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L-Deprenyl as an inhibitor of menadione-induced permeability transition in liver mitochondria

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Abstract

L-Deprenyl, an inhibitor of mitochondrial monoamine oxidase B (MAO B), inhibits the swelling of liver mitochondria induced by the pro-oxidant 2-methyl-1,4-naphtoquinone with a K_i dependent on quinone concentration. L-Deprenyl also inhibits the collapse of membrane potential, cation efflux, pyridine nucleotide oxidation and cytochrome *c* release, all events which accompany the osmotic change and are typical of membrane permeability transition induction, thus emphasizing the inhibitory effect of the drug on this phenomenon. Results show that this inhibition is not due to the effect of L-deprenyl on monoamine oxidase activity but is most likely due to a direct interaction of the drug with the pore forming structures. It is here proposed that L-deprenyl, being a propargylamine, at physiological pH has a protonated amino group able to interact with critical aromatic or anionic amino acidic residues. As a consequence, the opening of the transition pore is prevented. These results indicate a more generalized protective effect of L-deprenyl on mitochondrial functions, involving the inhibition of membrane permeability transition induced not only by the oxidation of substrates of MAO B, but also by pro-oxidant agents such as 2-methyl-1,4-naphtoquinone, which does not involve MAO B activity.

Keywords: L-Deprenyl; Menadione; Mitochondria; Permeability transition; Reactive oxygen species; Propargylamines

1. Introduction

L-Deprenyl, also called selegiline (Scheme 1), is a selective and irreversible inhibitor of mitochondrial MAO B [1], which is a major enzyme in the catabolism of catecholamines. L-Deprenyl has been reported to have a neuroprotective activity. It is currently used for the treatment of Parkinson's disease, in which it has been shown to delay the progression of symptoms in patients [2,3] and in Alzheimer's disease in which it improves the performance of patients [4,5]. It has been proposed that this neuroprotective effect is due to the prevention of apoptosis [6–8]. This proposal is also supported by observations, in animal models, in which L-deprenyl protects neurons against oxidative stress-induced apoptotic cell death [3,9,10]. Furthermore, the drug also protects cultured catecholamine cells from apoptotic death induced by deprivation of neurotrophic factor [3,9,11]. A number of mechanisms have been proposed to explain these effects. An underlying premise is that L-deprenyl may prevent oxidative stress due to the increased turnover of dopamine [12]. In fact, the inhibition of MAO B impairs the degradation of catecholamines, dopamine in particular, which results in reduced production of hydrogen peroxide formed during the reaction and consequently less damage [13]. In this regard, it would appear that L-deprenyl is able to protect Parkinson patients against the oxidative stress of increased dopamine turnover [12].

Some authors have proposed that the protective effect of L-deprenyl is cell-type specific due to the general

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Abbreviations: DTE, dithioerythritol; MAO, monoamine oxidase; menadione, 2-methyl-1,4-naphtoquinone; MPT, mitochondrial permeability transition; RLM, rat liver mitochondria; TPP^+, tetraphenylphosphonium; $\Delta\psi$, mitochondrial membrane potential.

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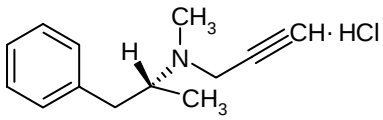

Scheme 1. Structural formula of the L-deprenyl (selegiline hydrochloride).

observations that its action is evident at the level of neuronal cells [14,15]. However, other investigations point to drug activity in extra-brain tissues, an activity that involves anti-tumorigenic as well as immunomodulatory effects [16]. Furthermore, some reports emphasize that the protective effects exhibited by L-deprenyl do not appear to involve the inhibition of MAO B [17,18]. In this view it has been suggested that the drug regulates the expression of apoptosis related genes [17]. Moreover, L-deprenyl reduces PC12 cell apoptosis at concentrations too low to inhibit MAO and the cell protection is likely due to the induction of new protein synthesis [14].

Other authors have proposed that L-deprenyl inhibits caspase-3, an enzyme involved in the pro-apoptotic process [19]. In the same paper it is also reported that high L-deprenyl concentrations exhibit pro-apoptotic action. In addition, the study also pointed out that the agent responsible for these effects is a putative metabolite of L-deprenyl [19]. Stabilization of the mitochondrial membrane and influencing the balance between the Bcl-2 and Bax genes were also considered as important factors in the mechanism of action of L-deprenyl [18]. Furthermore, it has also been reported that the drug is able to inhibit the mitochondrial permeability transition (MPT) induced by dopamine oxidation in brain mitochondria [20].

The MPT is a phenomenon strictly associated with apoptosis and is due to the opening of a protein pore mediated by the presence of supraphysiological Ca^{2+} concentrations and other effectors such as pro-oxidant agents. The opening of the transition pore induces non-specific solute traffic across the mitochondrial membrane that leads to the bioenergetic collapse of the organelle (for a review of MPT see Ref. [21]). This is the case of dopamine oxidation, which produces free radicals and quinone [22], powerful activators of MPT. This finding indicates that the inhibitory effect of L-deprenyl on MPT may be accomplished by the inhibition of mitochondrial MAO.

These observations regarding L-deprenyl protection, some of which are controversial (e.g. the tissue specificity and the involvement of MAO activity), together with other observations not yet well clarified (the role of MPT), have led us to investigate the effect of L-deprenyl on liver mitochondrial functions affected by 2-methyl-1,4-naphtoquinone (menadione). Quinones and their phenol precursors are common in the human diet [23] and are quite toxic since they can act as electrophiles or single electron acceptors [24] to yield the semiquinone radical [25].

Menadione triggers oxidation of endogenous pyridine nucleotides, cyanide-insensitive O_2 consumption and a transient decrease in mitochondrial membrane potential ($\Delta\psi$) in intact mitochondria [26]. In the presence of intramitochondrial Ca^{2+}, the menadione-induced oxidation of pyridine nucleotides is accompanied by their hydrolysis. Ca^{2+} is released from mitochondria, and they remain unaffected, provided excessive Ca^{2+} cycling is prevented [26]. The aim of this paper is to ascertain if menadione behaves as a typical MPT inducer and if L-deprenyl is able to act as an inhibitor of this phenomenon without requiring the activity of MAO B for its induction.

2. Materials and methods

2.1. Materials

HEPES, EGTA, rotenone, dithioerythritol (DTE), L-deprenyl hydrochloride and menadione were purchased from Sigma-Aldrich; purified mouse anti-cytochrome *c* monoclonal antibody 556433 from BD Biosciences. Anti-mouse IgG-horseradish peroxidase, enhanced chemiluminescence reagents and Hyperfilm were obtained from Amersham. All other reagents were of the highest quality available.

2.2. Mitochondria isolation and standard incubation procedures

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM HEPES (pH 7.4) and 1 mM EGTA [27]; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with BSA as a standard [28].

Mitochondria (1 mg protein/mL) were incubated in a water-jacketed cell at 20°. The standard medium contained 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 50 μM Ca^{2+} and 1.25 μM rotenone. Variations and/or other additions are given with each experiment.

2.3. Determination of mitochondrial functions

Mitochondrial swelling was determined by the change in the absorbance of mitochondrial suspensions at 540 nm using a Kontron Uvikon mod. 922 spectrophotometer equipped with thermostatic control.

The membrane potential was calculated on the basis of the distribution of the lipid-soluble cation tetraphenylphosphonium (TPP^+) across the inner membrane, measured with a TPP^+-specific electrode [29].

Ca^{2+} and Mg^{2+} contained in the supernatants were measured by atomic absorption spectroscopy, using a Perkin-Elmer 1100B spectrometer.

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed in a Aminco-Bowman 4-8202 spectrofluorometer operating at excitation

and emission wavelengths of 366 and 450 nm, respectively, with a slit of 5 nm.

2.4. Detection of cytochrome c release

Mitochondria (4 mg/mL) were incubated for 15 min at 20° in the standard medium with the appropriate additions. The reaction mixtures were then centrifuged at 13,000 *g* for 10 min at 4° to separate mitochondrial pellets from supernatant fractions, that were further spun at 100,000 *g* for 15 min at 4° to eliminate mitochondrial membrane fragments. Proteins of the supernatant fractions were separated by electrophoresis on 12% polyacrylamide/SDS vertical slab gel, transferred to nitrocellulose and immunoblotted using a mouse anti-cytochrome *c* monoclonal antibody and visualized using an enhanced chemiluminescence Western blotting detection system.

3. Results

RLM suspended in standard medium as described in Section 2, in the presence of 100 μM menadione and 50 μM Ca^{2+}, exhibit an apparent absorbance decrease at 540 nm of about 1.1 units in comparison with controls (Fig. 1A). This observation is indicative of the occurrence of a large amplitude colloid-osmotic swelling of RLM. Menadione effect is dose-dependent. Lower concentrations induce slower rates of swelling (results not reported). The induction of such an osmotic effect is paralleled by a complete collapse of $\Delta\psi$ (Fig. 1B). In fact, as can be observed in this figure, RLM normally exhibiting a $\Delta\psi$ value of about 180 mV (see control curve), when treated with menadione in the presence of Ca^{2+}, undergo a rapid drop in membrane potential. Furthermore, these mitochondrial alterations are accompanied by endogenous cation efflux (Fig. 2) and the oxidation of pyridine nucleotides (Fig. 3). Endogenous Mg^{2+} (28 nmol/mg protein) is almost completely released after 15 min of incubation (Fig. 2A), while exogenous Ca^{2+}, after being accumulated in the matrix is also released to a large extent at the same time (Fig. 2B).

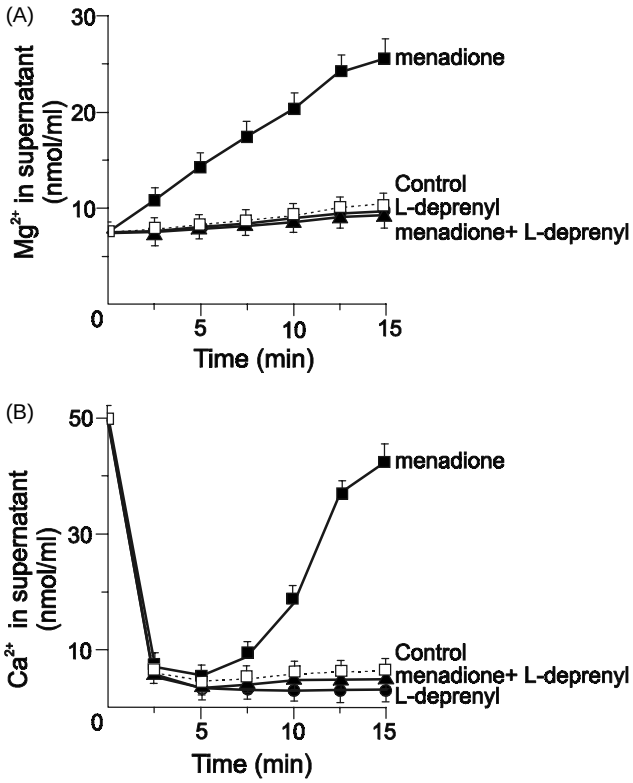

Fig. 2. Efflux of endogenous Mg^{2+} (A) and accumulated Ca^{2+} (B) induced by menadione; prevention by L-deprenyl. Experimental conditions and reagent concentrations as in legend to Fig. 1. The initial endogenous concentrations of Mg^{2+} and Ca^{2+} were 28 ± 1.45 nmol/mg protein and 10 ± 0.51 nmol/mg protein, respectively. Values are means of four observations ±SD.

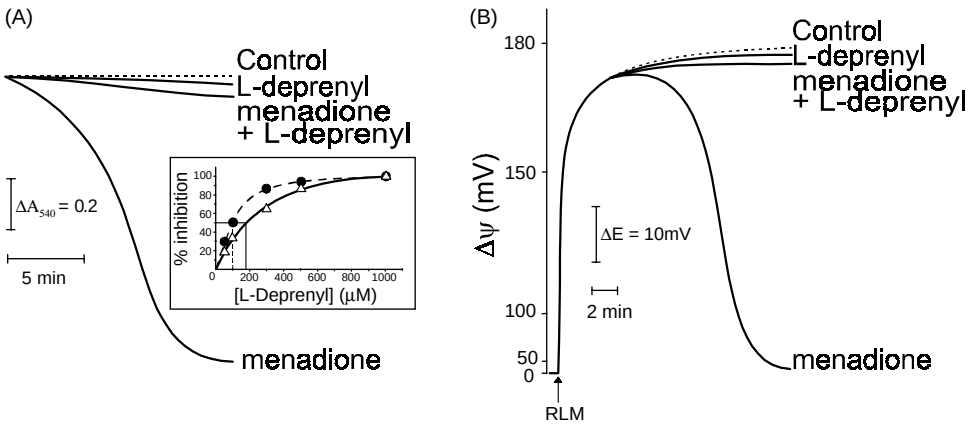

Fig. 1. Mitochondrial swelling (A) and collapse of $\Delta\psi$ (B) induced by menadione and their inhibitions by L-deprenyl. RLM were incubated in standard medium under the conditions indicated in Section 2. The control trace, also for the subsequent experiments (dashed lines in all the figures) refers to mitochondria incubated in standard medium in the absence of L-deprenyl. Where indicated, 100 μM menadione and 500 μM L-deprenyl were present. Panel A: downward deflection indicates mitochondrial swelling. The inset shows the calculation of IC_{50} values of L-deprenyl inhibition on swelling induced by 100 μM (continuous line) or 50 μM (dashed line) menadione. Panel B: 1 μM TPP^+ was added for $\Delta\psi$ measurements. ΔE is the electrode potential. Data are representative of five similar experiments.

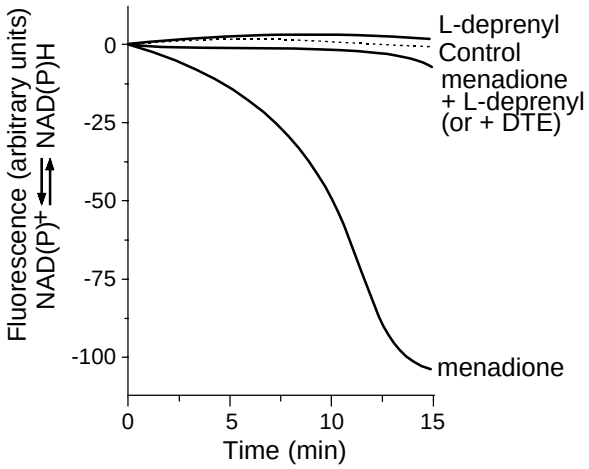

Fig. 3. Oxidation of endogenous pyridine nucleotides by menadione; inhibition by L-deprenyl or DTE. Experimental conditions and reagent concentrations as in legend to Fig. 1. When present, DTE concentration was 5 mM. The experiment began with the addition of menadione. A typical experiment is reported. Three additional experiments gave comparable results.

L-Deprenyl alone does not exhibit any effect on membrane integrity (see Figs. 1–3). It should also be emphasized that it does not affect the instantaneous efflux (7–8 nmol/mg protein) of Mg^{2+}, taking place at time-zero which is due to the equilibration of Mg^{2+} present in the intermembrane space, with the incubation medium (Fig. 2A). Furthermore, the drug does not alter the rate nor the extent of Ca^{2+} accumulation in mitochondria (Fig. 2B).

The incubation of mitochondrial suspensions with menadione in the presence of Ca^{2+} also induces a large decrease in fluorescence, indicating oxidation of the $NAD^+/NADP^+$ pool (Fig. 3). All these events are completely blocked by the immunosuppressant cyclosporin A (results not reported). The mitochondrial changes brought about by menadione plus Ca^{2+} and the protection exhibited by cyclosporin A are typical events of MPT induction [21].

When L-deprenyl is present in the incubation medium, at a concentration of 500 μM it exhibits a complete prevention of all the observed alterations as is the case of cyclosporin A. In fact, the drug is able to strongly inhibit any osmotic effect (Fig. 1A), maintains $\Delta\psi$ at about 180 mV (Fig. 1B), hampers Mg^{2+} and Ca^{2+} loss (Fig. 2), and maintains endogenous pyridine nucleotides in the reduced state (Fig. 3). The IC_{50} value of L-deprenyl's inhibitory effect on 100 and 50 μM concentrations of menadione has been calculated to be about 180 and 100 μM, respectively (see inset in Fig. 1A).

The onset of MPT is strictly associated with the release of some mitochondrial soluble factors (e.g. cytochrome *c* [30]), an event most likely due to the stretching of the inner membrane during mitochondrial swelling and the rupture of the outer membrane, and considered the first step of the pro-apoptotic pathway [30]. The results reported in Fig. 4 show that menadione plus Ca^{2+} induces the loss of a

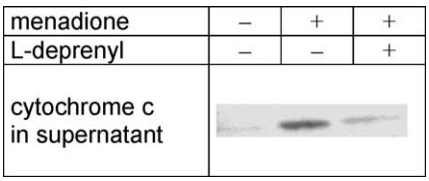

Fig. 4. Release of cytochrome *c* induced by menadione and prevention by L-deprenyl. The result of Western blotting of supernatant fractions is shown. Experimental conditions and reagent concentrations as indicated in Section 2.

consistent amount of cytochrome *c* from mitochondria after 15 min of incubation, the same time in which the other typical mitochondrial alterations of MPT induction can be observed. Instead, in the presence of L-deprenyl, cytochrome *c* is retained by mitochondria.

All the effects induced by menadione are completely prevented by cyclosporin A (results not reported).

Experiments performed in an isotonic KCl-medium have given similar results (results not reported).

4. Discussion

The results reported here demonstrate that liver mitochondria loaded with supraphysiological Ca^{2+} concentrations treated with menadione, without preventing excessive Ca^{2+} cycling which is promoted in this condition, undergo the phenomenon of MPT (see Figs. 1–3). The presence of L-deprenyl in the incubation medium completely prevents all the effects induced by the quinone (Figs. 1–3) by exhibiting IC_{50} values depending on menadione concentrations (inset in Fig. 1A).

The MPT is the result of the oxidative stress caused by the semiquinone radical generated by the interaction of menadione with the respiratory chain. The semiquinone radicals participate in a redox cycle of superoxide radical production by transfer of electrons to O_2 [31,32]. Superoxide radical and its metabolic product H_2O_2 may form hydroxyl radicals, which are toxic due to their extreme and indiscriminate activity. The results reported in Fig. 3 showing the oxidation of pyridine nucleotides sensitive to the reducing agent DTE, account for the oxidative stress induced by menadione.

The precise mechanism by which pro-oxidants induce MPT remains difficult to understand. Previous studies indicate that two sites contribute to MPT induction and modulation [33]. The first site, called the "S" site, has been identified as a membrane dithiol [33,34] whose oxidation appears to be controlled by the reactive oxygen species produced by the pro-oxidants [34]. The second site, called the "P" site, remains chemically undefined, and the mechanism of its action is unknown. However, apart from the precise mechanism of MPT induction, it is clear that MAO B activity is not involved in this process, since the oxidative catabolism of menadione is not catalyzed by

MAO B as mentioned above [32]. The results reported in this paper are indicative of a mechanism different from that of MAO B inhibition exhibited by L-deprenyl when it prevents MPT induction. This likelihood was discussed elsewhere, when the effects of other MAO inhibitors, clorgyline and pargyline on MPT induced by Ca^{2+} plus phosphate [35] were studied.

A mechanism that might explain the protective effect of L-deprenyl can be construed by considering its chemical structure (see Scheme 1). L-Deprenyl is a propargylamine compound with a *tertiary* amino group and an acetylene group. It differs from the others propargylamine derivatives for the presence of a chiral carbon that gives the characteristic stereospecificity. The presence of a *tertiary* amino group having pK_a of about 9.20 makes the molecule of L-deprenyl protonated for about 98.5% at physiological pH so that it behaves as a monovalent cation. It follows that L-deprenyl can act in the same way as several other molecules having one or more amino groups able to inhibit MPT (i.e. polyamines, monoamines, ruthenium red, carnitine and tetracaine) [21]. This effect can be explained as an almost general inhibitory effect of these amines observed on most K^+-channels [36–38]. Most likely, the transition pore has preserved structural features of the archetypal K^+-channel [39] that permits interaction with amines, in this case with L-deprenyl, resulting in MPT inhibition.

The interaction of the monovalent cationic drug with critical amino acid residues present in the pore forming structures can be responsible for the observed inhibition. In this regard, L-deprenyl can interact with the ring of aromatic residues by means of cation-π interactions [40] or with carboxylate ions by means of weak electrostatic interactions [41].

The results reported in Fig. 2 showing that L-deprenyl induces a protective effect on MPT without affecting the instantaneous efflux of Mg^{2+} from the intermembrane space (Fig. 2A) and without affecting Ca^{2+} transport (Fig. 2B), further confirm a direct action of the drug on the pore forming structures. The presence of Mg^{2+} in the intermembrane space would constitute a barrier against the opening of the transition pore [42]. An inhibition of Ca^{2+} transport would take away one of the main causes of MPT induction [21].

The results reported in this paper provide new insight to the previous proposal about the protective effects of L-deprenyl on apoptosis [14,17,19]. The observations that L-deprenyl is able to protect RLM against oxidative stress by a mechanism which differs from that of MAO B activity inhibition, demonstrates a more generalized protective effect of the drug that involves inhibition of MPT and that can be observed not only in brain but also in liver as confirmed here, and this is most likely true for other tissues as well. The inhibition of MPT induction by L-deprenyl also accounts for the protective effect observed in the cases of mitochondria-mediated apoptosis not induced by dopamine oxidation.

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