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Entacapone, a novel catechol-*O*-methyltransferase inhibitor for Parkinson's disease, does not impair mitochondrial energy production

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Abstract

Entacapone, a novel mainly peripherally acting catechol-*O*-methyltransferase inhibitor used in the treatment of Parkinson's disease, was evaluated for its possible uncoupling activity in cell culture, in rat liver mitochondria, and in isolated guinea-pig heart. Entacapone did not stimulate respiration in the L1210 murine T cell lymphoma cell line at the concentrations studied (5–40 μM). Furthermore, entacapone neither increased mitochondrial respiration nor impaired cardiac function at pharmacologically relevant concentrations (< 10 μM). In fact, the threshold concentration for increased mitochondrial oxygen consumption was 20 μM and half-maximal stimulation of respiration was not detected until 58 μM. Surprisingly, tolcapone, another catechol-*O*-methyltransferase inhibitor, which acts both peripherally and centrally, stimulated respiration in L1210 cells at the lowest concentration studied (5 μM). In addition, 1 μM tolcapone increased mitochondrial respiration, indicating that it caused uncoupling at a much lower concentration than that of 2,4-dinitrophenol, a well-known uncoupler of oxidative phosphorylation. Tolcapone also impaired the mechanical function and oxygen consumption of the isolated guinea-pig heart at 1 μM. These results show that peripherally acting entacapone, unlike the brain-penetrating tolcapone, is a safe catechol-*O*-methyltransferase inhibitor for the treatment of Parkinson's disease, since it does not interfere with mitochondrial energy metabolism at pharmacologically effective concentrations.

Keywords: Catechol-*O*-methyltransferase; (Uncoupling); Oxidative phosphorylation; Mitochondria; Entacapone

1. Introduction

Entacapone, a mainly peripherally acting catechol-*O*-methyltransferase inhibitor, is being investigated in clinical trials as an adjunct drug to levodopa in the treatment of Parkinson's disease (Merello et al., 1994). Entacapone and tolcapone, another catechol-*O*-methyltransferase inhibitor that acts centrally are structurally related to 2,4-dinitrophenol, which is a well-known uncoupler of oxidative phosphorylation (Stryer, 1981). Oxidative phosphorylation is the major mechanism by which aerobic cells produce ATP by means of a respiratory assembly that is located in the inner mitochondrial membrane. Uncoupling of oxidative phosphorylation leads to increased respiration without the production of ATP and the energy created is released as heat (Hemker, 1964).

It is now generally accepted that the mitochondrial respiratory chain functions abnormally in patients with Parkinson's disease. The mitochondrial respiratory chain comprises four multimeric enzyme complexes (I–IV) which together with ATPase (complex V) form the system for oxidative phosphorylation. Complexes I–IV are responsible for electron transport and reduction of oxygen, and complexes I, III and IV are fundamental for the generation of the electrochemical proton gradient that drives ATP synthesis (Hatefi, 1985). The first indication that aberrant mitochondrial function may be involved in the destruction of dopaminergic neurones in Parkinson's disease came from studies of the mechanism of action of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces parkinsonism in humans (Langston et al., 1983). 1-Methyl-4-phenylpyridine (MPP^+), the active metabolite of MPTP, is accumulated in dopaminergic neurones and then concentrated in mitochondria, where it has been shown to be a specific inhibitor of complex I (Mizuno et al., 1988).

The inhibition of complex I activity leads to a decrease in ATP production that ultimately results in cell death (Di Monte et al., 1986). The decrease in complex I activity in

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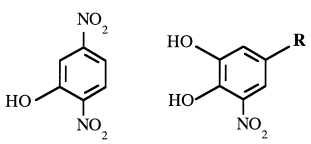
Parkinson's disease was first shown in the substantia nigra by a direct enzymatic assay (Schaphira et al., 1989) and by immunoblotting (Mizuno et al., 1989), and the available evidence suggests that this deficit is not present elsewhere in the parkinsonian brain (Schaphira et al., 1990). Studies of complex I activity in peripheral tissues such as skeletal muscle (Bindoff et al., 1989; Mann et al., 1992), platelets (Parker et al., 1989; Mann et al., 1992) and lymphocytes (Yoshino et al., 1992; Martín et al., 1996) have yielded controversial results, but they do not exclude the possibility of a deficit in the periphery.

Since one of the mechanisms which may cause nigral cell death in Parkinson's disease is the energy crisis due to mitochondrial respiratory failure, any drug that is likely to interfere with mitochondrial energy production should be evaluated carefully before it is used clinically. In addition, cardiac function is critically dependent on mitochondrial respiration through oxidative ATP production. We therefore studied the effects of entacapone on the mitochondrial respiratory chain, cellular respiration and cardiac function.

2. Materials and methods

2.1. Materials

Entacapone (Fig. 1), [(*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*,*N*-diethyl-2-propenamide] and tolcapone (3,4-dihydroxy-4′-methyl-5-nitrobenzophenone) were synthesized at Orion Pharma Research Center (Espoo). 2,4-Dinitrophenol was from Aldrich (Steinheim). EDTA, EGTA, L-glutamic acid monosodium salt, maleic acid monosodium salt, 3-(*N*-morpholino)propane sulfonic acid (MOPS) and oligomycin were purchased from Sigma Chemical (St. Louis, MO). Sodium succinate was from BDH (Dorset). AMP, ADP and ATP were from Boehringer Mannheim (Mannheim). All other chemicals were of the highest grade available from commercial sources. The stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO). No effects of the solvent at the concentrations used ($< 0.5\%$) in these experiments were observed.



Dinitrophenol **Entacapone: R = -CH=C(CN)-CO-N(C_2H_5)$_2$**
Tolcapone: R = -CO-C_6H_4-CH_3

Fig. 1. Chemical structures of 2,4-dinitrophenol, entacapone and tolcapone

2.2. Cell culture

The murine T cell lymphoma cell line L1210 (ATCC, Rockville, MD) was cultivated at $+37$°C in a humidified atmosphere of 95% air with 5% CO_2. The culture medium was Dulbecco's minimum essential medium with high glucose (4.5 g/l) and GlutaMax-I (Gibco, Paisley), supplemented with 10% fetal calf serum (Biological Industries, Beth Haemek), 100 IU/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). For measurement of oxygen consumption the cells were harvested by centrifugation and suspended in Hank's balanced salt solution (HBSS; Flow Laboratories) + 4 mM glutamine (Gibco) at a density of 2.5×10^6 cells/ml.

2.3. 2.3. Mitochondrial preparations

Male rats of the outbred Han:Wistar strain were fasted overnight and used as liver donors. Mitochondria were isolated from fresh livers by homogenization and differential centrifugation. Livers were removed from decapitated rats and homogenized (1:4 w/vol) in cold 250 mM sucrose, 2 mM Tris, 0.1 mM EDTA, pH 6.8. The homogenate was centrifuged at $1000 \times g$ for 10 min at $+4$°C, and the supernatant was further centrifuged at $8200 \times g$ for 10 min at $+4$°C. The precipitate, which contained the mitochondria, was washed with the homogenization buffer, collected by centrifugation and suspended in 2 ml of homogenization buffer at a concentration of about 10 mg protein/ml and stored in ice. The protein content of the mitochondrial preparation was estimated by using the Bio-Rad protein determination method (Bio-Rad Laboratories, Hercules, CA).

2.4. Measurement of respiratory rates

Cellular and mitochondrial respiration was measured as oxygen consumption at $+37$°C, using a polarographic technique and Clark oxygen electrode (YSI 5331 Oxygen Probe, Yellow Springs Instruments, Yellow Springs, OH) fitted in a thermostated glass chamber. For measurement of cellular respiration 2.3 ml of cells in HBSS + Gln was pipetted into the chamber. The cells and mitochondria were stirred constantly throughout the experiment. For the measurement of mitochondrial respiration an aliquot (40–50 μl, ca. 2 mg protein/ml) of the mitochondrial preparation was added to 1.8 ml of the respiration buffer consisting of 250 mM sucrose, 5 mM sodium phosphate, 10 mM MOPS, 2 mM magnesium chloride, 1 mM EGTA, pH 7.0, containing 5 mM succinate or 5 mM glutamate/malate as the substrate for respiration. To ensure that mitochondria were tightly coupled before the experiments, the ability of ADP (300 μM) to stimulate respiration was tested. Respiration was allowed to stabilize for 1–2 min or 4–10 min after the addition of mitochondria or L1210 cells, respectively. Thereafter the test compound was added to the

mixture. The change in oxygen concentration in the chamber was recorded with the oxygen probe, supplied with a voltage of 0.8 V, connected to an amplifier manufactured according to the instructions of the electrode supplier. The amplifier was connected to a chart recorder to graphically record the oxygen concentration versus time. The recorder was zero calibrated by the addition of sodium dithionite ($Na_2S_2O_4$) to the cells to deplete oxygen. Mitochondrial oxygen consumption is expressed as nmol oxygen/min × mg protein and was derived from authentic recordings of polarographic traces. The EC_{50}-values were calculated from the concentrations that gave 50% of the maximal increase in mitochondrial oxygen consumption. The respiratory control ratios were calculated from the state 3/state 4 respiration rates. The cellular respiration results are expressed as nmol oxygen/min × ml cell suspension (2.5×10^6 cells/ml) and as the change (%) in oxygen consumption after the addition of a test compound.

2.5. Cardiac function studies

Male and female guinea-pigs of the outbred Mol:duha were used as heart donors. The guinea-pigs were killed by a blow on the skull and after decapitation the heart was quickly excised. The heart was rinsed with ice-cold oxygenated perfusion buffer and a cannula was inserted into the aorta. Retrograde perfusion of the heart was begun with a modified Tyrode buffer as soon as the heart was placed in thermostatically controlled moist chamber of the Langendorff apparatus (Hugo Sachs Elektronik KG, March-Hugstetten). After the start of perfusion, the opening tract of the pulmonary artery was cannulated and a Clark type polarographic micro-oxygen probe (YSI Model 5357) and a chamber (YSI Model 5356) were placed in the perfusate and connected to an oxygen detector (YSI 5300 Biological Oxygen Monitor Micro System). This allowed continuous through-flow monitoring. The whole system was adjusted so that there was no afterload in the right ventricle. For the measurement of cardiac performance, a fluid-filled latex balloon was inserted into the left ventricle to record the intraventricular pressure. The balloon was connected to a pressure transducer (Isotec, Healthdyne Cardiovascular, Irvine, CA), which was linked to an acquisition system. The volume of the balloon was adjusted to create an initial diastolic pressure of about 5 mm Hg. A computerized program (Acquisition v3.1 program, Koskinen, Orion, Espoo) was used to calculate the following parameters of cardiac performance: heart rate, left ventricular systolic and end-diastolic pressures, and the first derivative (positive and negative) of the systolic pressure (dP/dt_{max}) as an index of myocardial contractility. Coronary flow (ml/min) was continuously recorded by an electromagnetic flow meter Narcomatic RT-500 (Narco Bio-System, Houston, TX). The signals from the flow meter and oxygen monitor were also collected by the acquisition v3.1 program on different computer channels. Results from 5–6 experiments per group were combined and expressed as a mean change from the initial level.

2.6. Determination of myocardial adenine nucleotides

Hearts were freeze-clamped with precooled tongs after 1 h of perfusion with or without 10 μM entacapone or 1 μM tolcapone. The frozen hearts were homogenized in cold 0.6 M perchloric acid and the homogenates were neutralized and centrifuged. The supernatants were stored at −20°C until the adenine nucleotides were analyzed, usually within three days. Concentrations of the adenine nucleotides AMP, ADP and ATP in supernatant were measured with a Waters high-performance liquid chromatography system (600E multidelivery system controller + pump, 717 autosampler, 996 Photodiode Array Detector and Millenium 2010 chromatography manager, Waters, Milford, MA) by a minor modification of the method described by Carter and Müller (1990). Briefly; a 20 μl aliquot was injected onto an ion-exchange column (250 × 3.2 mm, 5 μm Phenosphere SAX, Phenomenex, Torrance, CA) and eluted with a linear gradient of 0.005–0.75 M potassium phosphate buffer pH 4.5 at a flow rate of 2 ml/min.

2.7. Statistical analysis

Results are expressed as means ± S.E.M. and in cardiac function studies the difference between treatment versus control was evaluated with the analysis of variance (ANOVA) followed by Dunnett's *t*-test.

3. Results

3.1. Mitochondrial respiration

Entacapone was a rather weak uncoupler of oxidative phosphorylation (Fig. 2). Succinate- supported oxygen consumption was fully stimulated only in the presence of

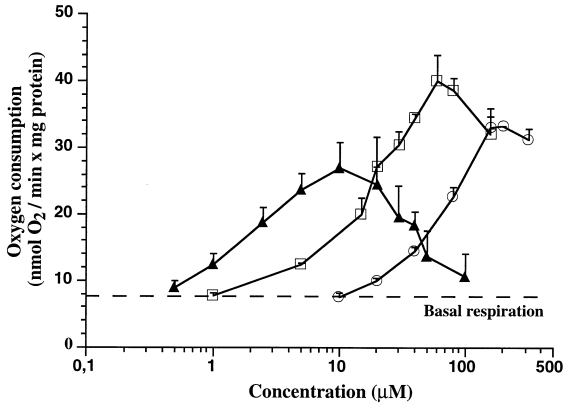


Fig. 2. Effect of entacapone (○), 2,4-dinitrophenol (□) and tolcapone (▲) on succinate (5 mM)-supported mitochondrial oxygen consumption in the respiration buffer. Mitochondria (ca. 2 mg protein) were preincubated 1–2 min before the addition of the test compound. The results are means ± S.E.M. of 3–5 experiments.

Table 1
The values for half-maximal stimulation (EC_{50}) of succinate-supported mitochondrial respiration

Compound	EC_{50}-values (μM)
Entacapone	58.0
2,4-dinitrophenol	12.5
Tolcapone	2.6

The EC_{50}-values for stimulation of succinate (5 mM)-supported mitochondrial respiration were calculated from Fig. 2.

entacapone at concentrations higher than 100 μM. The positive control, a well-known uncoupler 2,4-dinitrophenol, stimulated mitochondrial respiration at low micromolar concentrations. Surprisingly, the other catechol-*O*-methyltransferase inhibitor tolcapone increased mitochondrial respiration even at 1 μM (Fig. 2). The tight coupling of the mitochondria was always confirmed before the experiments by the addition of 0.3 mM ADP, which greatly stimulated respiration. Similar effects were detected when glutamate/malate was used as the respiration substrate, e.g. electrons were fed through complex I.

The concentrations that caused a 50% increase (EC_{50}) in succinate-supported respiration, i.e. uncoupling of oxidative phosphorylation, are listed in Table 1. These values further confirmed that entacapone was a weak uncoupler. It was five times weaker than the classical uncoupler 2,4-dinitrophenol and 20 times weaker than the other catechol-*O*-methyltransferase inhibitor tolcapone. Entacapone did not decrease the respiratory control until high micromolar concentrations were used, while tolcapone sharply decreased respiratory control at low micromolar concentrations, much lower than those of the classical uncoupler 2,4-dinitrophenol (Fig. 3). Entacapone did not release oligomycin-inhibited state 3 respiration at concentrations at which tolcapone was able to do so, signifying the weak uncoupling potential of entacapone (Fig. 4). State 3 respiration was 22 nmol oxygen/min/mg protein and

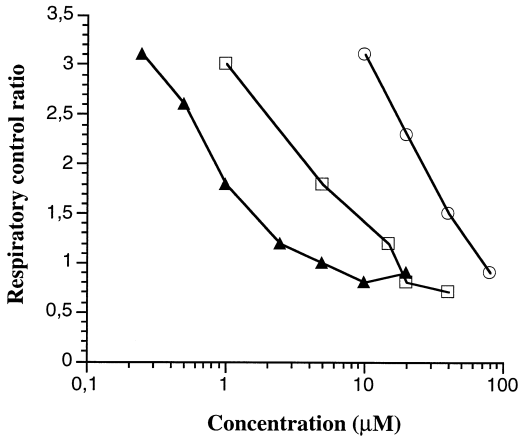


Fig. 3. Effect of entacapone (○), 2,4-dinitrophenol (□) and tolcapone (▲) on succinate (5 mM)-supported respiratory control ratio. The experimental conditions were the same as those reported in Fig. 2.

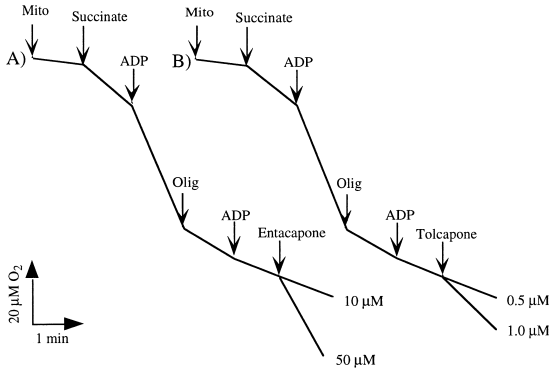


Fig. 4. Effect of entacapone, trace A, and tolcapone, trace B on oligomycin-inhibited state 3 respiration. The experimental conditions were the same as those reported in Fig. 2. ADP, oligomycin and the test compounds were added as indicated by the arrows.

was not affected by the test compounds until concentrations were used that also caused inhibition of state 4 respiration (see Fig. 2).

3.2. Cellular respiration

The cell culture experiments (Table 2) also showed that entacapone at the tested concentrations (5–40 μM) did not increase oxygen consumption by L1210 cells. The uncoupler 2,4-dinitrophenol concentration dependently increased the respiration rate and the other catechol-*O*-methyltransferase inhibitor tolcapone increased the respiration rate by almost 50% already at the lowest concentration used (5 μM).

When the test compounds were added successively three times to the cell suspension, entacapone (final cumulative concentration 60 μM) did not stimulate or inhibit cellular respiration. Both 2,4-dinitrophenol and tolcapone

Table 2
Effect of a single addition of entacapone, 2,4-dinitrophenol or tolcapone on the rate of oxygen consumption by L1210 cells

Compound	(μM)	Increase in rate (nmol O_2/min)	Change (%)
Entacapone	5	−0.20 (from 2.81 to 2.61)	−7
	10	−0.25 (from 2.80 to 2.55)	−8
	20	0.05 (from 2.41 to 2.46)	2
	40	0.08 (from 1.70 to 1.78)	5
Tolcapone	5	1.42 (from 3.10 to 4.52)	46
	10	1.35 (from 2.77 to 4.12)	48
	20	1.76 (from 2.69 to 4.45)	65
Dinitrophenol	10	0.18 (from 2.91 to 3.09)	6
	20	0.89 (from 2.18 to 3.07)	41
	40	1.76 (from 2.15 to 3.91)	81

The L1210 cells (2.5×10^6 cells/ml) were preincubated in the respiration buffer at 37°C for 4–10 min, and then the test compound was added and the rate of oxygen consumption was recorded and calculated as nmol O_2/min/ml. The percentage of change compared to that of the corresponding control was also calculated. The results are the means of 2–9 measurements.

behaved like uncouplers, stimulating respiration after the first and second addition. Furthermore the third addition of tolcapone, resulting in high cumulative concentration (60 μM), inhibited respiration (results not shown).

3.3. Cardiac function

In the cardiac function study the mechanical parameters, i.e. left ventricular systolic and end diastolic pressure, positive and negative dP/dt_{max} of the guinea-pig heart remained stable when entacapone up to 30 μM was added in the perfusion buffer. Entacapone (0.3–30 μM) seemed to stabilize the mechanical parameters e.g. left ventricular systolic pressure (Fig. 5), which otherwise slowly deteriorated in the control group during the experiment. In comparison, 10 μM tolcapone had a marked negative effect on the left ventricular systolic pressure (Fig. 5). Furthermore, 3 μM tolcapone increased left ventricular end diastolic pressure two-fold and inotropy was reduced by about 70%, leading to impaired relaxation in the presence of 10 μM tolcapone (results not shown). The heart rate remained at the control level during the experiments after the addition of entacapone and tolcapone.

These changes were preceded by an increase in oxygen consumption already after the addition of 1 μM tolcapone, whereas 30 μM entacapone was needed to produce a marked increase in oxygen consumption (Fig. 6). The increase in cardiac oxygen consumption seen after addition of 1–10 μM tolcapone and after 30 μM entacapone was reflected by an equivalent increase in coronary flow (results not shown).

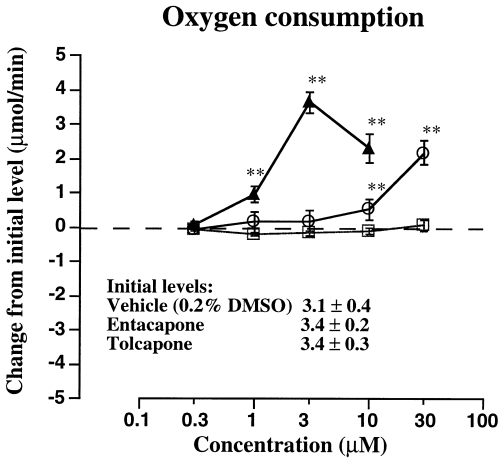


Fig. 6. The concentration–response curve for entacapone (○) and tolcapone (▲) on the change in the oxygen consumption of the isolated guinea-pig heart. Conditions were the same as those described in Fig. 4. The results are means ± S.E.M. of five experiments. Statistical significance: $^{**}P < 0.001$ versus vehicle (□).

The concentration–response study showed that the threshold concentration for inducing uncoupling related changes, i.e. increase in oxygen consumption, was 10 μM for entacapone and 1 μM for tolcapone, and so the isolated heart was exposed to these concentrations for 60 min. Thereafter the adenine nucleotide concentrations and energy charge in drug- and vehicle-treated hearts were measured. Myocardial ATP and total nucleotide content remained at the control level both in entacapone- and tolcapone-treated groups. Consequently the energy charge stayed at the control level. At this concentration (10 μM) entacapone did not impair the mechanical function of the heart. However, 1 μM tolcapone started to increase the

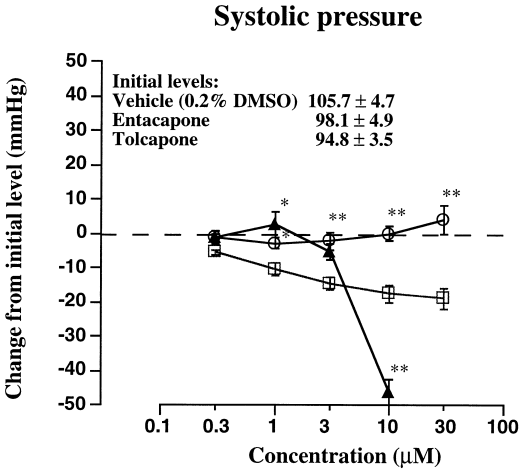


Fig. 5. The concentration–response curve for entacapone (○)- and tolcapone (▲)-induced change in the left ventricular systolic pressure of the isolated guinea-pig heart. After a 15 min stabilization period, entacapone (0.3–30 μM) or tolcapone (0.3–10 μM) was added to the perfusion solution by increasing the concentration at 15 min intervals. In the control (□) group, vehicle (0.2% DMSO) was added instead of the test compounds. The results are means ± S.E.M. of five experiments. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.001$ versus vehicle.

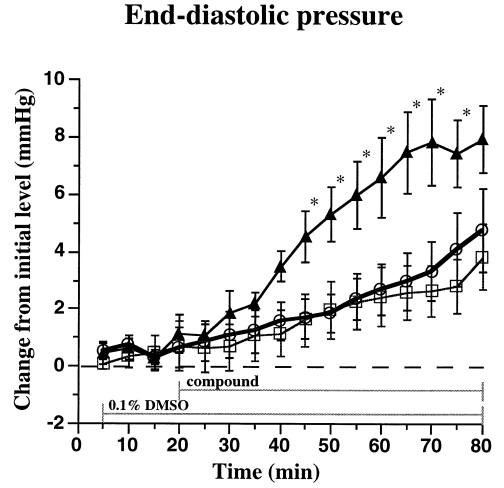


Fig. 7. The effect of entacapone (○) and tolcapone (▲) on end-diastolic pressure in isolated guinea-pig heart. After a 20 min stabilization period entacapone (10 μM) or tolcapone (1 μM) was added to the perfusion buffer. In the control (□) group, vehicle (0.1% DMSO) was added instead of the test compound. The recordings are means ± S.E.M. of six experiments. Statistical significance: $^{*}P < 0.01$ versus vehicle.

end-diastolic pressure 20 min after the drug was added to the perfusate. The change compared to that seen with vehicle was about four-fold greater at about 45 min (Fig. 7). Furthermore, the addition of 1 μM tolcapone to the perfusate increased markedly coronary flow and oxygen consumption, while 10 μM entacapone had no clear effect on these parameters (results not shown).

4. Discussion

It is well documented in the literature that weak acids like 2,4-dinitrophenol can increase proton transfer across energy-transducing membranes, e.g. mitochondrial membranes. This protonophoric effect abolishes the link between substrate oxidation and ATP synthesis. Consequently, uncouplers release respiratory control, which leads to suppression of all energy-linked reactions such as ATP synthesis and ion transport (Terada, 1981). The major ATP-utilizing reactions in mammalians on a whole-body basis are myosin ATPase, Na^+/K^+-ATPase and protein synthesis (Brown, 1992). Myosin ATPase is important for the contractile function of the heart and Na^+/K^+-ATPase has a major role in the regulation of ion fluxes. In addition, Na^+/K^+-ATPase is the major ATPase in brain and kidney. Also gluconeogenesis and urea synthesis in the liver are dependent on ATP supply. Since these critical cellular functions depend on a steady supply of ATP, any compound that is likely to interfere with ATP production at pharmacologically relevant concentrations should be evaluated very carefully for its uncoupling properties.

Entacapone, as suggested by its planar structure, was a weak uncoupler of oxidative phosphorylation. Its uncoupling potential in mitochondrial preparations was not detected until high micromolar concentrations were used. In contrast, tolcapone, which is structurally and functionally closely related to entacapone, was a very powerful uncoupler, being five times more effective than the reference compound 2,4-dinitrophenol. The greater potential of tolcapone to induce uncoupling of oxidative phosphorylation is most likely due to the fact that tolcapone is a more lipophilic compound than entacapone. Consequently, tolcapone can penetrate better through biological membranes and disturb mitochondrial proton flux. The lipid solubility of phenolic compounds has been documented and such compounds are known to increase the uncoupling effect for a long time (Hemker, 1962). Unlike tolcapone, entacapone did not behave like the uncoupler 2,4-dinitrophenol by first stimulating respiration and then at higher concentrations inhibiting uncoupled respiration, which is indicative of inhibition of ATP-synthetase activity (Hemker, 1964).

Mitochondrial preparations are commonly used to assess the uncoupling potential of drugs and other chemicals. However, isolated mitochondria alone do not prove that an uncoupler has serious consequences on the vitality of a cell or on the function of an organ. This is the case especially in the heart, where the ATP supply is extremely crucial for the proper performance of the organ. The energy for the heart is provided by mitochondrial respiration and by glycolysis and is stored/shuttled as creatine phosphate. Consequently, changes in ATP levels are not seen unless the oxygen supply is shut off for a long time, since glycolysis continues under anaerobic conditions as well (Opie, 1991). In the Langendorff heart model, the oxygen supply is high and the perfusion buffer also contains glucose, which means that it may take longer than the 1 h used in our isolated heart experiments to bring the energy charge into unbalance. However, we noticed a markedly increased end-diastolic left ventricular pressure and also an inappropriately high oxygen consumption and coronary flow with respect to mechanical performance, especially after 1 μM tolcapone during the 1 h perfusion experiment. These changes were not detected in the presence of 10 μM entacapone. The significantly elevated end diastolic pressure (impaired relaxation) after 1 μM tolcapone is likely to be due to leakage of calcium ions from the mitochondria to the cytosol, which is typical during uncoupling (Orrenius et al., 1989). Similar findings, namely an increase in myocardial oxygen consumption without detectable loss of ATP, have also been reported by others and attributed to mitochondrial dysfunction, i.e. uncoupling of oxidative phosphorylation (Leidtke et al., 1988; Kingsley-Hickman et al., 1990). It is important to notice that energy produced by glycolysis is specifically coupled to ion transport, whereas ATP produced by oxidative metabolism is coupled to contractile energy requirements (Opie and Bricknell, 1979).

Cell culture studies confirmed the findings for the mitochondrial preparations and isolated heart that entacapone, unlike tolcapone, does not stimulate respiration at pharmacologically relevant concentrations. Actually, entacapone had no effect on cellular respiration even at the highest concentration (60 μM) studied. This is also likely to be due to the lower lipid solubility of entacapone, which results in a poorer penetration of the drug through the cell membrane and thus a lower concentration of the compound interacts with the mitochondria. However, entacapone is a very effective peripherally acting catechol-*O*-methyltransferase inhibitor, causing a reduction in catechol-*O*-methyltransferase activity at low nanomolar concentrations (Nissinen et al., 1992). The higher lipophilicity of tolcapone and consequent inhibition of brain catechol-*O*-methyltransferase activity does not increase its efficacy as an enhancer of levodopa therapy (Männistö and Tuomainen, 1991).

In human trials, entacapone up to the highest tested dose (800 mg) has proven to be a safe catechol-*O*-methyltransferase inhibitor alone or when combined with levodopa + carbidopa (Keränen et al., 1994; Ahtila et al., 1995). After single oral doses of 200–800 mg of entacapone the peak plasma concentrations of entacapone are

about 4–15 μM in humans, respectively. These values are much lower than the concentrations needed to induce uncoupling in the various test systems used here, and in addition more than 90% of entacapone is bound to plasma proteins. However, single oral doses of 200–800 mg of tolcapone have been reported to produce peak plasma levels of about 27–89 μM (Dingmanse et al., 1995), which are an order of magnitude higher than the concentrations of tolcapone which caused uncoupling-related effects in our experiments.

There is also increasing evidence for oxidative damage in the substantia nigra of patients with Parkinson's disease at the time of death. During aerobic metabolism the respiratory chain is a significant source of superoxide ions, and inhibition of complex I results in an increase in superoxide production (Cleeter et al., 1992). Enhanced free radical formation can also impair respiratory chain function; however, a specific complex I deficiency is not suggested to be caused by free radicals, but rather free radical damage is due to complex I deficiency. Interestingly, there are also reports that catecholamines such as dopamine and levodopa can cause changes in brain mitochondrial complex I activity in vitro (Jackson-Lewis et al., 1991). In addition, chronic levodopa treatment has been shown to decrease complex I activity in the rat brain (Przedborski et al., 1993).

Recently it has been reported that inhibition of catechol-*O*-methyltransferase activity in the brain should be avoided, since *O*-methylation of catecholamines and melatonin may have an important protective role in the brain's defence against oxidative stress (Miller et al., 1996). If Parkinsonian patients are treated with brain-penetrating catechol-*O*-methyltransferase inhibitors, which are uncouplers especially in combination with levodopa, chronic treatment may lead to unpredictable adverse effects due to decreased ATP production and possibly increased free radical damage.

Along with the possible disturbance of heart and brain function, uncouplers are likely to cause gastrointestinal and kidney dysfunction. Oxygen consumption is extremely high in the kidney and is only exceeded by that of the heart. Since most of the ATP in the kidney is produced by oxidative metabolism, the renal mitochondria have a crucial role in supporting ATP-dependent processes, most importantly sodium reabsorption. This makes the kidney very sensitive to the toxic action of agents which may interfere with mitochondrial ATP production (Mingatto et al., 1996). Likewise, in the gastrointestinal tract ATP is needed by Na^+/K^+ATPase for the maintenance of Na^+/K^+ and cellular osmotic balance. Disturbance of the osmotic balance may contribute to the occurrence of diarrhoea, which is a common adverse effect seen in Parkinsonian patients during catechol-*O*-methyltransferase inhibitor and levodopa + carbidopa therapy (Spencer and Benfield, 1996). Loss of respiratory control would also lead to secondary damage as a result of increased uncontrolled leakage of Ca^{2+} from the mitochondria, which in the intestine could cause the generation of reactive oxygen species, proteolysis and activation of Ca^{2+}-sensitive enzymes (Orrenius et al., 1989; Somasundaram et al., 1995).

As a conclusion, entacapone was not an uncoupler of oxidative phosphorylation at pharmacologically relevant concentrations (< 10 μM) in mitochondrial preparations, in cell cultures or in isolated hearts indicating that it does not interfere with mitochondrial ATP production. This suggests that the peripherally acting catechol-*O*-methyltransferase inhibitor entacapone is a safe extension to levodopa + carbidopa therapy for the treatment of Parkinson's disease.

References

Ahtila, S., Kaakkola, S., Gordin, A., Korpela, K., Heinävaara, S., Karlsson, M., Wikberg, T., Tuomainen, P., Männistö, P.T., 1995. Effect of entacapone, a COMT inhibitor, on the pharmacokinetics and metabolism of levodopa after administration of controlled-release levodopa–carbidopa in volunteers. Clin. Neuropharmacol. 18, 46–57.

Bindoff, L.A., Birch-Machin, M., Cartlidge, N.E.F., Parker, W.D. Jr., Turnbull, D.M., 1989. Mitochondrial function in Parkinson's disease. Lancet 1, 49.

Brown, G.C., 1992. Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem. J. 284, 1–13.

Carter, A.J., Müller, R.E., 1990. Application and validation of an ion-exchange high-performance liquid chromatographic method for measuring adenine nucleotides, creatine and creatine phosphate in mouse brain. J. Chromatogr. 527, 31–39.

Cleeter, M.J.W., Cooper, J.M., Schaphira, A.H.V., 1992. Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: Evidence for free radical involvement. J. Neurochem. 58, 786–789.

Di Monte, D., Jewell, S.A., Ekström, G., Sandy, M.S., Smith, M.T., 1986. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridine (MPP^+) cause rapid ATP depletion in isolated hepatocytes. Biochem. Biophys. Res. Commun. 137, 310–315.

Dingmanse, J., Jorga, K., Zürcher, G., Schmitt, M., Sedek, G., Da Prada, M., Van Brummelen, P., 1995. Pharmacokinetic–pharmacodynamic interaction between the COMT inhibitor tolcapone and single-dose levodopa. Br. J. Pharmacol. 40, 253–262.

Hatefi, Y., 1985. Mitochondrial electron transport and oxidative phosphorylation system. Ann. Rev. Biochem. 54, 1015–1069.

Hemker, H.C., 1962. Lipid solubility as a factor influencing the activity of uncoupling phenols. Biochim. Biophys. Acta 62, 46–54.

Hemker, H.C., 1964. Inhibition of adenosine diphosphatase and respiration of rat liver mitochondria by dinitrophenols. Biochim. Biophys. Acta 81, 1–8.

Jackson-Lewis, V., Naini, A.B., Przedborski, S., Simonetti, S., Fahn, S., Cadet, J.L., 1991. Catecholamines inhibit mitochondrial complex I activity in rat brain. Soc. Neurosci. Abstr. 17, 1276.

Keränen, T., Gordin, A., Karlsson, M., Korpela, K., Pentikäinen, P.J., Rita, H., Schultz, E., Seppälä, E., Wikberg, T., 1994. Inhibition of soluble catechol-*O*-methyltransferase and single-dose pharmacokinetics after oral and intravenous administration of entacapone. Eur. J. Clin. Pharmacol. 46, 151–157.

Kingsley-Hickman, P.B., Sako, E.Y., Ugurbil, K., From, A.H.L., Foker, J.E., 1990. ^{31}P NMR measurement of mitochondrial uncoupling in isolated rat hearts. J. Biol. Chem. 265, 1545–1550.

Langston, J.W., Ballard, P., Tetrud, J.W., Irwin, I., 1983. Chronic

parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979–980.

Leidtke, A.J., DeMaison, L., Eggleston, A.M., Cohen, L., Nellis, S.H., 1988. Changes in substrate metabolism and effects of excess fatty acids in reperfused myocardium. Circ. Res. 62, 535–542.

Mann, V.M., Cooper, J.M., Krige, D., Daniel, S.E., Schaphira, A.H.V., Marsden, C.D., 1992. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. Brain 115, 333–342.

Martín, M.A., Molina, J.A., Jiménez-Jiménez, F.J., Benito-León, J., Ortí-Pareja, M., Campos, Y., Arenas, J., 1996. Respiratory-chain enzyme activities in isolated mitochondria of lymphocytes from untreated Parkinson's disease patients. Neurology 46, 1343–1346.

Merello, M., Lees, A.J., Webster, R., Bovingdon, M., Gordin, A., 1994. Effect of entacapone, a peripherally acting catechol-*O*-methyltransferase inhibitor, on the motor response to acute treatment with levodopa in patients with Parkinson's disease. J. Neurol. Neurosurg. Psychiatry 57, 186–189.

Miller, J.W., Selhub, J., Joseph, J.A., 1996. Oxidative damage caused by free radicals produced during catecholamine autooxidation: Protective effects of *O*-methylation and melatonin. Free Radiat. Biol. Med. 21, 241–249.

Mingatto, F.E., Santos, A.C., Uyemura, S.A., Jordani, M.C., Curti, C., 1996. In vitro interaction of nonsteroidal anti-inflammatory drugs on oxidative phosphorylation of rat kidney mitochondria: Respiration and ATP synthesis. Arch. Biochem. Biophys. 334, 303–308.

Mizuno, Y., Suzuki, K., Sone, N., Saitoh, T., 1988. Inhibition of mitochondrial respiration by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mouse brain in vivo. Neurosci. Lett. 91, 349–353.

Mizuno, Y., Ohta, S., Tanaka, M., Takamiya, S., Suzuki, K., Sato, T., Oya, H., Ozawa, T., Kagawa, Y., 1989. Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. Biochem. Biophys. Res. Commun. 163, 1450–1455.

Männistö, P.T., Tuomainen, P., 1991. Effect of high single doses of levodopa and carbidopa on brain dopamine and its metabolites: Modulation by selective inhibitors of monoamine oxidase and/or catechol-*O*-methyltransferase in the male rat. Naunyn–Schmiedeberg's Arch. Pharmacol. 344, 412–418.

Nissinen, E., Lindén, I.-B., Schultz, E., Pohto, P., 1992. Biochemical and pharmacological properties of a peripherally acting catechol-*O*-methyltransferase inhibitor entacapone. Naunyn–Schmiedeberg's Arch. Pharmacol. 346, 262–266.

Opie, L.H., 1991. ATP synthesis and breakdown; adenosine and response to ischemia. In: Opie, L. (Ed.), The Heart, Physiology and Metabolism. Raven Press, New York, NY, pp. 247–276.

Opie, L.H., Bricknell, O.L., 1979. Role of glycolytic flux and the effect of glucose in decreasing fatty acid induced release of lactate dehydrogenase from isolated coronary ligated rat heart. Cardiovasc. Res. 13, 693–702.

Orrenius, S., McConkey, D.J., Bellomo, G., Nicotera, P., 1989. Role of Ca^{2+} in toxic cell killing. Trends Pharmacol. Sci. 10, 281–285.

Parker, W.D. Jr., Boyson, S.J., Parks, J.K., 1989. Abnormalities of the electron transport chain in idiopathic Parkinson's disease. Ann. Neurol. 26, 719–723.

Przedborski, S., Jackson-Lewis, V., Muthane, U., Jiang, H., Ferreira, M., Naini, A.B., Fahn, S., 1993. Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. Ann. Neurol. 34, 715–723.

Schaphira, A.H.V., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B., Marsden, C.D., 1989. Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1, 1269.

Schaphira, A.H.V., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clark, J.B., Marsden, C.D., 1990. Anatomic and disease specificity of NADH CoQ1 reductase (Complex I) deficiency in Parkinson's disease. J. Neurochem. 55, 2142–2145.

Somasundaram, S., Hayllar, H., Rafi, S., Wrigglesworth, J.M., Macpherson, A.J.S., Bjarnason, I., 1995. The biochemical basis of non-steroidal anti-inflammatory drug-induced damage to the gastrointestinal tract: A review and a hypothesis. Scand. J. Gastroenterol. 30, 289–299.

Spencer, C.M., Benfield, P., 1996. Tolcapone: New drug profile. CNS Drugs 5, 475–481.

Stryer, L., 1981. Oxidative phosphorylation. In: Stryer, L. (Ed.), Biochemistry. W.H. Freeman and Company, New York, NY, pp. 307–331.

Terada, H., 1981. The interaction of highly active uncouplers with mitochondria. Biochem. Biophys. Acta 639, 225–242.

Yoshino, H., Nakagawa-Hattori, Y., Kondo, T., Mizuno, Y., 1992. Mitochondrial complex I and II activities of lymphocytes and platelets in Parkinson's disease. J. Neural. Transm. 4, 27–34.