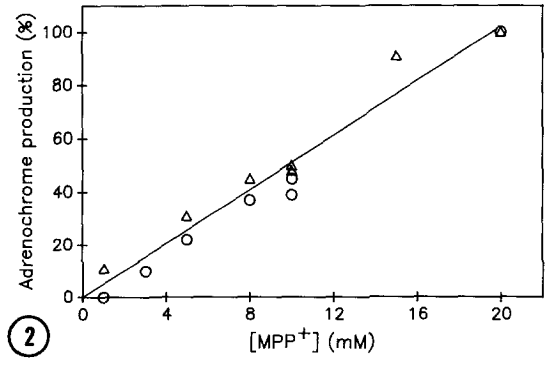


Fig.1. TPB^- enhances the inhibition of NADH oxidase by MPP^+. ETP (0.8 mg protein/ml) in 0.25 M sucrose-50 mM K phosphate, pH 7.6, was incubated at 25° C with MPP^+ alone (□) or with MPP^+ plus 10 μM TPB^- (○) for 5 min and assayed for NADH-oxidase activity immediately. The assay cuvette contained the same concentration of inhibitor. Another sample was incubated at 25° C with MPP^+ alone for 3 hours (△) before assay. Each point is the average of at least two assays.

Fig.2. $O_2^{\bullet-}$ production induced in ETP by $MPP^+ \pm TPB^-$. ETP (0.5 mg protein/ml) was in incubated at 25°C for 5 min with no additions (reference cuvette), 0.8 μM rotenone (to give the 100% value), or MPP^+ (○) or MPP^+ + 10 μM TPB^- (△). Adrenalin was added and the cuvettes equilibrate to 37° C for 5 min when NADH was added to initiate oxyradical production.

This slow penetration is of importance in the context of the data to be presented. It may also be the underlying cause of the variability reported for the degree of the inhibition of NADH oxidation by a given MPP^+ derivative by different laboratories, as well as for the reversal of the inhibition. Since the inhibition by MPP^+ and its analogs results from non-covalent binding to the enzyme, it is fully reversible. However, reversal of the inhibition by MPP^+ and its analogs on dilution is a slow process, like the development of the inhibition in the absence of TPB^- (Fig. 1), as shown by the fact that the diffusion of the inhibitor from the hydrophobic pocket is time-dependent. A report (5) that 52% of the inhibition attained on prolonged incubation of MPP^+ with submitochondrial particles was not reversed by washing, was very likely due to the short time (20 min) between dilution of the inhibited particles and assay, quite insufficient for complete dissociation of the inhibitor. In our hands, centrifugation, incubation with buffer alone at 4° for 2 hrs, followed by recentrifugation always results in complete reversal of the inhibition by MPP^+. The time-dependence of the development of the inhibition on preincubation of the particles with MPP^+ and the dramatic effect of TPB^- on the process were used to explore the correlation between the degree of inhibition of NADH oxidation and the amount of oxyradical generated. As seen in Fig. 2, TPB^- has no effect on $O_2^{\bullet-}$ generation induced by MPP^+, nor on lipid peroxidation (Table 1), in contrast to its effect on the rate and extent of the inhibition of NADH oxidation (Fig.1). Moreover, while the inhibition develops very slowly during preincubation in the absence of TPB^- (Fig. 1), $O_2^{\bullet-}$ generation does not increase after 5 min preincubation. Under the conditions of Fig. 1, the amount of adrenochrome formed 5 min after the addition of 5 mM MPP^+ to the particles was 35% of that given by excess rotenone and it did not increase after 2.8 hrs incubation but, instead, *decreased* to 22%. Malondialdehyde formation also failed to increase after 5 min. Although the amount of superoxide formed after the addition of NADH and adrenaline does not seem to depend on the time of preincubation of the particles with the inhibitor prior to assay, it varies linearly with MPP^+ concentration with or without TPB^- over a wide range of concentrations, except at very low levels of MPP^+ where reduced Q present in the preparation may quench the $O_2^{\bullet-}$ produced before it has a chance to react with adrenaline. These observations indicate that the degree of inhibition elicited by MPP^+ does not parallel the MPP^+-induced generation of superoxide or attendant lipid peroxidation.

Superoxide generation and lipid peroxidation initiated by rotenone and piericidin A. Rotenone, piericidin A, MPP^+ and its analogs all inhibit electron transport between the high potential Fe-S cluster of NADH-Q oxidoreductase and the Q pool (19,20). They are thought to bind in the same general region of this complex enzyme, because they displace each other competitively from the binding site. Thus, MPP^+ and its analogs have been shown to prevent the inhibition of NADH oxidation by piericidin A and rotenone (20,21). Among these piericidin A is by far the most potent inhibitor. Rotenone and piericidin are highly lipophilic and thus bind to mitochondrial components without saturation. Only a very small fraction, and presumably a

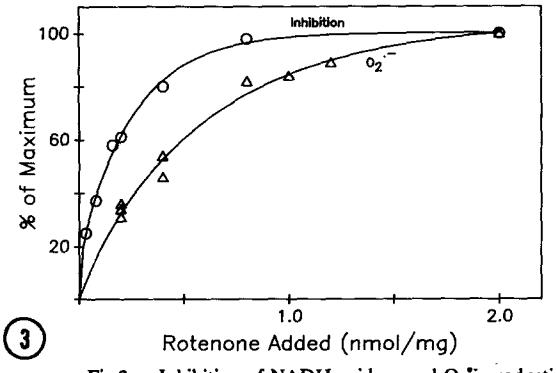
Table 1. TPB does not enhance lipid peroxidation induced by MPP^+ and ETP

	Malondialdehyde (nmol/mg)	
Inhibitor	Alone	+ TPB^-
Rotenone	1.75	1.85
MPP^+ (5 mM)	0.28	0.21
(20 mM)	0.44	0.48

ETP (3 mg/ml) was preincubated with rotenone (2 nmol/mg protein) or MPP^+ as indicated for 5 min at 25° C before assaying for lipid peroxidation in the presence of the same amount of inhibitor.

stoichiometric amount, is bound to the specific site responsible for the inhibition. The rest is spuriously bound, contributing little, perhaps not at all, to the inhibition. Washing the inhibited particles with 2% BSA - 0.25 M sucrose removes all the unspecifically bound rotenone and piericidin A, without dislodging piericidin from the specific site or reversing the inhibition. The less tightly bound rotenone is dissociated to a minor extent from the specific site by repeated washing with BSA-sucrose, but most of the inhibition remains (14). These specific and non-specific binding properties have not been taken into consideration in other laboratories (1,4). Fig. 3 is a rotenone titration, simulating conditions used by other workers: the inhibitor was present in the assay cuvette, and thus, the results represent the contribution of both specifically and unspecifically bound rotenone. It is seen that titration curves for $O_2^{\cdot-}$ generation and for inhibition do not coincide, although both effects increase with rotenone concentration. Fig. 4 shows the same titration curve after washing with BSA to remove spuriously bound rotenone. As seen, most of the inhibition remained despite removal of all unspecifically bound rotenone, but $O_2^{\cdot-}$ generation ceased completely. Lipid peroxidation was practically unaffected by repeated washing with BSA (not shown). It is clear that superoxide generation is not due to the binding of rotenone at the specific inhibition site but to other site(s) apparently unrelated to the primary inhibition, while lipid peroxidation seems to be the consequence of the inhibition of the enzyme. These conclusions are supported by similar experiments with the more tightly bound piericidin A. Fig. 5 shows that after complete removal of unspecifically bound piericidin A, NADH-Q oxidoreductase remains completely inhibited, malondialdehyde synthesis remains unimpaired but $O_2^{\cdot}$ generation is absent.

Further evidence that superoxide generation is not the main source of lipid peroxidation in the inner mitochondrial membrane is provided by the effects of succinate, superoxide dismutase and catalase. Succinate, by reducing Q, prevents $O_2^{\cdot-}$ accumulation but leaves malondialdehyde formation unaffected (97% of value with rotenone alone). Superoxide dismutase also destroys all $O_2^{\cdot-}$ but leaves the majority of malondialdehyde formation unaffected (66%). Catalase also partially inhibits malondialdehyde formation (51% remaining). The



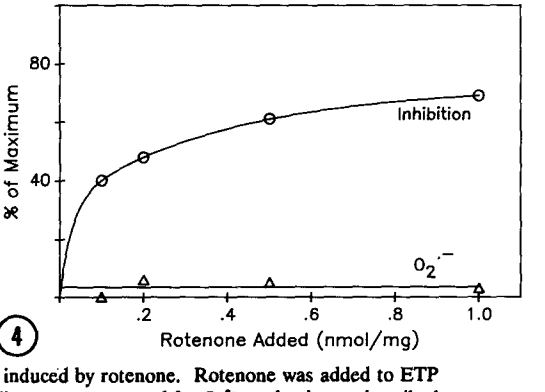


Fig.3. Inhibition of NADH oxidase and $O_2^{\cdot-}$ production induced by rotenone. Rotenone was added to ETP (3 mg/ml) and preincubated for 5 min at 25° C. Aliquots were assayed for $O_2^{\cdot-}$ production as described in Fig. 2 with 0.5 mg/ml ETP and rotenone at the same amount per mg protein, as in the preincubation and for NADH oxidase activity as described in Fig. 1.

Fig.4. Inhibition of NADH oxidase and $O_2^{\cdot-}$ production induced by specifically bound rotenone. Rotenone was added to ETP (3 mg protein/ml) followed by 0.2 mM NADH and incubated on ice for 10 min. The samples were chilled and BSA was added to 2% (w/v) concentration. After 1 hour on ice, tubes were centrifuged at 146,000 g for 20 min. The pellets were resuspended in 1 ml 0.05 M phosphate buffer (pH 7.4) containing 2% BSA and left on ice for 2 hours before centrifuging again. The pellets were resuspended in buffer to the original volume. Aliquots were assayed for NADH oxidase activity and adrenochrome formation as described in the Methods.

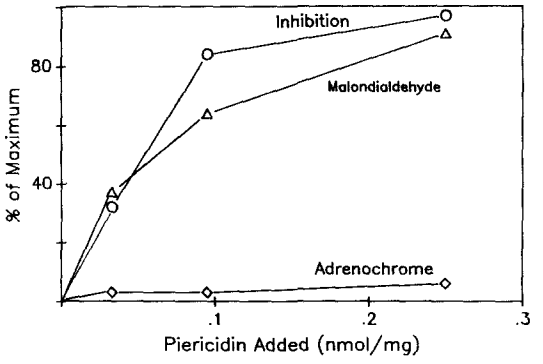


Fig.5. NADH oxidase inhibition, $O_2^{\bullet-}$ production and malondialdehyde formation induced by piericidin A. Piericidin A was incubated with ETP and the non-specifically bound inhibitor removed as described for rotenone in Fig.4. The washed ETP were assayed for NADH oxidase activity (shown as % inhibition, (O), adrenochrome formation (◇), and for malondialdehyde generated (Δ))as described in the Methods.

different effects of these and of the various treatments in Figs. 4 and 5 on superoxide formation and lipid peroxidation are not surprising, in view of the fact that lipid peroxidation may be initiated by several agents besides superoxide (22).

DISCUSSION

Superoxide generation and lipid peroxidation initiated by oxyradicals is thought to occur both in the NADH dehydrogenase (1-3) and succinate dehydrogenase (23) segments of the respiratory chain in the presence of the appropriate substrate and an inhibitor which blocks electron flux to Q. Lipid peroxidation in the former system is usually increased in the presence of ADP-Fe^{3+} chelate, whereas in the latter system it also occurs in the absence of an added iron chelate (24). In the Complex I region both NADH and NADPH induce $O_2^{\bullet-}$ generation and lipid peroxidation (2,3,24) but while the radical generation is thought to be initiated with either substrate reacting with the substrate site of NADH dehydrogenase, the subsequent enzymatic pathway may be different for the two nucleotides (24). A further complication is the report that in heart mitochondria, a major source of $O_2^{\bullet-}$ generation is due to an external NADH dehydrogenase associated with the inner membrane, which is thought to link cytoplasmic NADH to the respiratory chain (25).

Although there are conflicting reports about the requirements for oxygen radical formation at other sites, there seems to be general agreement that radical formation in the NADH dehydrogenase region induced by NADH requires rotenone or another inhibitor blocking specifically the "rotenone site". This fact and the availability of a wide range of inhibitors reacting at that site prompted us to try to delineate more closely the site of oxygen radical formation in this segment. When an inner membrane preparation from heart (ETP) was fully inhibited by rotenone or piericidin A and the spuriously bound inhibitor was removed from unspecific binding sites by washing with BSA, leaving the NADH oxidation inhibited, superoxide formation was absent. This suggested that the action of rotenone and piericidin in initiating $O_2^{\bullet-}$ generation was not related to inhibition of NADH oxidation but to binding at other site(s). This was in accord with the differences in the appearance of enzyme inhibition and of $O_2^{\bullet-}$ formation on titration with rotenone (Fig. 3).

Unlike superoxide generation, lipid peroxidation is related to the extent of inhibition of electron transport from NADH dehydrogenase to the Q pool (Fig. 5). Although this experiment and the differential effect of various treatments on $O_2^{\bullet-}$ generation and lipid peroxidation suggest that $O_2^{\bullet-}$ is not the primary source

of oxygen radicals for lipid peroxidation, interestingly, neither process is enhanced by increasing the inhibitory effect of TPB^- on NADH oxidation (Fig. 2 and Table 1).

The observations described in this paper suggest that oxygen radical formation is not directly related to the inhibition of electron flux from NADH dehydrogenase to the respiratory and the consequent reaction of the reduced prosthetic groups of the enzyme with molecular O_2.

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