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Clorgyline and other propargylamine derivatives as inhibitors of succinate-dependent H_2O_2 release at NADH:UBIQUINONE oxidoreductase (Complex I) in brain mitochondria

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Abstract Complex I is the main O_2^- producer of the mitochondrial respiratory chain. O_2^- release is low with NAD-linked substrates and increases strongly during succinate oxidation, which increases the QH_2/Q ratio and is rotenone sensitive. We show that the succinate dependent O_2^- production (measured as H_2O_2 release) is inhibited by propargylamine containing compounds (clorgyline, CGP 3466B, rasagiline and TVP-1012). The inhibition does not affect membrane potential and is unaffected by ΔpH modifications. Mitochondrial respiration is similarly unaffected. The propargylamines inhibition of O_2^-/H_2O_2 production is monitored also in the presence of the Parkinson's disease toxin dopaminochrome which stimulates O_2^- release. Propargylamine-containing compounds are the first pharmacological inhibitors described for O_2^- release at Complex I.

Keywords Reactive oxygen species · Brain mitochondria · Complex I · Succinate · Reverse electron flow · Propargylamine containing compounds · Dopamine-derived dopaminochrome

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Abbreviations

ROS	reactive oxygen species
O_2–	superoxide
MAO	monoamine oxidase
NO	nitric oxide
SOD	superoxide dismutase
CoQ	coenzyme Q
$\Delta\Psi$	mitochondrial membrane potential
ΔpH	mitochondrial pH gradient
Δp	mitochondrial proton motive force
PD	Parkinson disease
MOPS	4-morpholinepropanesulfonic acid
BSA	bovine serum albumin
HRP	horse-radish peroxidase
DACHR	dopaminochrome
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Introduction

Several reactive oxygen species (ROS) generators are recognized in mammalian cells including NAD(P)H oxidases, monoamine oxidases (MAO), mitochondrial respiration, xanthine oxidase, auto-oxidation of catecholamines and uncoupled NO synthase (NOS). Among these mitochondrial respiration is the major production system and superoxide appears to be the primary ROS produced as the result of single electron reduction of O_2. Mitochondrial Complex I is one of the three proton pumps in the respiratory chain; furthermore it is the main site of O_2^- production in the mammalian respiratory chain. O_2^- is produced intramitochondrially and is dismutated into H_2O_2

by Mn–SOD: the permeable H_2O_2 is measured in the extramitochondrial space (St-Pierre et al. 2002). The process of H_2O_2 production is highly controlled and levels of H_2O_2 generation vary by approximately ten times in coupled substrate-treated brain mitochondria. Specifically, H_2O_2 generation with NAD-linked substrates (glutamate/malate or pyruvate/malate) is low, while the release with succinate is much higher. H_2O_2 production with succinate (a substrate whose electrons reach CoQ from Complex II, without passing through Complex I) is nevertheless attributed to Complex I, since it is strongly inhibited by the Complex I inhibitor rotenone. The succinate effect was attributed to a reverse mode of electron flow from reduced CoQ into Complex I, driven by the membrane potential ($\Delta\Psi$) generated by succinate oxidation (Turrens and Boveris 1980; Hansford et al. 1997; Liu et al. 2002; Korshunov et al. 1997; Votyakova and Reynolds 2001; Han et al. 2003). The succinate-driven H_2O_2 production is induced by low submillimolar (physiological) succinate concentrations. Furthermore such H_2O_2 generation is not significantly inhibited by the co-oxidation of NAD-linked substrates in mM concentration (also physiological). Besides, succinate does not inhibit fully the forward electron flow in Complex I, since α-ketoglutarate production from glutamate/malate (G/M) is only decreased (but not stopped) by succinate. These properties led us to conclude (Zoccarato et al. 2007) that succinate and G/M appear to compete for oxidation (i.e. there is no net reverse electron flow) while the succinate capability to form high H_2O_2 is not strongly depressed. We tentatively interpreted these results (i.e. the high succinate- induced H_2O_2 generation) as a consequence of the succinate-induced increase of the QH_2/Q ratio rather than of reverse electron transfer at Complex I (Zoccarato et al. 2007). Indeed also G/M alone, without succinate, can stimulate a high H_2O_2 production rate in cytochrome c depleted mitochondria, where the slowing of electron transfer to cytochrome c oxidase also determines an increase of the QH_2/Q ratio (Kushnareva et al. 2002).

The importance of ROS removal from the mitochondrial matrix is demonstrated by the poor survival of Mn–SOD-null mice (Melov et al. 1999) and by the increased life span of mice expressing mitochondrial catalase (Schriner et al. 2005). While ROS have been considered important in various pathologies (Halliwell and Gutteridge 1999) and aging (Cadenas and Davies 2000), it is now recognized that ROS are physiological mediators and signalling molecules (Allen and Tresini 2000; Paravicini and Touyz 2006). Also mitochondrial Complex III is known to produce O_2^- (mainly on the cytosolic space). However this O_2^- release is largely dependent on the electron transfer inhibitor antimycin A (Turrens et al. 1985).

Clorgyline is a propargylamine derivative, known as a strong irreversible inhibitor of MAO A (Youdim et al. 2006). MAO inhibitors are used pharmacologically in the treatment of Parkinson's disease (PD), to decrease the loss of dopamine typical of that condition, and as antidepressants. We show in this study that clorgyline and other propargylamine derivatives are inhibitors of succinate-dependent H_2O_2 release independently of their effect as MAO inhibitors.

Materials and methods

Reagents Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was from Molecular Probes. HRP (horseradish peroxidase; grade I;EC 1.11.1.7), nigericin, clorgyline, pargyline and deprenyl were supplied from Sigma. Rasagiline and ladostigil (TVP-1012) were supplied from TEVA Pharmaceuticals Industries, Netanya (Israel) and CGP 3466B from Novartis, Basel (Switzerland). All other reagents were of analytical grade.

Preparation of rat brain mitochondria Brain mitochondria were isolated as described in (Zoccarato et al. 2004). Briefly, the cerebral cortices of two 6–7-week-old rats were rapidly removed into 20 ml of ice-cold isolation medium (320 mM sucrose, 5 mM MOPS, 0.5 mM EDTA and 0.05 mM EGTA, pH 7.3) and homogenized. The homogenate was centrifuged at 900×*g* for 5 min at 4°C, and the supernatant spun again at 8,500×*g* for 15 min. The resulting pellet was resuspended in 2 ml of isolation medium, layered on a discontinuous gradient consisting of 4 ml of 6% Ficoll, 1.5 ml of 9% Ficoll, and 4 ml of 12% Ficoll (all prepared in isolation medium) and centrifuged at 75,000×*g* for 30 min. The myelin, synaptosomal, and free mitochondrial fractions formed above the 6% layer, as a doublet within the 9% layer, and as a pellet, respectively. The pellet was resuspended in 250 mM sucrose and 10 mM K-MOPS, pH 7.3, and centrifuged at 8,500×*g* for 15 min before being resuspended in this last medium to 10–20 mg of protein per milliliter by the Gornall protein assay. Only mitochondrial preparations that exhibited a respiratory control ratio greater than 5 were used in this study.

Standard incubation method Mitochondria (0.5–0.7 mg/ml) were incubated at 30°C in 125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 500 μg/ml defatted BSA, 20 mM Mops, pH 7.3 (adjusted with KOH) and 100 μM EGTA. Further additions were as specified in the figure legends.

H_2O_2 measurements H_2O_2 was measured with 7 μM Amplex Red and 15 μg/ml HRP (3.75 units) included in the incubations. H_2O_2 was detected by the formation of the fluorescent Amplex Red oxidation product resorufin using excitation and emission wavelengths of 563 and 587 nm respectively on a Shimadzu RL-5000 spectrofluorometer in

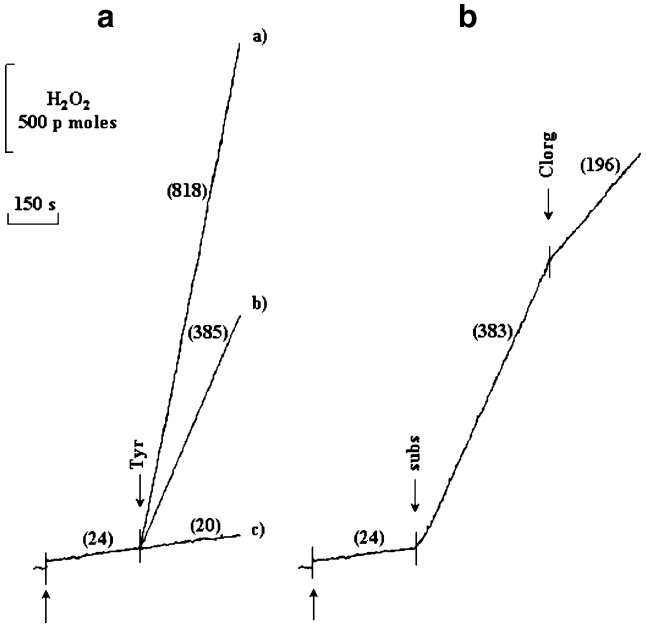

Fig. 1 Clorgyline inhibition of metabolic substrates induced H_2O_2 release is unrelated to MAO inhibition. **a** (*trace a*) MAO-dependent H_2O_2 production with 75 μM tyramine (Tyr); (*trace b*) 10 μM clorgyline was preincubated 5 min with mitochondria before tyramine addition; (*trace c*) pargyline (10 μM) was preincubated 5 min with mitochondria, before tyramine addition. **b** Mitochondria were pre-incubated 5 min with pargyline (10 μM), followed by tyramine (75 μM). Then 1 mM glutamate, 1 mM malate and 0.4 mM succinate were added (*subs*) and finally clorgyline (10 μM) was added. *Arrows* indicate mitochondria additions. In *parenthesis* the values of H_2O_2 in picomoles × per milligram × per minute. Traces are representative of duplicate traces from at least four independent experiments

a stirred cuvette (Zoccarato et al. 2005). H_2O_2 release was linear at least 6–8 min. The H_2O_2 calibration scale is linear in the 0–5 μM range, and, at the end of each assay, traces were calibrated by the addition of H_2O_2 (500 pmol).

Alternatively H_2O_2 was measured essentially as in (Bortolami et al. 2008) using a Fluoroskan Ascent FL plate reader in 24 wells plates. The reaction was started with addition of the Amplex red 20 μM. H_2O_2 was detected by the formation of the fluorescent product resorufin (544/590 nm ex/em wavelengths). Internal standard of 1 nmol H_2O_2 was added at the end of each assay. The wells were read every minute and mean reading of every well was considered. The first 10 min rates were calculated.

Measurements of mitochondrial membrane potential (ΔΨ) ΔΨ was measured using fluorescence quenching of the cationic dye safranine (3 μM) at 495 nm excitation and 586 nm emission as in (Zoccarato et al. 2004).

Results

The clorgyline inhibition of the succinate-induced H_2O_2 production is independent of MAO inhibition

Clorgyline and pargyline are two powerful inhibitors of H_2O_2-generating MAO. Clorgyline is more specific for MAO A and pargyline for MAO B (Youdim et al. 2006). Mitochondria also contain respiratory chain (mainly Complex I) dependent $O_2^{\cdot-}/H_2O_2$ generation whose rate with succinate is very high (being rotenone-sensitive is to be ascribed to Complex I), and with NAD-linked substrates is much lower. While performing experiments on Complex I-dependent H_2O_2 generation we noticed that the succinate-induced (high) peroxide was strongly inhibited by clorgyline (and much less so by pargyline). The independence of the clorgyline effect on its action on MAO is shown in

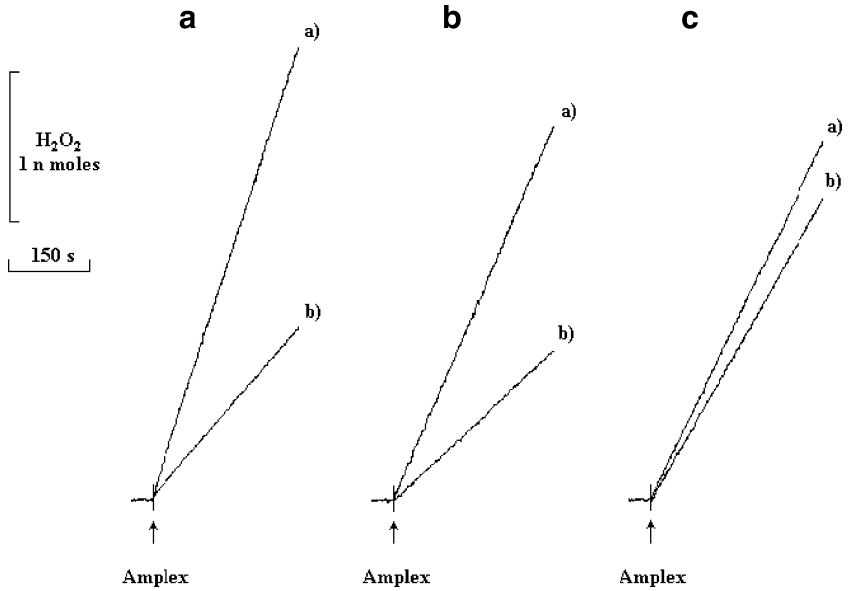

Fig. 2 The clorgyline inhibition of respiratory chain dependent H_2O_2 release is reversible. **a** H_2O_2 release with glutamate/malate and succinate in the absence (*trace a*) and in the presence (*trace b*) of clorgyline (10 μM). **b** Mitochondria minus clorgyline (*trace a*) and plus clorgyline (*trace b*) were spun down and resuspended in the supernatant. H_2O_2 was measured as in **a**. **c** Mitochondria (without and with clorgyline) were spun down and resuspended with a fresh medium supplied with substrates in the absence of clorgyline. Traces are representative of duplicate traces from at least three independent experiments

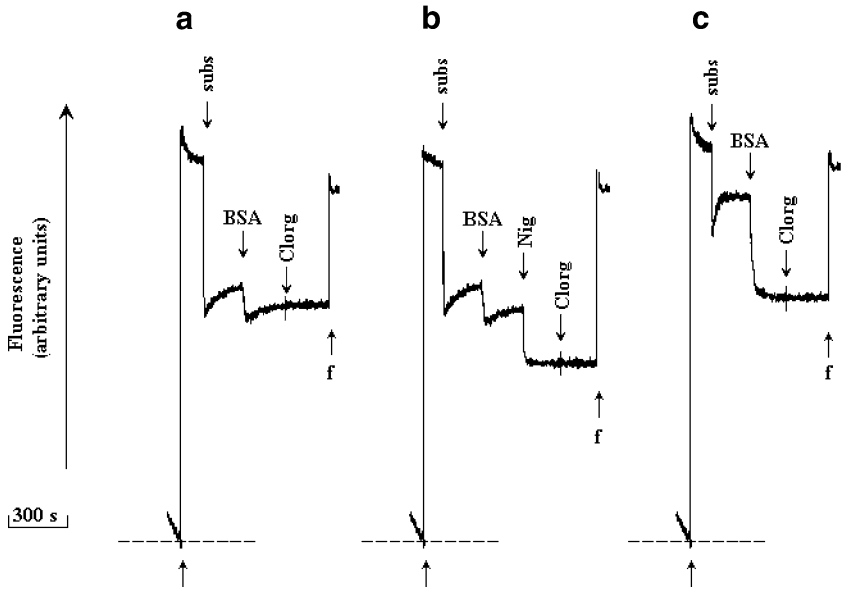

Fig. 3 Clorgyline does not modify mitochondrial ΔΨ. **a** Safranine (*arrow*) was 3 μM, glutamate/malate 1 mM each and succinate 0.4 mM (*subs*) were added as indicated, followed by BSA (500 μg/ml), clorgyline (20 μM) and 3 μM FCCP (*f*). **b** Same as **a**, with the further addition of nigericin (100 nM). **c** Same as **a** in a phosphate free medium. Traces are representative of duplicate traces from at least four independent experiments

Fig. 1. As expected a high rate of H_2O_2 production was activated by the monoamine tyramine in brain mitochondria (Fig. 1). This peroxide was completely inhibited by a short preincubation with pargyline and only partially inhibited by clorgyline, in line with the notion that brain mitochondria contain mainly MAO B. Importantly, H_2O_2 generation was reactivated by adding succinate (alone or plus G/M) to the tyramine plus pargyline-treated mitochondria. This peroxide generation in turn was strongly depressed by the addition of clorgyline. This shows that the respiration-dependent H_2O_2 is MAO-independent (insensitive to pargyline). The sensitivity to clorgyline is therefore a MAO-independent effect. Furthermore, the MAO inhibition by propargylamine-derived compounds is irreversible and depends on a covalent binding between MAO and the inhibitors (Youdim et al. 2006). Instead the clorgyline induced inhibition of the respiratory chain derived H_2O_2 was removed if clorgyline-treated mitochondria were washed prior to testing for substrate activated peroxide generation (Fig. 2). Collectively, these results show that the clorgyline activity on respiratory chain dependent peroxide generation is independent of its action on MAO.

The clorgyline inhibition of succinate-induced H_2O_2 production is unrelated to variations of the proton motive force (Δp)

The respiratory chain linked H_2O_2 production (particularly the succinate-dependent component) is strongly inhibited by even low decreases of the mitochondrial membrane potential ΔΨ. BSA has indeed to be included in the incubation medium in order for H_2O_2 release to be monitored. This property has been recently studied in detail by the group of Adam Vizi (Tretter et al. 2007), who detected a decreased membrane potential in BSA-free incubations, that was removed by BSA addition coincident with the activation of H_2O_2 generation. The BSA effect was tentatively ascribed to the removal of the uncoupling action of endogenous fatty acids. The possibility that clorgyline could inhibit H_2O_2 generation by inducing a fall in membrane potential was studied in the experiment reported in Fig. 3a which shows that the increase in membrane potential induced by the addition of BSA was unmodified by the subsequent addition of clorgyline. This result shows that the clorgyline effect is not dependent on variations of membrane potential. Furthermore, it was recently reported (Lambert and Brand 2004) that the ΔpH component of the mitochondrial proton motive force (the sum of ΔΨ and ΔpH), is the major controller of succinate-induced H_2O_2 generation, while ΔΨ has a minor function. We therefore analysed if the clorgyline effect is related to the control exerted by ΔpH on H_2O_2 generation. The lower H_2O_2

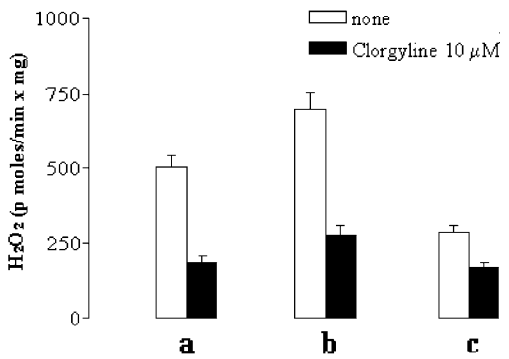

Fig. 4 The clorgyline inhibition of H_2O_2 release in the standard incubation medium (*a*), in a medium without phosphate (*b*) and in the standard medium supplied with 100 nM nigericin (*c*). Results are means±SD for duplicate incubations from five preparations

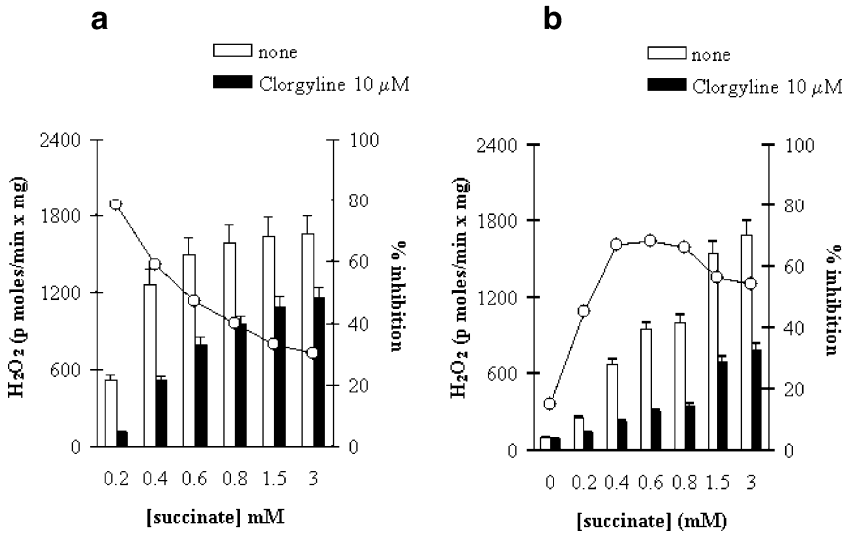

Fig. 5 Inhibition by clorgyline (10 μM) of succinate induced H_2O_2 release (**a**) and of glutamate/malate (1 mM each) plus succinate induced H_2O_2 release (**b**). (o) values represent the percentage inhibition. Results are means±SD for at least five independent experiments

generation with nigericin (which operates a 1:1 exchange of K^+ for H^+ leading to the removal of ΔpH if the mitochondria are suspended in a high K^+ medium) and the higher H_2O_2 generation in the absence of P_i (which quenches ΔpH via a P_i/H^+ antiporter) were nevertheless inhibited by clorgyline to a similar extent as in the previous experiments (Fig. 4). The effect of nigericin and of the absence of P_i on membrane potential and the effect of subsequent clorgyline addition are reported in Fig. 3b, c. The addition of clorgyline after BSA was without effect on ΔΨ in all cases. As expected, the addition of nigericin induced an increase of ΔΨ (to compensate for the decrease of ΔpH) and omitting phosphate decreased ΔΨ owing to increased ΔpH. These results show that the clorgyline inhibition is superimposed to the normal controls of H_2O_2 release. Clorgyline (20–30 μM) had no significant effect on mitochondrial respiration both with G/M plus succinate and with succinate alone.

We reported recently that palmityl CoA is a natural inhibitor of H_2O_2 release at Complex I (Bortolami et al. 2008). This compound is a strong inhibitor of succinate-dependent NAD(P) reduction via inhibition of energy-dependent transhydrogenase and apparently also of reverse electron transfer. Unlike palmityl CoA, clorgyline had no effect on succinate-driven NAD(P) reduction (not shown). Furthermore, palmityl CoA slightly stimulated the low level of G/M induced H_2O_2 generation, while clorgyline acted as a slight inhibitor (see below). Thus, the mode of action of the two inhibitors appears to be different.

Clorgyline inhibits differently the G/M and succinate induced H_2O_2 productions

The percent inhibition by clorgyline (10 μM) was low (10±2%) on the low H_2O_2 generation induced by G/M (Fig. 5b). The effect of clorgyline (10 μM) was much more evident on succinate-induced H_2O_2 generation. Inhibition was highest (80±5%) at low (0.2 mM) succinate and decreased progressively with increasing succinate concentration (Fig. 5a). Interestingly, when G/M (1 mM each) and succinate were present together (the physiological situation)

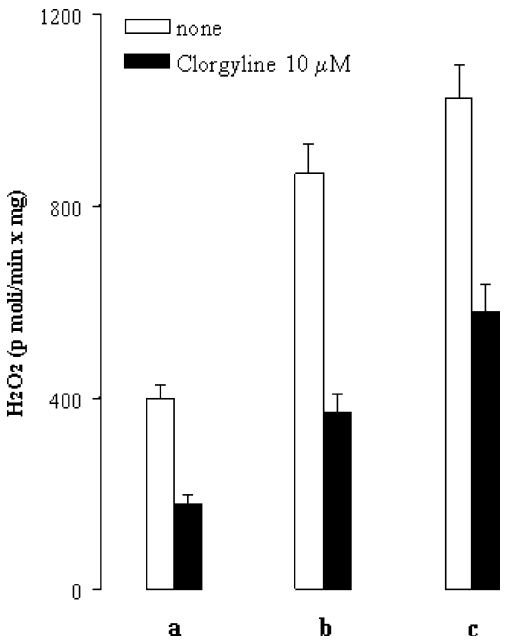

Fig. 6 Inhibition by clorgyline of dopaminochrome stimulated H_2O_2 release and on its increase by rotenone. DACHR was generated in the cuvette prior to the addition of mitochondria by 5 min preincubation in standard medium containing tyrosinase (45 U/ml) and (when present) dopamine (1.5 μM). The final concentration of DACHR, determined by the absorbance at 470 nm [19], was 1.3 μM. Then mitochondria were added, in the presence of G/M (1 mM) and succinate (0.4 mM), with or without clorgyline. *a* no DACHR, *b* DACHR 1.5 μM, *c* DACHR and rotenone (10 nM). Results are means±SD for at least three independent experiments

the clorgyline (10 μM) inhibition increased to a maximum (about 75±5%) in the succinate concentration range 0.4–0.8 mM. It is interesting that this is the range of the physiological succinate concentrations (Fig. 5b). As mentioned above, at zero succinate, a low level of inhibition was observed.

The DACHR-induced increase of H_2O_2 production is inhibited by clorgyline

One important characteristic of Parkinson's disease (PD) appears to be a decreased activity of Complex I. This conclusion derived from the observation that MPP^+, the active metabolite of the PD toxin MPTP, is a Complex I inhibitor. The observation that low doses of the Complex I inhibitor rotenone also induce PD-like symptoms confirmed this thesis (Sherer et al. 2002). Furthermore, a decrease of Complex I activity is described in platelets of PD patients (Smigrodzki et al. 2004). We reported recently that dopamine-derived dopaminochrome (DACHR), a possible toxin present in PD, induces an increment of H_2O_2 release at Complex I, and that this H_2O_2 production was further increased by low rotenone concentrations (Zoccarato et al. 2005). We initiated a study of the clorgyline effect on DACHR-stimulated H_2O_2 release and on its increase by rotenone. As shown in Fig. 6 clorgyline inhibited partially both the DACHR-induced H_2O_2 production and its stimulation by rotenone.

Other propargylamines act similarly to clorgyline

Deprenyl is a propargylamine derivative, neuroprotective and also a MAO B inhibitor which is used in the treatment of PD (Youdim et al. 2006). Another propargylamine derivative is CGP 3466B (Youdim et al. 2006; Sagot et al. 2000), also neuroprotective but devoid of MAO inhibition. Rasagiline (Youdim et al. 2005; Guay 2006) is a recently introduced propargylamine derivative used in the treatment of PD. The effect of these compounds on the "physiological" respiratory chain-linked H_2O_2 generation

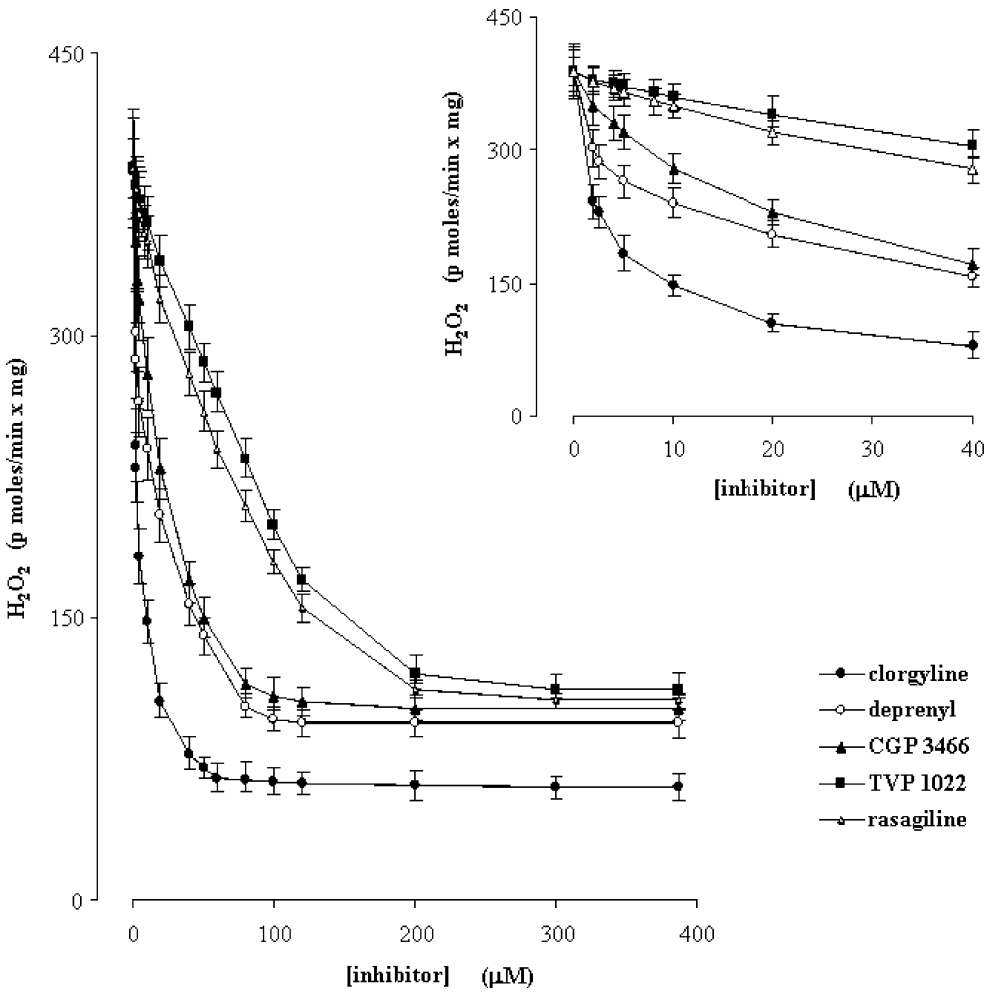

Fig. 7 Titration of the inhibition of H_2O_2 release induced in glutamate/malate (1 mM each) plus succinate (0.4 mM) with the various propargylamine derivatives. Results are means±SD for at least seven independent experiments

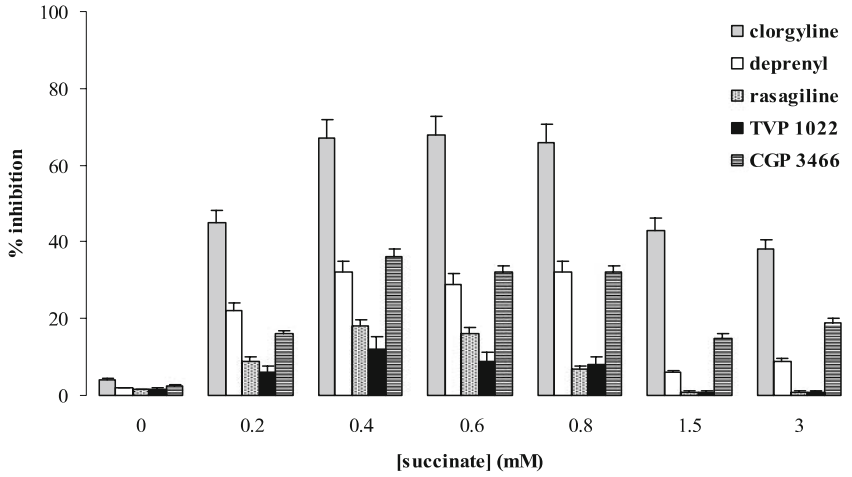

Fig. 8 Inhibition of H_2O_2 release by the propargylamine derivatives (10 μM) in the presence of glutamate/malate (1 mM each) and variable succinate. Results are means±SD for at least seven independent experiments

(G/M plus succinate), is reported in Fig. 7. It is shown that clorgyline is the most powerful inhibitor, followed by deprenyl and CGP 3466B, and by rasagiline and TVP-1022 (Youdim et al. 2005). The latter compound is an S-enantiomer of rasagiline, which is also reported to be neuroprotective, but is a poor MAO inhibitor.

Similarly to clorgyline the other propargylamines had no effect on mitochondrial $\Delta\Psi$ and on respiration. Their inhibition of H_2O_2 release by G/M (1 mM) as a function of succinate concentration is reported in Fig. 8 and was similar to the behaviour of clorgyline. Similar to the effect of clorgyline was also their effect on the inhibition of DACHR stimulated H_2O_2 release (Fig. 9).

Discussion

O_2^- production by Complex I and the subsequent SOD-dependent formation of H_2O_2 (followed by its release to the extramitochondrial space) are strongly stimulated by succinate, apparently via the increase of the QH_2/Q ratio induced by this substrate (Zoccarato et al. 2007). We showed recently that succinate stimulated H_2O_2 generation is specifically inhibited by long chain acyl-CoA (Bortolami et al. 2008). The results reported in this study identify a new class of compounds capable of inhibiting H_2O_2 production at Complex I of the mitochondrial respiratory chain, apparently without modifying any other known aspect of mitochondrial physiology. Such a specific negative modulation of H_2O_2 release identifies a potentially important pharmacological tool. It is likely that decreasing specifically peroxide release without any other apparent effect may be beneficial. In this context it may be significant that data have been reported repetitively on the activity of deprenyl in increasing life span (Kitani et al. 2005). Furthermore, the increase of life span upon removing the protein p66Shc which was reported to be responsible for a form of mitochondrial H_2O_2 production, has been attributed to the decrease of oxidative stress (Giorgio et al. 2005; Pinton et al. 2007).

Clinically deprenyl and rasagiline are used in the cure of PD. Deprenyl is a rather strong inhibitor of peroxide release, and rasagiline is less powerful. Whether in vivo derivatives of these chemicals are formed with increased H_2O_2 inhibitory power is presently not known.

CGP 3466B, deprenyl and rasagiline are known to bind to glyceraldehydes-3-phosphate dehydrogenase (GADPH) and are negatively involved in the translocation of this enzyme (in its nitrosylated form) to the nucleus, thus preventing a proapoptotic activity of GADPH translocation (Hara et al. 2006). The affinity of CGP 3466B and deprenyl for GADPH is extremely elevated. However in (Hara et al. 2006) the protective effect of deprenyl on etoposide induced cytotoxicity was shown to be in the 10–100 μM range, thus suggesting the possible involvement of the inhibition of O_2^-/H_2O_2 production in the mechanism of action of these substances. The inhibition of MAO activity

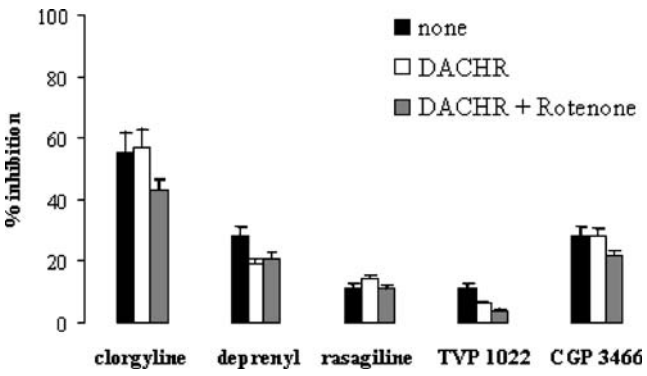

Fig. 9 Inhibition by various propargylamine derivatives of DACHR stimulated H_2O_2 release and on its increase by rotenone. Propargylamines were 10 μM. Conditions as in Fig. 6. Results are means±SD for at least three independent experiments

has been ruled out as the major responsible for protection, so at present the two remaining possibilities are the GADPH-mediated antiapoptosis and the inhibition of Complex I-dependent H_2O_2 release. It is possible that propargylamines share the two activities. Indeed, in mouse brain mitochondria, the efficacy of deprenyl in inhibiting mitochondrial H_2O_2 production has recently been reported to be significantly higher than in rat mitochondria (Czerniczyniec et al. 2006).

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