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Effects of entacapone and tolcapone on mitochondrial membrane potential

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Abstract

Catechol-*O*-methyl transferase (COMT) inhibitors, entacapone and tolcapone, are used as an adjunctive treatment to L-dopa in Parkinson's disease. Based on their catechol structure, both inhibitors are potential uncoupling agents, but only tolcapone shows this effect in vitro at clinically relevant concentrations. This study was designed to evaluate the direct uncoupling effects of the two COMT inhibitors in vitro and in vivo. In isolated rat liver mitochondria, entacapone had no effect on the membrane potential at therapeutical concentrations, but both tolcapone and the reference compound 2,4-dinitrophenol disrupted the potential at low μM concentrations. Since protein binding is speculated to decrease the uncoupling effects in vivo, the COMT inhibitory effect of entacapone and tolcapone as a surrogate for the overall activity of these inhibitors was evaluated in vitro with or without serum. The COMT inhibitory activity of entacapone was reduced to half, while tolcapone had only about 1/10 of its activity left in the presence of serum. Further, uncoupling is known to induce an increase in the body temperature in vivo, and these effects were evaluated in the rat by a possible hyperthermic response to the treatment with entacapone or tolcapone in combination with L-dopa (10 mg/kg) and carbidopa (20 mg/kg). This combination with entacapone (400 mg/kg) had no effect on the rectal body temperature. In contrast, tolcapone (50 mg/kg) caused an elevation in the body temperature together with L-dopa and carbidopa ($P < 0.01$). Both in vitro and in vivo results indicate that entacapone does not impair energy metabolism related to uncoupling of oxidative phosphorylation.

Keywords: COMT inhibitor; Entacapone; Tolcapone; Uncoupling; Parkinson's disease

1. Introduction

The membrane potential across the mitochondrial inner membrane is the driving force for phosphorylation of ADP in the process of oxidative phosphorylation. Mitochondrial membrane potential consists of an inward proton gradient that couples the transport of electrons down the respiratory chain to the oxidative synthesis of ATP (Brown, 1992). Uncoupling agents disrupt the membrane potential by acting as proton carriers that allow protons to flow into mitochondrial matrix without passing through the transmembrane protein complex that synthesises ATP from ADP. Uncoupling of oxidative phosphorylation leads to elevated mitochondrial oxygen consumption and to decreased ATP production as a compensatory function of the cell. Since the formed energy is liberated as heat, this results in a rise of the body temperature (Hemker, 1964).

It has been shown that the catechol-*O*-methyl transferase (COMT) inhibitor entacapone expresses effects of uncoupling of oxidative phosphorylation at concentrations higher than 100 μM in rat liver mitochondria in vitro (Nissinen et al., 1997). Tolcapone, another COMT inhibitor, has similar uncoupling effect at significantly lower concentrations (2.6 μM) (Nissinen et al., 1997). Since the evidence for the interference of entacapone and tolcapone with mitochondrial membrane potential was evaluated only as ATP/ADP ratio and oxygen consumption (Haasio et al., in press), this study was carried out to estimate directly the effects of entacapone and tolcapone on the mitochondrial membrane potential in vitro. It has also been claimed that the strong binding of tolcapone to serum proteins could prevent in vivo the uncoupling of oxidative phosphorylation effect caused by tolcapone in vitro in isolated mitochondria (Borroni et al., 1999, 2001). Since strong protein binding diminishes the concentration of the free pharmacologically active drug in plasma, we studied COMT inhibition as a surrogate for the overall activity of the COMT inhibitors with and without serum proteins in vitro.

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To study the effects on uncoupling in vivo, the rectal body temperature of rats was measured after a single oral administration of entacapone or tolcapone combined with L-dopa and a dopa decarboxylase inhibitor, carbidopa. As a positive reference substance, a known uncoupler 2,4-dinitrophenol was used. 2,4-Dinitrophenol is a potent uncoupling agent which is known, at the cellular level, to disrupt the mitochondrial membrane potential (Sibille et al., 1998) and to increase the body temperature in man (Parascandola, 1974). 2,4-Dinitrophenol has also been used as a tool to induce a nonimmune-mediated hyperthermia in experimental animals (Anari and Renton, 1993).

2. Materials and methods

2.1. Test compounds

Entacapone, tolcapone and L-dopa were synthesised at Orion Pharma (Espoo, Finland) (Bäckström et al., 1989; Bernauer et al., 1987; Borgulya et al., 1991). The drug substances were identified by comparing them to standard using infrared spectra. The impurities in both entacapone and tolcapone, determined by HPLC, were under 0.1%. 2,4-Dinitrophenol was purchased from Aldrich (Steinheim, Germany), and it served as a positive reference compound. Carbidopa was synthesised at Egis Pharmaceuticals (Budapest, Hungary). For membrane potential studies, fresh stock solution of each test substance was made in dimethylsulfoxide (DMSO, Aldrich) for each experiment. Serial dilutions were made in DMSO to prepare the stock solutions for addition to the assay mixture. Disodium succinate hexahydrate was obtained from BDH (BDH Chemical, Poole, England). Safranine *O* was purchased from Aldrich and dissolved in water (3 mM). In protein binding studies, *S*-adenosyl-L-methionine was purchased from Aldrich, and 3,4-dihydroxybenzoic acid from Sigma (USA). For in vivo studies, the test compounds were suspended in 0.5% methylcellulose (low substitution, BDH).

2.2. Mitochondrial membrane potential studies

2.2.1. Measurement of mitochondrial membrane potential

Male rats of outbred Crl:CD®BR stock (Sprague–Dawley origin), supplied by Charles River Wiga (Sulzfeldt, Germany), served as liver donors. Mitochondria were isolated with differential centrifugation and suspended in suspension buffer (0.22 M mannitol, 0.05 M sucrose, 5 mM KCl, 5 mM KH_2PO_4, 1 mM Na_2-EDTA, 3 mM $MgCl_2$, 10 mM MOPS, pH adjusted to 7.4 with KOH) (Nissinen et al., 1997).

Mitochondrial membrane potential was measured using a fluorometric method originally described by Åkerman and Wikström (1976) and slightly modified by Kauppinen and Hassinen (1984) and Fromenty et al.(1990). Rotenone was not present in the assay mixture. Fluorescence with excitation at 520 nm and emission at 570 nm (bandpasses 5 nm) was measured in a Hitachi F-4010 fluorometer (Hitachi, Japan) equipped with a thermostatted cuvette holder and magnetic stirrer. The contents of the cuvette were kept homogenous throughout the measurement by stirring with a teflon-coated magnet. In brief, 10 μl of mitochondrial suspension (protein concentration 20–30 mg/ml) was added to thermostatted (30 °C) cuvette containing 1.5 ml of suspension buffer and 5 μl of 3 mM safranine *O* (final concentration, 10 μM). The mixture was allowed to equilibrate for 10–15 min during which the fluorescence increased to maximal value that corresponds to zero membrane potential observed in the absence of respiratory substrate (or after complete disruption of membrane potential by an uncoupling agent). Fluorescence recording was started and the measurement was initiated by adding 10 μl of 1.5 M succinate (final concenctration, 10 mM) to allow the formation of membrane potential, which took 1–2 min. Thereafter, a test compound was added in 5 μl of DMSO (final concentration of DMSO 0.3%), and the fluorescence was allowed to reach steady-state or near steady-state level before the next addition of the test compound. Fluorescence readings for calculation of results were taken at time points just before the next addition of the test compound to the cuvette. One to five additions of each test compound (in 5 μl DMSO) were made during the total time of 8–12 min used for fluorescence recording.

2.2.2. Calculation of the membrane potential

Mitochondrial membrane potential was calculated as percentage of membrane potential in fully energised (with succinate) mitochondria using the following formula: Membrane potential (percentage of maximum)=$(F_{max}\text{-}F_n)/(F_{max}\text{-}F_{min}) \times 100$, where F_{max} is the fluorescence maximum (zero membrane potential) the reading was taken at the start of the measurement before addition of succinate; F_{min} is the fluorescence minimum (maximal membrane potential); the reading was taken after the addition of succinate; and F_n is the fluorescence after the addition of the test compound.

The results were expressed as the concentration, which induced 50% reduction in membrane potential (EC_{50}).

2.3. Measurement of COMT activity

COMT activity in vitro was determined, as described earlier, using a soluble COMT preparation from rat liver (Nissinen et al., 1992). In brief, the effect of serum on COMT inhibition was studied by incubation of various concentrations of entacapone or tolcapone (0.1, 0.5, 1, 2, 5, 10 μM) with or without 10% rat serum for 30 min at 37 °C. Control samples were prepared without COMT inhibitors. Thereafter, the enzymatic reaction was carried out adding buffer, COMT enzyme, cofactor *S*-adenosyl-L-methionine and the substrate 3,4-dihydroxybenzoic acid to the mixture. The

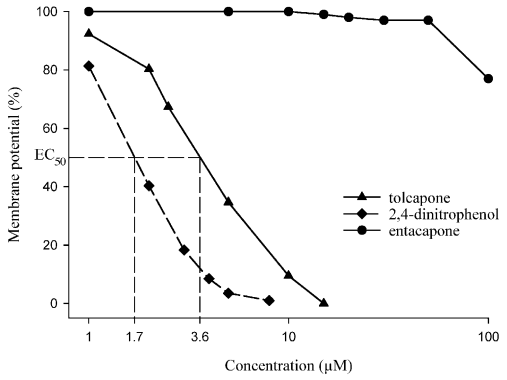

Fig. 1. Concentration dependency for the effect of entacapone, tolcapone or 2,4-dinitrophenol on mitochondrial membrane potential (percentage of maximum). $N = 3$-5, except at concentrations where membrane potential is below 5% of the maximum $N = 1$-2. EC_{50} is the concentration, which induces 50% reduction in membrane potential.

incubation was continued for 15 min at 37 °C, and the reaction was stopped by the addition of perchloric acid. The formed 3-*O*-methoxy-benzoic acid was determined from the supernatant using HPLC, as described earlier (Nissinen et al., 1992). The results were expressed as the concentration, at which the COMT inhibition is 50% of the maximum (IC_{50}).

2.4. Body temperature measurement studies

Male Wistar rats (Mol:Wist), weighing 180–220 g and supplied by Mollegaard Breeding Center (Ejby, Denmark) were used. The rats were acclimatised to the laboratory conditions 5 days before allocation into treatment groups. The study was conducted in a semibarriered, limited access animal room. Commercially available rodent SDS diet RM1 (E) SQC (Special Diet Services, Witham, Essex, England) and tap water from the public supply, filtered twice, were available ad libitum. The animal care was performed according to the regulations of the Council of Europe and the National Research Council, USA (Council of Europe, 1990; National Research Council, 1996).

The rats, 10 animals per group, were fasted overnight prior to the treatment. Entacapone 400 mg/kg or tolcapone 50 mg/kg were administered orally 1 h prior to oral carbidopa 20 mg/kg and L-dopa 50 mg/kg. The control group received vehicle (0.5% methylcellulose). The dose volume was 5 ml/kg. The dose selection was based on the previous toxicity studies (Haasio et al., 2000, 2001) and on single-dose studies performed prior to the actual study. The selected tolcapone dose, 50 mg/kg, was used, as it was the lowest dose rising the body temperature in rats. Rectal body temperature was measured several times prior to the dosing period to adapt the animals to the measurements. A digital electronic thermometer with a stainless probe (S. Brannan and Sons, Cleator Moor, Cumbria, England) was used, and the rectal body temperature was measured 1, 2 and 4 h after dosing of carbidopa with L-dopa. The experiment was approved by the Animal Ethics Committee of Orion Corporation.

Analysis of variance followed by the Dunnett's test or Fisher's PLSD test was used for the statistical analysis of the data. The change from the initial body temperature values was used in calculations.

3. Results

3.1. Membrane potential

Repeated stepwise additions of various concentrations of entacapone, starting from 5, 10, or 50 μM concentration, had no effect on mitochondrial membrane potential when the cumulative final concentration of entacapone remained under 100 μM (Fig. 1). Addition of several doses of tolcapone, each dose increasing the concentration of tolcapone in mitochondrial suspension by 1.0 or 2.5 μM, gradually disrupted the membrane potential. The concentration required to decrease the membrane potential by 50% was 3.6 μM (Fig. 1). The membrane potentials measured in the presence of higher than 5 μM, total concentration of tolcapone were consistently below 20% of the initial maxium value. With final tolcapone concentrations of 12–20 μM, the membrane potential decreased to 0–7% of the initial value. 2,4-Dinitrophenol caused a concentration-dependent decrease of mitochondrial membrane potential at slightly lower concentrations than tolcapone. The concentration required to decrease the membrane potential by 50% was 1.7 μM. At concentrations of 5 μM or above 2,4-dinitrophenol suppressed the membrane potential to 1–7% of the initial value (Fig. 1).

The data demonstrate a close resemblance of tolcapone and 2,4-dinitrophenol in terms of gradually short-circuiting the mitochondrial membrane potential at concentrations below 10 μM, while at least 100 μM concentration of entacapone is required for any significant effect to be observed.

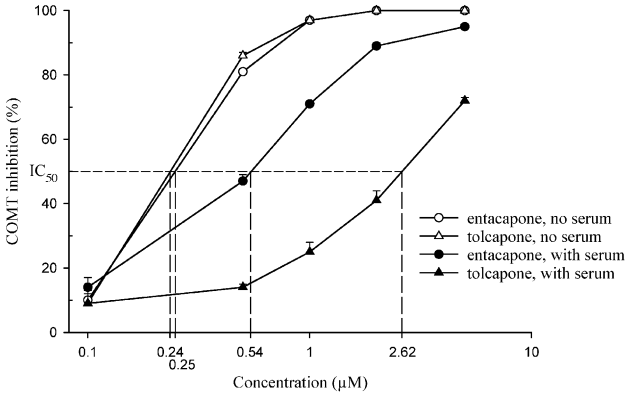

Fig. 2. The effect of addition of rat serum on the COMT inhibition activity (%) of entacapone and tolcapone. $N = 3$. IC_{50} indicates the concentration, at which the COMT inhibition is 50% of the maximum. Mean ± S.E.M.

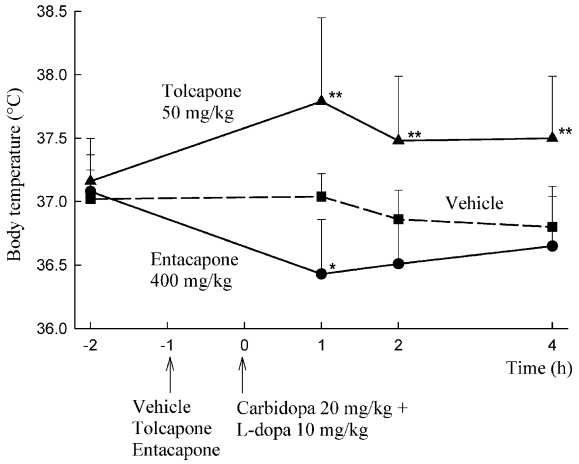

Fig. 3. The effect of combined treatment with entacapone or tolcapone with carbidopa and L-dopa on the body temperature of rats. Mean ± S.D., $N=10$, * $P<0.05$, ** $P<0.01$.

3.2. COMT activity

When COMT activity was measured without preincubation in rat serum, both entacapone and tolcapone inhibited COMT at equal concentrations (IC_{50} values of 0.25 and 0.24 μM, respectively Fig. 2). However, when the compounds were preincubated in 10% rat serum for 30 min, the COMT inhibitory activity of entacapone was reduced to some extent (IC_{50} value 0.54 μM), while that of tolcapone was shifted to micromolar level (IC_{50} value 2.62 μM).

3.3. Body temperature

The combination of carbidopa and L-dopa did not induce any changes in the body temperature. When entacapone (400 mg/kg) was added to the treatment, the temperature decreased slightly ($P<0.05$). Tolcapone (50 mg/kg) increased the body temperature even in rats treated with carbidopa/L-dopa ($P<0.01$) (Fig. 3).

4. Discussion

One of the characteristics of uncoupling agents of oxidative phosphorylation is disruption of mitochondrial membrane potential. In a normal cell, high-energy electrons are passed to oxygen by means of the respiratory chain producing ATP. The membrane potential increases as the electron flows down the respiratory chain to oxygen. Uncouplers of oxidative phosphorylation in mitochondria inhibit the coupling between the electron transport and phosphorylation reactions; thus, they also inhibit ATP synthesis without directly affecting the respiratory chain. Since mitochondrial membrane potential is disrupted and ATP is no longer formed from ADP, the energy is liberated as heat (Terada, 1990). Miscellaneous compounds are known to be uncouplers; however, weakly acidic uncoupling agents are the potent ones, and they are considered to produce uncoupling by their protonophoric action on the mitochondrial membrane. As a weak acid, the uncoupling effect of a known uncoupler, 2,4-dinitrophenol, is attributable to its protonophoric nature (Terada, 1990).

In the present study, entacapone did not have any effect on membrane potential at concentrations lower than 100 μM. In contrast, tolcapone caused a disruption of mitochondrial membrane potential at the same concentration range as the classical uncoupling agent 2,4-dinitrophenol, half-maximal concentration for disruption (EC_{50}) being lower than 5 μM for both compounds. This result is consistent with the earlier findings that entacapone influences cellular respiration and mitochondrial oxygen consumption only at high concentrations and thus does not uncouple oxidative phosphorylation by disrupting the mitochondrial membrane potential. Nissinen et al. (1997) also reported previously that EC_{50} for stimulation of succinate-supported mitochondrial respiration of tolcapone was 2.6 μM, which is in the same concentration range as the concentration for disruption of membrane potential in the present study. These results are consistent with those reported by Borroni et al. (2001). The reason for the greater potential of tolcapone to cause uncoupling and disruption of membrane potential might relate to its lipid solubility (Dingemanse, 1997). Since tolcapone is a more lipophilic compound than entacapone, it penetrates more easily through the cell membrane and further across the mitochondrial bilayer than the less lipid soluble entacapone.

Entacapone is in vitro 98% and tolcapone 99.9% bound to plasma proteins (Entacapone Product Monograph, 1999; Tasmar Product Monograph, 1997). Under in vitro conditions, using rat liver soluble COMT preparation without added rat serum, they are equally potent COMT inhibitors with an IC_{50} value of ca. 0.25 μM. When serum is added to the incubation mixture, 10-fold concentration of tolcapone (2.62 μM) is needed to achieve similar IC_{50} value of COMT inhibition, whereas only twice as much entacapone (0.54 μM) is needed for 50% COMT inhibition in the presence of serum. However, in clinical use, both agents have been proved to be potent COMT inhibitors despite extensive protein binding (Kaakkola, 2000). This is not in agreement with Borroni et al. (2001) that protein binding decreases noticeably the pharmacological activity of the compounds in vivo. In clinical use, the unbound concentration of entacapone ($C_{max}=0.118$ μM) and tolcapone ($C_{max}=0.023$ μM) should thus be twice and 10 times higher, respectively, to achieve effective COMT inhibition (Borroni et al., 2001; Dingemanse et al., 1995; Keränen et al., 1994). There is equilibrium between the bound and free fraction of a drug in plasma, and the free fraction penetrates the tissues, e.g. liver, where the drug accumulates if it is not efficiently metabolised, e.g. by glucuronidation or sulfation. Gasser and Smit (2001) have reported that 5.7% of patients ($N=3848$) receiving 200 mg t.i.d. of tolcapone have experienced liver function abnormalities. This could be due to the fact that

intracellular concentrations of tolcapone are increased in the liver tissue of patients, whose glucuronidation rate is poor. The liver concentrations of both tolcapone and entacapone at doses of 300 or 500 mg/kg/day after 7 days' oral dosing of rats are equally high (Haasio et al., in press), indicating that both compounds can penetrate the liver, although they are highly bound to plasma proteins. Thus, the binding of a drug to serum proteins does not directly affect tissue concentrations.

The uncoupling phenomenon is reflected in vivo as a rise of the body temperature, when the formed energy in mitochondria is liberated as heat. We have shown earlier that tolcapone induces a rise in the body temperature when administered alone, but entacapone has no effect on the body temperature (Haasio et al., 2001). Tolcapone treatment with L-dopa and carbidopa induced body temperature elevation at exposure in the present study, which does not exceed the exposure in clinical use more than four times (AUC_{rat} 200 mg/kg/day is 325 h·μg/ml, AUC_{man} at maximal recommended dose is 75 h·μg/ml, exposure factor 4) (Dingemanse et al., 1995; Haasio et al., 2001; Keränen et al., 1994). The entacapone concentrations needed to induce uncoupling in vitro are far higher than the peak plasma concentrations in clinical use. Entacapone induces the peak plasma concentrations of about 4–14 μM after single oral doses of 200–800 mg in man (Keränen et al., 1994), while after same doses of tolcapone, the peak concentrations are about 27–89 μM (Dingemanse et al., 1995). The disruption of membrane potential occurred with entacapone at concentrations higher than 100 μM, and with tolcapone at the concentration lower than 5 μM, indicating that under clinical conditions entacapone is not likely to affect the membrane potential.

Dopamine agonists are known to induce a decrease in the body temperature through stimulation of dopamine D_2-receptors (Cox and Tha, 1975; Faunt and Crocker, 1987). In the present study, L-dopa administered together with carbidopa did not induce any changes in the body temperature at the selected dose. However, when entacapone was added to the treatment, there was a slight decrease in the temperature. As the concomitant dosing of COMT inhibitors with dopa decarboxylase inhibitors increases the bioavailability of L-dopa, the decrease in the body temperature may be due to the higher dopamine concentration in the brain. In contrast, tolcapone induced a rise in body temperature even in the presence of L-dopa and carbidopa. Dagani et al. (1991) have shown earlier that L-dopa treatment does not interfere with mitochondrial respiration in rat skeletal muscle. This indicates that the mechanism by which tolcapone induces a rise in the body temperature is not related to dopamine and dopamine receptors, but may be a result of uncoupling of oxidative phosphorylation.

The results of our study correlate with the in vitro results obtained by Nissinen et al. (1997) that tolcapone and 2,4-dinitrophenol were substantially more potent uncoupling agents of oxidative phosphorylation than entacapone. Signs of uncoupling were also observed in vivo as hyperthermia even after a single dose of tolcapone in the presence of L-dopa and carbidopa, while entacapone induced no signs of uncoupling in vivo. Thus, the data indicate that entacapone does not impair energy metabolism related to uncoupling of oxidative phosphorylation.

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