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Dose-related inversion of cinnarizine and flunarizine effects on mitochondrial permeability transition

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Abstract

We investigated the effects of cinnarizine and flunarizine on mitochondrial permeability transition, ATP synthesis, membrane potential and NAD(P)H oxidation. Both drugs were effective in inhibiting the mitochondrial permeability transition induced either by Ca^{2+} alone or in the presence of *tert*-butylhydroperoxide. This protective effect occurred at low concentrations ($< 50\ \mu M$) of these drugs and was accompanied by the inhibition of NAD(P)H oxidation and the restoration of the mitochondrial membrane potential decreased by a high concentration of Ca^{2+} ($25\ \mu M$). However, at higher concentrations ($> 50\ \mu M$) of cinnarizine and flunarizine and in the absence of both *tert*-butylhydroperoxide and Ca^{2+}, their effects on the mitochondria were reversed as follows: mitochondrial permeability transition was generated, mitochondrial NAD(P)H was oxidized and membrane potential collapsed. These deleterious effects were not antagonized by cyclosporine A, the most potent inhibitor of the mitochondrial permeability transition, but by 2,6-di-*tert*-butyl-4-methylphenol, a known antioxidant agent. This mitochondrial effect was neither accompanied by an increase in malondialdehyde production nor by an increase in H_2O_2 generation, which attested that the effect of both drugs was not due to an increase in reactive oxygen species production. The dual effects of both cinnarizine and flunarizine on mitochondrial functions is discussed with regard to both the protective effect afforded by these drugs against ischemia–reperfusion injury and their side effect observed in some therapeutic situations where an overdosage seems likely. © 1998 Elsevier Science B.V.

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1. Introduction

Mitochondrial Ca^{2+} overload leads to mitochondrial membrane permeability transition, which is associated with the opening of a mitochondrial membrane permeability transition pore (Haworth and Hunter, 1979). The opening of the latter can be enhanced by various agents including prooxidants, thiol cross-linking agents, inorganic phosphate and uncouplers (Zoratti and Szabo, 1995). The pore allows equilibration of solutes with molecular weight up to 1200 Da and corresponds to the mitochondrial mega-channel previously identified in patch-clamp studies on single mitoplasts (Kinnally et al., 1989). Multiple regulatory steps are involved in the modulation of the mitochondrial permeability transition activity. Indeed mitochondrial permeability transition can be inhibited by a wide variety of agents ranging from Me^{2+} ions, local anesthetics to scavengers of radical oxygen species and many other compounds with no common structural features (for a review see Zoratti and Szabo, 1995). It is interesting to note that cyclosporine A is the most potent mitochondrial permeability transition inhibitor described to date (Fournier et al., 1987; Broekemeier et al., 1989).

Mitochondrial permeability transition induced by prooxidants, for instance *tert*-butylhydroperoxide, is triggered by Ca^{2+}-stimulated production of reactive oxygen species which accumulated due to the exhaustion of mitochondrial antioxidants glutathione and NAD(P)H (Richter and Schlegel, 1993; Halestrap, 1994). The attack of reactive oxygen species to membrane protein thiols produces

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cross-linking reactions (Vercesi, 1993) that may open membrane pores upon Ca^{2+} binding. However, the precise nature of the pore is still unknown.

Cinnarizine and its fluorine derivative flunarizine are selective Ca^{2+} blockers widely used in the treatment of cerebral and vascular insufficiency (Singh et al., 1985). Recently, both drugs have been shown to protect isolated perfused rat liver against warm ischemia and reperfusion (Konrad et al., 1995) but also to be neuroprotective (De Haan et al., 1993; Gunn et al., 1994). Paradoxically, the same drugs have been implicated in the induction of Parkinson's disease (Veich and Hue, 1994; Negrotti and Calzetti, 1997). Their effects appeared to be similar to that of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP^+), a selective inhibitor of mitochondrial respiratory complex I, especially in dopaminergic cells of substantia nigra (Javitch et al., 1985). As mitochondrial permeability transition has been suggested to be involved in this neurodegenerative disease as well as in ischemia–reperfusion injury (Mizuno et al., 1995; Nicolli et al., 1995), we have investigated the effects of both cinnarizine and flunarizine on the mitochondrial permeability transition induced by oxidative stress condition. Our data show that at low concentrations ($< 50\ \mu M$) both drugs inhibited the mitochondrial permeability transition induced by Ca^{2+} and *tert*-butylhydroperoxide whereas at high concentrations and in the absence of both agents they were able to induce it. These findings might explain the dual effects of cinnarizine and flunarizine observed in therapeutic and toxic situations.

2. Materials and methods

2.1. Isolation of mitochondria

All animal procedures used in this study are in strict accordance with the French Agency's policies about animal experimentation.

Rat liver mitochondria were isolated as described by Johnson and Lardy (1967). Briefly male Wistar rats weighing approximately 250 to 300 g were decapitated, liver were excised rapidly and placed in a medium containing 250 mM sucrose, 10 mM tris and 1 mM EGTA, pH 7.2 at 4°C. The tissue was scissor minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at $600 \times g$ for 10 min (Sorvall® RC 28 S). The supernatant was centrifuged for 5 min at $15\,000 \times g$ to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at $15\,000 \times g$ for 5 min.

The mitochondrial pellet was washed with medium from which the EGTA was omitted and centrifuged for 5 min at $15\,000 \times g$ resulting in a final pellet containing approximately 50 mg of protein per milliliter. The mitochondrial suspension was stored on ice before use. The protein content was determined by the method of Lowry et al. (1951).

2.2. Mitochondrial swelling measurements

Mitochondrial swelling was assessed by measuring the change in absorbance of their suspension at 520 nm by using a Hitachi® model U-3000 spectrophotometer. Mitochondrial swelling induced by *tert*-butylhydroperoxide was measured according to Halestrap and Davidson (1990), slightly modified by Elimadi et al. (1997).

When swelling was induced either by Ca^{2+} or cinnarizine or flunarizine alone, mitochondria (4 mg) were added to 3.6 ml of the phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, 3 mM succinate and 2 μM rotenone, pH 7.2 at 25°C). 1.8 ml of this suspension was added to both sample and reference cuvettes in the presence or absence of different inhibitors. After 1 min of incubation at 25°C, the swelling was initiated by the introduction of either 25 μM of Ca^{2+} or a high concentration of cinnarizine or flunarizine to the sample cuvette only and the A_{520} scanning was started.

2.3. Optical monitoring of mitochondrial membrane potential

Rhodamine 123, a fluorescent dye, was used to monitor changes in membrane potential of isolated mitochondria according to Emaus et al. (1986). The excitation and emission wavelengths were 503 and 527 nm, respectively.

The following were added to the cuvette in the presence or absence of drugs: 1.8 ml of the phosphate buffer, 0.3 μM of rhodamine 123 and 25 μM of Ca^{2+}. Fluorescence scanning of the rhodamine 123 was monitored using a Perkin-Elmer® LS 50 B spectrofluorimeter. After 30 s, mitochondria (0.5 mg/ml) were added.

2.4. Determination of mitochondrial NAD(P)H oxidation

Mitochondrial pyridine nucleotides were monitored at 25°C according to Minezaki et al. (1994) by measuring their autofluorescence at an excitation and emission wavelength of 366 and 450 nm, respectively, in a Perkin-Elmer® LS 50 B spectrofluorimeter. Mitochondria (1 mg) were added to the phosphate buffer (1.8 ml) and incubated for 1 min at 25°C. The oxidation of NAD(P)H was started by adding 25 μM of Ca^{2+} or high concentrations of either cinnarizine or flunarizine.

2.5. Measurement of Ca^{2+} fluxes and respiration parameters

Ca^{2+} flux and mitochondrial respiration were simultaneously measured in a thermostat-controlled reaction chamber (4 ml) at 25°C. Mitochondrial respiration was measured by means of a Clark-type oxygen microelectrode fitted to an oxygen monitoring system (Hansatech®). Mi-

tochondrial respiration (1 mg of protein/ml) was induced by the addition of sodium succinate (6 mM final concentration) and oxidative phosphorylation was initiated by the addition of ADP to a final concentration of 0.1 mM. Oxygen consumption recordings allowed the calculation of V_3, the rate of state 3 (ADP-stimulated) respiration, of V_4, the rate of state 4 (non-ADP-stimulated) respiration, of the respiratory control ratio ($RCR = V_3/V_4$), and the P/O ratio (ADP used divided by oxygen (O_2) consumed in state 3 respiration).

The concentration of Ca^{2+} in the extramitochondrial medium was monitored using a specific Ca^{2+} electrode (Orion®) connected to the auxilliary output of the oxygen monitoring system via a 720 A Orion ionometer.

2.6. Assay of lipid peroxidation

Lipid peroxidation was assayed as the generation of malondialdehyde according to Braughler et al. (1986). Briefly, after the mitochondrial swelling reached its steady-state level, samples of 0.5 ml were taken and mixed with 1 ml of 30% trichloroacetic acid, 0.5 ml of desferrioxamine and 1.5 ml of 0.67% thiobarbituric acid. The mixture was then heated at 100°C for 1 h and recooled in ice for 10 min before centrifugation in an Eppendorf centrifuge. Results were expressed as micromolar of malondialdehyde per milligram of mitochondrial protein present at the end of the incubation period.

2.7. Data analysis and statistics

The initial rate of swelling (V_i) was expressed as change in absorbance per minute per milligram protein and the percentage of swelling inhibition rate (E) induced by different inhibitors was determined as follows:

$$E = \frac{V_{max} - V_i}{V_{max}} \times 100 \quad (1)$$

where V_{max} is the maximal swelling rate in the absence of the inhibitor.

Data of swelling inhibition experiments were fitted to the following equation:

$$E = \frac{E_{max} \cdot C}{IC_{50} + C} \quad (2)$$

where E is the swelling inhibition rate (in %) in the presence of a particular drug concentration (C), E_{max} the maximal effect and IC_{50} the concentration that inhibits 50% of the maximal effect.

All parameters were calculated by means of a nonlinear regression analysis using a commercially available software (Microphar INSERM 1990; Urien, 1995).

Statistical comparisons were made between two parameters by means of Student's two tailed unpaired *t*-test. A P value < 0.05 was considered statistically significant. All values are shown as means ± S.D. of at least three different experiments.

3. Results

3.1. Effects of cinnarizine and flunarizine on mitochondrial swelling

In the presence of the prooxidant *tert*-butylhydroperoxide (10 μM) and Ca^{2+} (100 μM) mitochondria swelled, which was attested by the increase in mitochondrial absorption at 520 nm. Cinnarizine inhibited the mitochondrial swelling in a concentration-dependent manner with an IC_{50} of 20.0 ± 0.7 μM. In the presence of 25 μM of Ca^{2+} alone, swelling of the energized mitochondria was also observed and cinnarizine inhibited it with an IC_{50} of 3.7 ± 0.5 μM (Fig. 1A). At high concentrations, however, cinnarizine was able to induce mitochondrial swelling by itself, i.e., in the absence of both *tert*-butylhydroperoxide and Ca^{2+} (Fig. 1B). The maximal effect of cinnarizine was observed at a concentration of 100 μM. In this case cyclosporine A which is the most potent inhibitor of mitochondrial swelling known today was unable to abrogate this swelling even at a concentration as high as 1 μM. Moreover, at this concentration, cyclosporine A potentiated the swelling induced by cinnarizine as shown in Fig. 1B. However, 150 μM 2,6-di-*tert*-butyl-4-methylphenol inhibited completely this mitochondrial swelling.

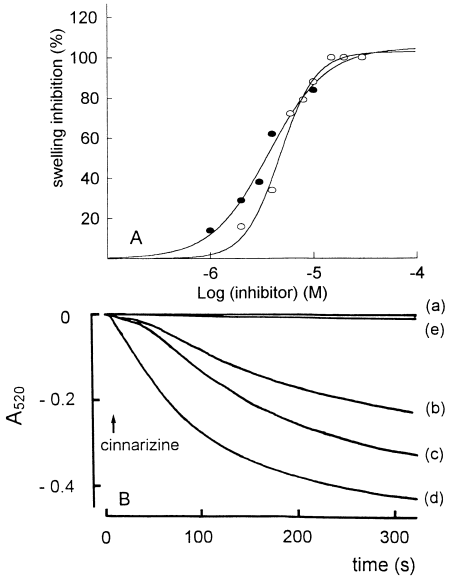

Fig. 1. Dual effect of cinnarizine and flunarizine on mitochondrial swelling. Swelling was assessed by measuring the change in absorbance of the mitochondrial suspension at 520 nm (A_{520}). (A) Inhibition of mitochondrial swelling by low concentrations of cinnarizine (●) and flunarizine (○). Swelling was induced by 25 μM Ca^{2+} in energized mitochondria. (B) Induction of mitochondrial swelling by high concentrations of cinnarizine, 50 μM (line b), 100 μM (line c). Line a: no addition of cinnarizine. Mitochondria were preincubated with either cyclosporine A (1 μM; line d) or 2,6-di-*tert*-butyl-4-methylphenol (150 μM, line e) and swelling was induced by 100 μM cinnarizine.

The same results were obtained with flunarizine, which inhibited the mitochondrial swelling induced by Ca^{2+} with an IC_{50} of 4.8 ± 0.3 μM (Fig. 1A) and induced it at higher concentrations (data not shown). As both cinnarizine and flunarizine behaved alike in all our mitochondrial tests and for reasons of space availability and clarity, we will present only the results obtained with cinnarizine.

3.2. Effects of cinnarizine on mitochondrial membrane potential

The mitochondrial membrane potential is another parameter which can be used to monitor the mitochondrial pore opening. Indeed, as shown in Fig. 2, cinnarizine at low concentrations restored the mitochondrial membrane potential collapsed by the addition of 25 μM of Ca^{2+} in a dose-dependent fashion. However, at high concentrations and in the absence of Ca^{2+} cinnarizine induced the collapse of the mitochondrial membrane potential which is in accordance with our first data. Again, cyclosporine A (1 μM) was unable to restore the mitochondrial membrane potential. As 2,6-di-*tert*-butyl-4-methylphenol interfered with the fluorescence of rhodamine 123, we could not test the effect of this antioxidant on mitochondrial membrane potential induced by high concentrations of cinnarizine.

3.3. Effects of cinnarizine on mitochondrial Ca^{2+} fluxes

Energized mitochondria can accumulate Ca^{2+} for a period of time. After addition of a low concentration of Ca^{2+} to the incubation medium, a rapid uptake was observed followed by a dynamic steady state corresponding to the equilibrium between the influx and the efflux of Ca^{2+}. When anaerobiosis was reached, this equilibrium was disrupted and Ca^{2+} was released. By addition of 25 μM Ca^{2+} to the incubation medium, the upsetting of the equilibrium occurred more rapidly before anaerobiosis (Fig. 3A, line a).

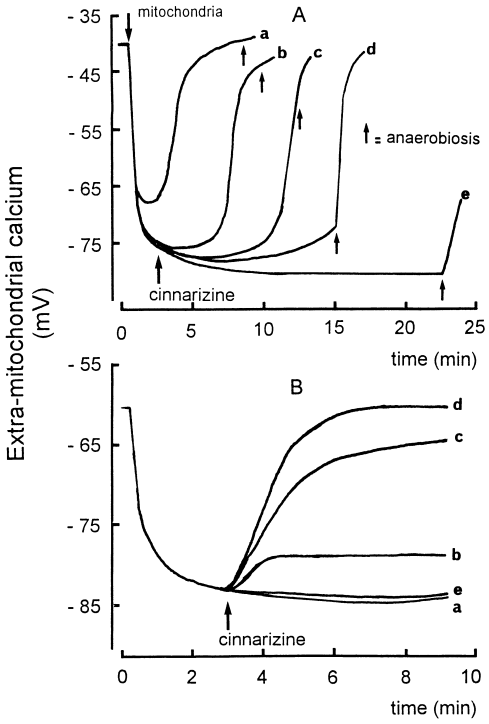

Fig. 3. Effect of cinnarizine on mitochondrial Ca^{2+} fluxes. Extramitochondrial Ca^{2+} was monitored using a specific Ca^{2+} electrode in a thermostat-controlled reaction chamber (4 ml) at 25°C containing respiration buffer and 6 mM succinate. The extramitochondrial Ca^{2+} concentration decreased rapidly when mitochondria (1 mg/ml) were added to the medium. (A) Increase of the Ca^{2+} steady state duration by low concentrations of cinnarizine, 1 μM (line b), 5 μM (line c), 10 μM (line d) and 25 μM (line e). Line a: Ca^{2+} (25 μM) alone. (B) Stimulation of Ca^{2+} release by high concentrations of cinnarizine 50 μM (line b) and 100 μM (line c). Line d: cinnarizine 100 μM + cyclosporine A 1 μM. Line e: cinnarizine 100 μM + 2,6-di-*tert*-butyl-4-methylphenol 150 μM. Line a: control, Ca^{2+} alone (2 μM).

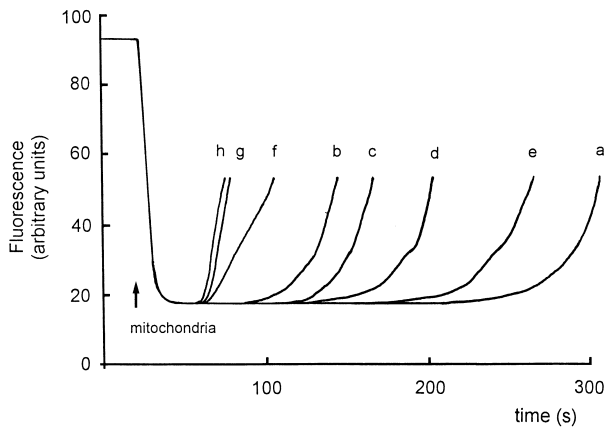

Fig. 2. Effect of cinnarizine on mitochondrial membrane potential. Rhodamine 123 was incubated at 25°C in 1.8 ml of phosphate buffer. After 30 s, mitochondria (0.5 mg/ml) were added (arrow). Line a: control. Line b: in the presence of 25 μM Ca^{2+}, mitochondrial potential is dissipated and restored by increasing concentrations of cinnarizine, 10 μM (line c), 12.5 μM (line d) and 15 μM (line e). Cinnarizine 50 μM (line f) and 100 μM (line g) collapse the membrane potential. Line h: cinnarizine 100 μM + cyclosporine A 1 μM.

In the presence of low concentrations of cinnarizine, the duration of the steady state is increased in a concentration-dependent manner (Fig. 3A). However, in the presence of high concentrations of cinnarizine, mitochondrial Ca^{2+} efflux was stimulated (Fig. 3B). This effect was totally abolished by 2,6-di-*tert*-butyl-4-methylphenol (150 μM) and reinforced by cyclosporine A (1 μM).

3.4. Effects of cinnarizine on NAD(P)H oxidation

In the presence of Ca^{2+} (25 μM), mitochondrial NAD(P)H was oxidized. As shown in Fig. 4A, cinnarizine at low concentrations antagonized this effect. However, at high concentrations and in the absence of Ca^{2+}, cinnarizine induced the oxidation of mitochondrial NAD(P)H, an

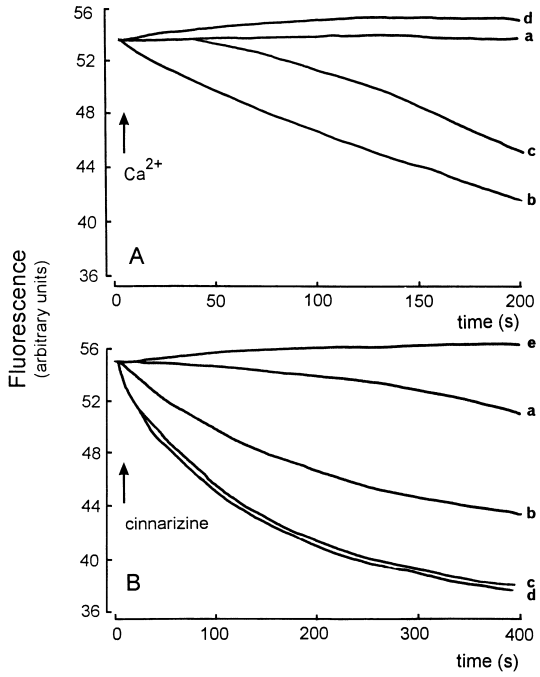

Fig. 4. Effect of cinnarizine on mitochondrial NAD(P)H level. Mitochondrial NAD(P)H was monitored by measuring its autofluorescence at an excitation and emission wavelength of 366 and 450 nm, respectively. Mitochondria (1 mg) were incubated for 1 min at 25°C in 1.8 ml phosphate buffer and Ca^{2+} (A) or cinnarizine (B) was added to the medium. (A) Ca^{2+} induced NAD(P)H oxidation was inhibited by low concentrations of cinnarizine. Line a, no Ca^{2+}; line b, Ca^{2+} 25 μM; Line c, Ca^{2+} 25 μM + cinnarizine 5 μM; Line d: Ca^{2+} 25 μM + cinnarizine 25 μM. (B) High concentrations of cinnarizine induced NAD(P)H oxidation in absence of Ca^{2+}. NAD(P)H oxidation was measured in the absence (line a) or in the presence of 50 μM (line b) or 100 μM (line c) cinnarizine. Line d: cinnarizine 100 μM + cyclosporine A 1 μM. Line e: cinnarizine 100 μM + 2,6-di-*tert*-butyl-4-methylphenol 150 μM.

effect which was totally abrogated by 2,6-di-*tert*-butyl-4-methylphenol (150 μM) but not by cyclosporine A (1 μM) (Fig. 4B).

Table 1
Alteration of mitochondrial functional parameters in the presence of increasing concentrations of cinnarizine

Cinnarizine (μM)	V_3	V_4	RC	P/O
0	0.61	0.15	4.07	1.82
1	0.69	0.17	4.03	1.80
2	0.67	0.16	4.19	1.72
5	0.59	0.14	4.17	1.86
10	0.57	0.16	3.53	1.60
15	0.58	0.17	3.37	1.72
20	0.48	0.14	3.48	1.50
30	0.44	0.15	2.98	1.46
50	0.30	0.12	2.56	1.25
65	0.24	0.12	2.01	1.09
85	0.20	0.20	1.00	–
100	0.31	0.31	1.00	–

Values in the table correspond to a typical experiment. V_3 and V_4 are expressed as nanomoles of O_2 per milligram of mitochondrial protein per second.

Table 2
Mitochondrial malondialdehyde level in the presence of different concentrations of cinnarizine

Agents	Concentrations (μM)	[malondialdehyde] (μM/mg of protein)
Cinnarizine	0	0.07 ± 0.04
	50	0.09 ± 0.02
	100	0.08 ± 0.02
	150	0.09 ± 0.03
	200	0.10 ± 0.03
Fe^{2+}/Fe^{3+}	50/150	0.45 ± 0.13[a]

[a] $P < 0.008$ versus control value (without cinnarizine).
Results are expressed as means ± S.D. of three separate experiments.

3.5. Effects of cinnarizine on mitochondrial oxidative-phosphorylation

Table 1 shows the effects of increasing concentrations of cinnarizine on V_3, V_4, RC and P/O, parameters reflecting the mitochondrial oxidative phosphorylation state.

For a concentration of 10 μM, cinnarizine inhibited mitochondrial ATP synthesis. Above $\approx$ 80 μM of cinnarizine, mitochondria were totally uncoupled.

3.6. Increasing concentrations of cinnarizine do not affect malondialdehyde level

Mitochondrial membrane lipid peroxidation was assessed as malondialdehyde generation. Our results show that of Fe^{2+}/Fe^{3+} (50 μM/150 μM) increased mitochondrial malondialdehyde level, however, neither cinnarizine nor flunarizine up to a concentration of 150 μM had any effect on the malondialdehyde production (Table 2).

The above results were further confirmed by measuring the production of H_2O_2 by mitochondria in the presence of cinnarizine. Indeed increasing concentrations of cinnarizine did not lead to an increase in H_2O_2 generation by mitochondria (results not shown).

4. Discussion

Our data show that cinnarizine and flunarizine at low concentrations (< 50 μM) inhibited mitochondrial swelling induced either by Ca^{2+} alone or in the presence of *tert*-butylhydroperoxide. Although both drugs were effective in inhibiting the ensued mitochondrial swelling, which is due to mitochondrial permeability transition generation, the IC_{50} by which both drugs inhibited mitochondrial permeability transition induced by *tert*-butylhydroperoxide was 5 times higher than that when the mitochondrial permeability transition was induced by Ca^{2+} alone. This difference in potency can be accounted for, at least partly, by the severity of the conditions under which the mitochondrial permeability transition is generated when *tert*-butylhydroperoxide was used. Furthermore, both drugs were able to restore mitochondrial membrane potential collapsed by the

addition of high concentrations of Ca^{2+} which further attested the preservation of mitochondrial membrane integrity by low concentrations of cinnarizine and flunarizine.

The mechanism of mitochondrial Ca^{2+} release has previously been linked to the oxidation of NAD(P)H and its subsequent hydrolysis to nicotinamide and ADP-ribose. The latter rapidly associates to a mitochondrial porin responsible for Ca^{2+} release (Richter and Schlegel, 1993). Our results show that the addition of low concentrations of both cinnarizine and flunarizine inhibited in a concentration-dependent manner the oxidation of mitochondrial NAD(P)H and Ca^{2+} release. The fact that the inhibition of mitochondrial permeability transition by both drugs parallels their inhibition of Ca^{2+} release suggests that mitochondrial permeability transition is responsible for Ca^{2+} release as stated by Bernardi and Petronilli (1996). However, a direct or indirect inhibition of the ADP-ribosylated porin which might be distinct from the mitochondrial permeability transition is also plausible (Richter, 1996). Indeed it is not easy to differentiate between the mitochondrial permeability transition and the hypothetic ADP-ribosylated porin. As observed with cinnarizine and flunarizine, any agent that inhibits the mitochondrial permeability transition opening inhibits Ca^{2+} release and inversely, any agent that promotes mitochondrial permeability transition opening, induces Ca^{2+} release (Zoratti and Szabo, 1995).

The results reported here do not provide evidence of the molecular mechanism by which cinnarizine and flunarizine at low concentrations exert their effects. It is possible that the inhibition of the mitochondrial permeability transition is the keystone by which these two drugs restored the mitochondrial normal functions. As both cinnarizine and flunarizine exert their mitochondrial effects with the same potency and effectiveness, we ruled out that the fluorine atoms take part in the mechanism of action of flunarizine.

Mitochondria have been involved in the mechanism of injury induced by chemical (Orrenius et al., 1989) or pathological situations, for instance ischemia–reperfusion injury (Gunter et al., 1994; Rosser and Gores, 1995). Indeed, many agents that preserve mitochondrial integrity, protect the whole cell against injury induced by ischemia–reperfusion. Recently, Konrad et al. (1995) by using a model of isolated rat liver, have shown that flunarizine protects liver cells against damage caused by warm ischemia and reperfusion. Furthermore, De Haan et al. (1993) have found that the neuroprotective effect afforded by flunarizine to fetal lambs subjected to asphyxia, is not a consequence of the effects of this drug on the regional blood flow, arterial blood pressure or fetal heart rate. Taken together, these data show that the beneficial effects of this drug cannot only ascribed to its effect on Ca^{2+} channel. According to our results, a mitochondrial component that might take part in the mechanism of action of both cinnarizine and flunarizine is also plausible.

Interestingly, in the absence of Ca^{2+} and *tert*-butylhydroperoxide, when the concentrations of both cinnarizine and flunarizine were increased, mitochondrial swelling occurs and this is accompanied by oxidation of mitochondrial NAD(P)H, Ca^{2+} release and mitochondrial membrane potential collapse. All these effects are not antagonized by cyclosporine A, the most potent inhibitor of the mitochondrial permeability transition, even at a concentration as high as 1 μM. Furthermore in the presence of cyclosporine A, mitochondrial swelling induced by cinnarizine and flunarizine is exacerbated. However, in the presence of 2,6-di-*tert*-butyl-4-methylphenol, a known antioxidant, the swelling induced by high concentrations of either cinnarizine or flunarizine is completely abolished. This suggests that the swelling induced by both drugs might be due to the generation of free radicals when their concentration is increased. Indeed, Veich and Hue (1994) have shown that increasing concentrations of flunarizine increase superoxide generation in submitochondrial particles. However, as cinnarizine and flunarizine even at high concentrations did neither increase malondialdehyde level nor mitochondrial H_2O_2 production, it is unlikely that the mechanism by which these two drugs at high concentrations induce the mitochondrial permeability transition, might be the increase in mitochondrial reactive oxygen species generation.

Recently, Gudz et al. (1997) have shown that 2,6-di-*tert*-butyl-4-methylphenol operates in inhibiting the mitochondrial permeability transition independently of its free radical scavenging activity. They have shown that 3,5-di-*tert*-butyltoluene, a derivative of 2,6-di-*tert*-butyl-4-methylphenol devoid of radical scavenging activity, exerts an analogous effect on the mitochondrial permeability transition. The authors concluded for the existence of antioxidant binding sites within the hydrophobic zone of the inner membrane or in the matrix space. Taken together, these results and ours suggest that cinnarizine and flunarizine might also exert their biphasic effects by binding to those hydrophobic sites, which might explain the competition observed between both drugs and 2,6-di-*tert*-butyl-4-methylphenol. This is strengthened by the fact that the induction of the mitochondrial permeability transition by cinnarizine and flunarizine at high concentrations is cyclosporine A independent. The same has been observed by Gudz et al. (1997). They have found that the induction of the mitochondrial permeability transition through the hydrophobic sites is not antagonized by cyclosporine A.

Although all our work was performed on isolated liver mitochondria, the same effects could be observed with mitochondria isolated from other tissues. This is based on the finding of Veich and Hue (1994) showing that the effects of both cinnarizine and flunarizine on mitochondria were not tissue-specific.

Many investigators have shown brain mitochondrial dysfunction in patients suffering from Parkinson's disease. This dysfunction has been suggested to be partly due to the

generation of mitochondrial permeability transition (Mizuno et al., 1995). It is interesting to stress that flunarizine interacts with dopamine receptors located in substantia nigra and so an accumulation of this drug, which is lipophilic in nature, within the cells of this region of the brain is likely to occur. Bearing in mind that it is not easy to extrapolate our in vitro results to clinical situations, we can hypothesize that in aged patients, cinnarizine and flunarizine might accumulate in substantia nigra, reaching concentrations that might trigger the mitochondrial permeability transition induction, thus leading to mitochondrial dysfunction and cell death. These toxic events might be mainly due to drug overdosages.

In conclusion, we have shown that cinnarizine and flunarizine possess a mitochondrial protective effect at low concentrations and a toxic effect associated with the induction of mitochondrial permeability transition at high concentrations. Both effects might be linked to the binding of these drugs to mitochondrial hydrophobic sites, the shift from the inhibition to the induction of the mitochondrial permeability transition being dependent on the concentration of cinnarizine or flunarizine that reaches these sites.

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