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S-15176 inhibits mitochondrial permeability transition via a mechanism independent of its antioxidant properties

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Abstract

Mitochondrial Ca^{2+} accumulation can induce a sudden increase in the permeability of the inner membrane. This phenomenon is due to the generation of a large nonselective ion channel, termed the permeability transition pore (PTP), which contributes to cellular injury during ischemia and reperfusion. Inhibition of PTP generation constitutes a relevant pharmacological target to protect a cell from death. In this study, we examined the effect of S-15176 ((*N*-[(3,5-di-tertiobutyl-4-hydroxy-1-thiophenyl)]-3-propyl-*N*′ -(2,3,4-trimethoxybenzyl)piperazine), a novel anti-ischemic agent, on PTP in rat liver mitochondria. S-15176 prevented PTP opening generated by various triggering agents, as attested by the concentration-dependent inhibition of mitochondrial swelling, of mitochondrial membrane potential dissipation and of NADPH oxidation. These effects were associated with an increase in the Ca^{2+} loading capacity of mitochondria. S-15176 was a strong inhibitor of lipid peroxidation, but experiments with another trimetazidine derivative devoid of antioxidant activity indicated that this activity was not essential to the inhibitory effect. Binding studies demonstrated that [^{3}H]S-15176 bound to mitochondrial binding sites, especially those localized in the inner membrane. These sites were shared by several well-known inhibitors of PTP opening. These results demonstrate that the mechanism by which S-15176 protects mitochondria against the deleterious effects of ischemia–reperfusion involves inhibition of PTP opening and provide evidence that the drug operates through low structural specificity binding sites located in the inner mitochondrial membrane.

Keywords: Permeability transition pore; Binding site; Specific; Antioxidant property; Mitochondrion; Liver; (Rat)

1. Introduction

There is an increasing amount of evidence suggesting that the mitochondrial inner membranes contain a non-specific pore, generally termed the permeability transition pore (PTP), which when opened causes membrane depolarization, the release of small molecules and ions (< 1500 Da) from the mitochondrial matrix, uncoupling of oxidative phosphorylation and swelling (Bernardi et al., 1994; Zoratti and Szabo', 1995; Crompton, 1999). PTP opening is favored by high phosphate concentrations, ATP and ADP depletion, Ca^{2+} overload and oxidative stress, conditions prevailing during ischemia–reperfusion. This is why several groups have suggested that pore opening might be a critical event in the genesis of the cell death associated with this condition (Crompton et al., 1987; Bernardi, 1996; Crompton, 1999; Lemasters et al., 1999). This hypothesis was reinforced by several observations: (1) mitochondria can control cell death by apoptosis and/or necrosis, and PTP is highly involved in these phenomena (Kroemer et al., 1998); and (2) cyclosporin A, which is the most potent inhibitor of PTP (Fournier et al., 1987; Broekemeier et al., 1989), protects cells from ischemia–reperfusion injury by inhibiting PTP during reperfusion (Halestrap et al., 1997; Friberg et al., 1998). Taken together, these data lead to the interesting idea that inhibition of pore opening might constitute a relevant pharmacological approach to protect cell a from death (Nicolli et al., 1995), and the search for novel PTP inhibitors should be considered with great interest (Morin et al., 2002).

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Recently, we demonstrated that a novel derivative of the anti-ischemic agent trimetazidine, S-15176 ((*N*-[(3,5-di-tertiobutyl-4-hydroxy-1-thiophenyl)]-3-propyl-*N*′-(2,3,4-trimethoxybenzyl)piperazine), was able to limit the hepatic injury induced by ischemia–reperfusion (Settaf et al., 2000). This protective effect was associated with the preservation of mitochondrial function and seemed to be related to the inhibition of PTP opening (Elimadi et al., 2001). In the present study, we analyzed the effect of S-15176 on mitochondrial function and demonstrate that PTP inhibition exhibited by S-15716 is not related to its scavenging properties against reactive oxygen species.

2. Materials and methods

2.1. Chemicals

Labelled and unlabelled S-15176 and S-00240 (Fig. 1) were kindly provided by Servier Laboratories (Neuilly-sur-Seine, France). Other chemicals were obtained from Sigma (St. Quentin Fallavier, France), Merck (Nogent-sur-Marne, France), Research Biomedical International/Bioblock Scientific (Illkirch, France) and Interchim (Montluçon, France) and were of the highest purity available.

2.2. Isolation of mitochondria

Rat liver mitochondria were isolated as described by Johnson and Lardy (1967). Briefly male Wistar rats weighing approximately 250–300 g were decapitated and the liver was 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 × *g* for 10 min (Sorvall RC 28 S), and the supernatant was centrifuged for 5 min at 15 000 × *g* to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at 15 000 × *g* for 5 min.

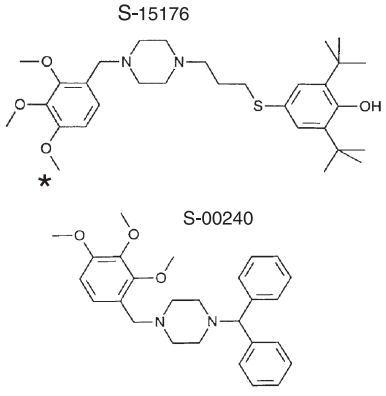

Fig. 1. Chemical structures of the trimetazidine derivatives used in this study. *Labelling position.

The mitochondrial pellet was washed with medium from which EGTA was omitted and centrifuged for 5 min at 15 000 × *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).

For binding experiments, mitochondria were purified according to Morin et al. (1998) and the submitochondrial localization of [^{3}H]S-15176 binding sites was determined using a digitonin solubilization method to separate the outer from the inner mitochondrial membrane, as described by Anholt et al. (1986). Briefly, freshly isolated mitochondria (8 mg/ml) were treated or not with 0.6 mg digitonin/mg/protein in 1 ml Tris–sucrose buffer for 15 min at 4 °C. After incubation, the mitochondrial suspension was diluted and centrifuged at 15 000 × *g* for 7 min. Cytochrome C oxidase activity, monoamine oxidase activity and [^{3}H]S-15176 (8 nM) binding were then assayed in the pellet.

2.3. Mitochondrial swelling measurements

Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 520 nm by using a Hitachi model U-3000 spectrophotometer. In a first approach, mitochondrial swelling was measured in de-energized mitochondria. Swelling was induced by pro-oxidant agents, phenylarsine oxide or *tert*-butylhydroperoxide, in the presence of Ca^{2+} and was measured according to the method of Halestrap and Davidson (1990) as modified by Elimadi et al. (1997a). Briefly, mitochondria (4 mg) were added to 3.6 ml of buffer containing 150 mM sucrose, 5 mM Tris, 0.5 μg of rotenone/ml at pH 7.4 at 25 °C. Then, 1.8 ml of this suspension was added to both sample and reference cuvettes in the presence or absence of different inhibitors. After 3 min of incubation at 25 °C, 100 μM of $CaCl_2$ was added to both cuvettes. Four minutes later, swelling was initiated by the introduction of 10 μM phenylarsine oxide or *tert*-butylhydroperoxide to the sample cuvette only and A_{520} scanning was started.

Mitochondrial swelling was also studied with energized mitochondria and was induced by two other triggering agents, inorganic phosphates or arachidonic acid, in the presence of Ca^{2+}. When Ca^{2+} and phosphate were used to induce swelling, mitochondria (4 mg) were added to 3.6 ml of a phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, 6 mM succinate and 2 μM rotenone, pH 7.2 at 25 °C), and 1.8 ml of this suspension was added to both sample and reference cuvettes in the presence or absence of inhibitor. After 1 min of incubation at 25 °C, swelling was initiated by the addition of 25 μM Ca^{2+} to the sample cuvette only.

When arachidonic acid was the trigger, the protocol described by Scorrano et al. (2001) was used. The incubation medium contained 250 mM sucrose, 5 mM Tris, 1 mM KH_2PO_4 and a mixture of glutamate/malate (2.5 mM) as a substrate. The experiment was started by the addition of 1

mg/ml mitochondria followed 1 min later by 20 μM Ca^{2+} and then swelling was induced with 40 μM arachidonic acid.

2.4. Optical monitoring of mitochondrial membrane potential

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

In total, 1.8 ml of the phosphate buffer, 0.3 μM of Rhodamine 123 and 25 μM of Ca^{2+} were added to the cuvette in the presence or absence of S-15176 and fluorescence scanning of Rhodamine 123 was monitored using a Perkin-Elmer® LS 50B spectrofluorimeter. After 30 s, mitochondria (0.5 mg/ml) were added.

2.5. Determination of mitochondrial NAD(P)H oxidation

Mitochondrial pyridine nucleotides were monitored at 25 °C by measuring their autofluorescence at excitation and emission wavelengths of 366 and 450 nm, respectively, in a Perkin-Elmer® LS 50B spectrofluorimeter according to the procedure described by Minezaki et al. (1994). 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 Ca^{2+}.

2.6. Measurement of Ca^{2+} fluxes and O_2 consumption

Ca^{2+} flux and mitochondrial O_2 consumption were simultaneously measured in a thermostat-controlled reaction chamber (4 ml) at 30 °C. Mitochondrial oxygen consumption was measured by means of a Clark-type oxygen microelectrode fitted to an oxygen monitoring system (Hansatech®). The concentration of Ca^{2+} in the extramitochondrial medium was monitored using a specific Ca^{2+} electrode (Orion®) connected to the auxiliary output of the oxygen monitoring system via a 720A Orion ionometer. Mitochondria (1 mg protein/ml) were preincubated for 1 min in a sucrose phosphate medium (250 mM sucrose, 5 mM KH_2PO_4, pH = 7.4 at 30 °C), and oxygen consumption was induced by the addition of a mixture of glutamate/malate (2.5 mM final concentration).

2.7. Assays of radical scavenging capacity, of lipid peroxidation and O_2^{O-} production

The free radical scavenging capacity of drugs was determined using diphenyl-2-picryl-hydrazyl stable free radical (Tamura et al., 1990; Brand-Williams et al., 1995). Drugs were added to an ethanol solution of diphenyl-2-picryl-hydrazyl (100 μM) and the decrease in absorbance was recorded against time in an Hitachi U-3000 spectrophotometer.

Lipid peroxidation was assayed as the generation of malondialdehyde according to Braughler et al. (1986). Briefly, mitochondria (1 mg/ml) were incubated in a total volume of 1 ml with a mixture of Fe^{2+}/Fe^{3+} (50 μM/150 μM) for 30 min. The reaction was stopped by adding 1 ml of 3% trichloroacetic acid and then 1.5 ml of 1% thiobarbituric acid was added. The mixture was heated at 100 °C for 1 h and cooled in ice for 10 min before centrifugation in an Eppendorf centrifuge. Then, supernatant was read at 530 nm. Results are expressed as micromoles of malondialdehyde/milligram of mitochondrial protein present at the end of the incubation period.

The generation of O_2^{O-} was measured as previously reported (Zini et al., 1999). Briefly, mitochondria (0.25 mg protein/ml) were incubated at 25 °C in a final volume of 1.5 ml of the sucrose phosphate medium containing 1 μM cyclosporin A and 100 μM nitroblue tetrazolium. Cyclosporin A was added to the medium to inhibit mitochondrial swelling, which slightly interfered with the spectroscopic detection of the reduction reaction. The reaction was started by adding 6 mM succinate and the rate of nitroblue tetrazolium reduction was measured at 560 nm.

2.8. Enzyme assays

Monoamine oxidase and cytochrome C oxidase activities were determined by spectrophotometric methods using an Hitachi® UV-3000 spectrophotometer. The determination of monoamine oxidase activity was performed according to the method of Bembenek et al. (1990), using kynuramine as a substrate and monitoring the formation of 4-hydroxyquinoline at 316 nm. Cytochrome C oxidase (EC 1.9.3.1) activity was assayed at 37 °C according to the method of Rustin et al. (1994) by monitoring the oxidation of ferrocytochrome C (prepared from type III horse heart cytochrome C (Sigma)) at 550 nm.

2.9. Binding experiments

Purified mitochondria were disrupted by freezing and sonication. They were suspended (2 mg protein/ml) in a Tris buffer (50 mM Tris, 250 mM sucrose, pH 7.4) and incubated with 4 nM [^{3}H]S-15176 (84 Ci/mmol) and 15–20 different concentrations of competing drug or Tris buffer for 30 min at 25 °C in a total volume of 250 μl. Specific binding was defined as the difference between total binding and binding in the presence of 10 μM S-15176.

At the end of the incubation period, bound and free ligands were separated by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.1% polyethylenemine). Each filter was washed twice with an additional 5 ml of ice-cold phosphate buffer (25 mM) and counted in a liquid scintillation counter Packard 1600 TR with an efficiency of 45%. The filtration process was rapid enough to avoid the dissociation of radiolabelled ligands from their binding sites. In addition, in all cases, radiolabelled ligand

binding to glass fiber filters was very low, less than 0.05% of the total ligand concentration.

2.10. Data analysis and statistics

The initial rate of swelling (V_i) is expressed as the change in absorbance/min/mg protein and the percentage inhibition of the swelling 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 from the swelling inhibition experiments were fitted to the following equation:

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

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 commercially available software (Micropharm INSERM 1990, Urien, 1995). The same software was used to calculate inhibition binding parameters (IC_{50}).

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. S-15176 inhibits mitochondrial swelling induced by different agents

It is well established that non-energized mitochondria supplemented with high Ca^{2+} concentrations swell in the presence of a pro-oxidant drug (Zoratti and Szabo', 1995). Fig. 2 (panel A) shows that 10 μM *tert*-butylhydroperoxide induced mitochondrial swelling, as attested by the decrease in absorbance of the mitochondrial suspension. The swelling was due to PTP opening since it was completely prevented by 1 μM cyclosporin A and 50 μM ubiquinone 0, two well-known inhibitors of this channel (Broekemeier et al., 1989; Fontaine et al., 1998), and by 150 μM the free radical scavenger 2,6-di-*tert*-butyl-4-methylphenol (BHT). Pretreatment of mitochondria with S-15176 also decreased the rate of mitochondrial swelling in a concentration-dependent manner with an IC_{50} of 45.7 ± 16.8 μM. In order to verify if the effect of S-15176 was instantaneous or took time to occur, we performed a kinetic study of the effect of the S-15176 on the swelling induced by *tert*-butylhydroperoxide in the presence of Ca^{2+}. The results showed that the inhibitory effect of S-15176 was rapid and remained stable for a long time (data not shown).

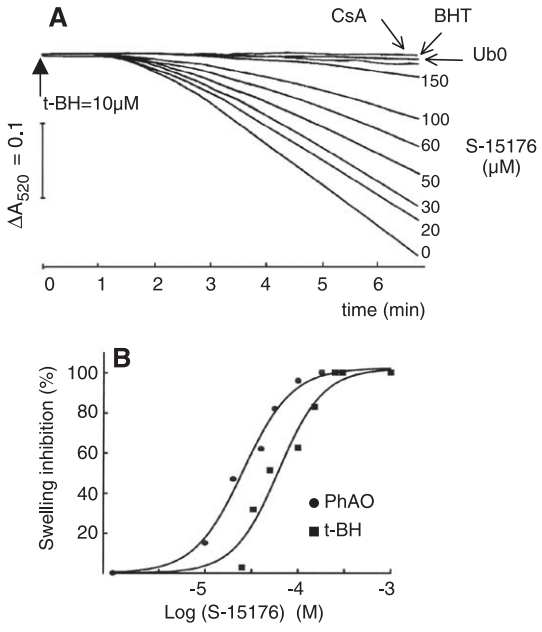

Fig. 2. Effect of S-15176 on mitochondrial swelling induced by *tert*-butylhydroperoxide or phenylarsine oxide under de-energized conditions. (panel A) Swelling was induced by the addition of 10 μM *tert*-butylhydroperoxide (t-BH) in the presence of 100 μM Ca^{2+}. It was inhibited by increasing concentrations of S-15176, 1 μM cyclosporin A (CsA), 50 μM ubiquinone (UB0) or 150 μM BHT. (panel B) Concentration-dependent effect of S-15176 on mitochondrial swelling induced by *tert*-butylhydroperoxide (*t*-BH) or phenylarsine oxide (PhAO). In these particular experiments, IC_{50} values were 30 and 61 μM for phenylarsine oxide and *tert*-butylhydroperoxide, respectively.

Other oxidant agents are able to induce PTP opening. For example, phenylarsine oxide, which promotes swelling by cross-linking thiol groups in the inner mitochondrial membrane (Zoratti and Szabo', 1995). Interestingly, the swelling induced by phenylarsine oxide did not depend on the presence of extramitochondrial Ca^{2+} because EGTA, even at a concentration of 2 mM, did not affect the initial rate of swelling. This is in accordance with the results of Bernardi (1992), indicating that phenylarsine oxide can modulate the process of PTP induction, independently of Ca^{2+}. Again, S-15176 was active in inhibiting this swelling, with an IC_{50} of 43.3 ± 12.7 μM (Table 1; Fig. 2, panel B).

S-15176 was also effective when experiments were performed with mitochondria energized either with succinate or glutamate/malate. Fig. 3 shows that swelling could be easily induced by 40 μM arachidonic acid and prevented by increasing concentrations of S-15176 with an IC_{50} value of 39.6 ± 10.6 μM. In the same way, S-15176 inhibited the swelling induced by inorganic phosphate in the presence of Ca^{2+}. S-15176 appeared potent in this test, with an IC_{50} value of 9.25 ± 3.6 μM. Table 1 summarizes these effects.

Table 1
IC_{50} values for inhibition by BHT and the trimetazidine derivatives S-15176 and S-00240 of mitochondrial swelling induced by different agents and NAD(P)H oxidation

	IC_{50} (μM)		
	S-15176	S-00240	BHT
Swelling			
t-BH	45.7 ± 16.8	47.5 ± 13.4	17.5 ± 3.5
PhAO	43.3 ± 12.7	92.6 ± 16.7	28.5 ± 3.5
Pi	9.25 ± 3.6	7.20 ± 3.10	10.0 ± 2.0
AA	39.6 ± 10.6	NS	NS
NAD(P)H			
Oxidation	10.0 ± 2.10	15.0 ± 3.20	18.0 ± 2.50

NS, not studied; Pi, inorganic phosphate; *t*-BH, *tert*-butylhydroperoxide; PhAO, phenylarsine oxide; AA, arachidonic acid.

3.2. S-15176 prevents the dissipation of mitochondrial membrane potential and the oxidation of NAD(P)H

Swelling, induced in energized mitochondria by high concentrations of Ca^{2+}, was accompanied by a dissipation of the mitochondrial membrane potential (Fig. 4, panel A) and an increase in NAD(P)H oxidation (Fig. 4, panel B). The presence of S-15176 inhibited both mitochondrial membrane potential dissipation and oxidation of NAD(P)H in a concentration-dependent manner.

3.3. Effect of S-15176 on mitochondrial Ca^{2+} loading

Another indicator of PTP opening and thus another way to investigate the interaction of drugs with PTP is to study their effect on mitochondrial Ca^{2+} retention. The loading protocol was derived from that described by Fontaine et al. (1998). Rat liver mitochondria energized with a mixture of glutamate/malate (2.5 mM) were incubated for 2 min in a sucrose phosphate buffer before the addition of successive 25 μM Ca^{2+} pulses. Fig. 5 shows that in control experiments, four successive additions of 25 μM Ca^{2+} (25 nmol/mg protein) were required to induce a fast release of Ca^{2+}. After each addition, rapid uptake was observed, followed by a dynamic steady state corresponding to the equilibrium between the influx and the efflux of Ca^{2+}. Each Ca^{2+} addition promoted an acceleration of oxygen consumption due to the transient drop in the membrane potential, which was immediately compensated for by an increase in respiration. When the maximal Ca^{2+} loading threshold was

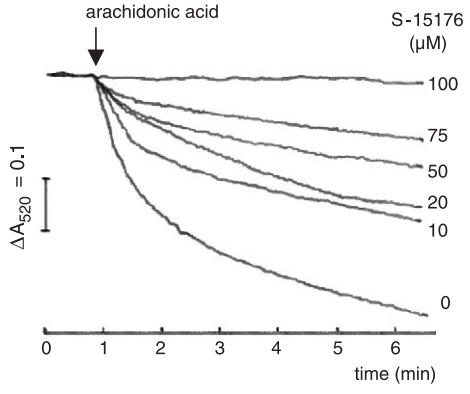

Fig. 3. S-15176 inhibits arachidonic acid-induced mitochondrial swelling under energized conditions. Swelling was induced by the addition of 40 μM arachidonic acid in the presence of increasing concentration of S-15176.

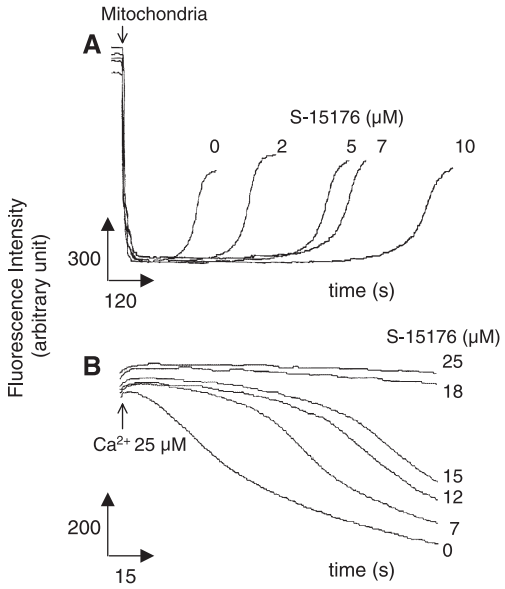

Fig. 4. Effect of S-15176 on mitochondrial membrane potential and NAD(P)H oxidation. (panel A) Mitochondrial membrane potential was measured after the addition of mitochondria (0.5 mg/ml). The incubation buffer contained 250 mM sucrose, 5 mM KH_2PO_4, 6 mM succinate, 25 μM Ca^{2+} and 0.3 μM of Rhodamine 123 supplemented or not (0) with increasing concentrations of S-15176. (panel B) The incubation buffer contained 250 mM sucrose, 5 mM KH_2PO_4, 6 mM succinate and mitochondria (0.5 mg/ml). NAD(P)H oxidation was induced by the addition of 25 μM Ca^{2+}.

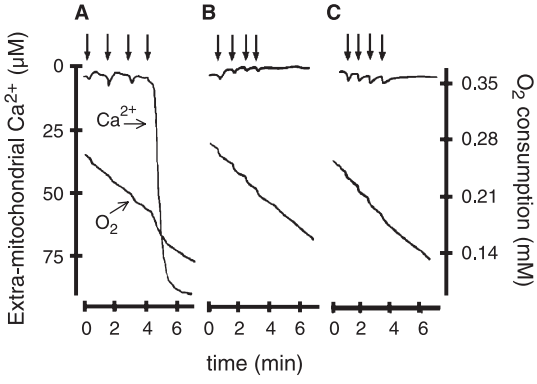

Fig. 5. Effect of S-15176 on Ca^{2+} fluxes and O_2 consumption. Mitochondria (1 mg/ml) were suspended in a phosphate buffer (sucrose 250 mM, KH_2PO_4 5 mM) supplemented or not (panel A: control) with either 1 μM cyclosporin A (panel B) or 10 μM S-15176 (panel C). Experiments were started by the addition of a mixture of glutamate/malate (2.5 mM) and four successive 25 μM Ca^{2+} pulses were added (arrows).

reached, this equilibrium was disrupted and Ca^{2+} was released. The release was blocked by prior addition of 1 μM cyclosporin A, indicating that it was due to PTP opening. Cyclosporin A greatly increased the amount of Ca^{2+} necessary for PTP opening from 100 to 375 nmol/mg protein. The same effect was observed with S-15176 but it was clearly less effective than cyclosporin A, as demonstrated by the concentration-dependent response depicted in Fig. 6. Interestingly, these curves were almost linear, indicating a possible relationship between Ca^{2+} and S-15176 effects.

3.4. The protective effect of S-15176 does not seem to be related to its antioxidant properties

We have previously shown that S-15176 is a strong inhibitor of lipid peroxidation of liver membranes (IC_{50} = 0.3 μM; Settaf et al., 2000). Fig. 7A confirms these data. S-15176 was as effective as the well-known free radical scavenger BHT but was more potent (BHT: IC_{50} = 7.20 ± 0.77 μM). S-15176 was also able to reduce slowly the stable free radical diphenyl-2-picryl-hydrazyl in ethanolic solution (Fig. 7, panel B). In order to evaluate the role of the antioxidant activity of the drug in mitochondrial protection, the same swelling experiments were performed with another trimetazidine derivative, S-00240, which is devoid of antioxidant activity (Fig. 7, panels A and B). The results are shown in Table 1 and compared to those of BHT. The two drugs displayed the same anti-swelling profile, indicating that the antioxidant activity was not necessary for the protective effect. The results obtained for O_2^{O-} produc-

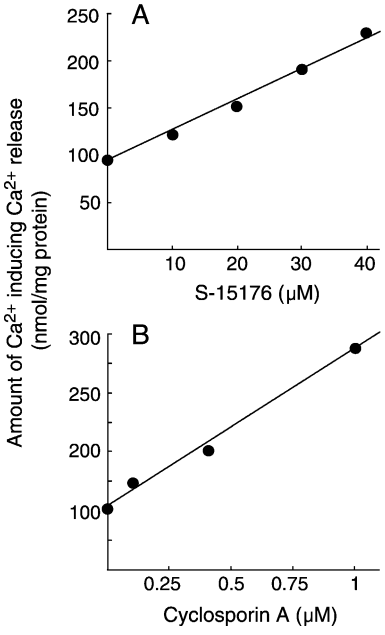

Fig. 6. Comparison of the effect of increasing concentrations of S-15176 and cyclosporin A on the amount of Ca^{2+} necessary to induce Ca^{2+} release from mitochondria. Experimental conditions were similar to those described in the legend to Fig. 5, except that mitochondria were supplemented or not with increasing concentrations of either S-15176 (panel A) or cyclosporin A (panel B). Experiments were started by the addition of a mixture of glutamate/malate (2.5 mM) and successive 25 μM Ca^{2+} pulses were added at 30-s intervals. For each concentration, the amount of Ca^{2+} necessary to induce Ca^{2+} release was determined.

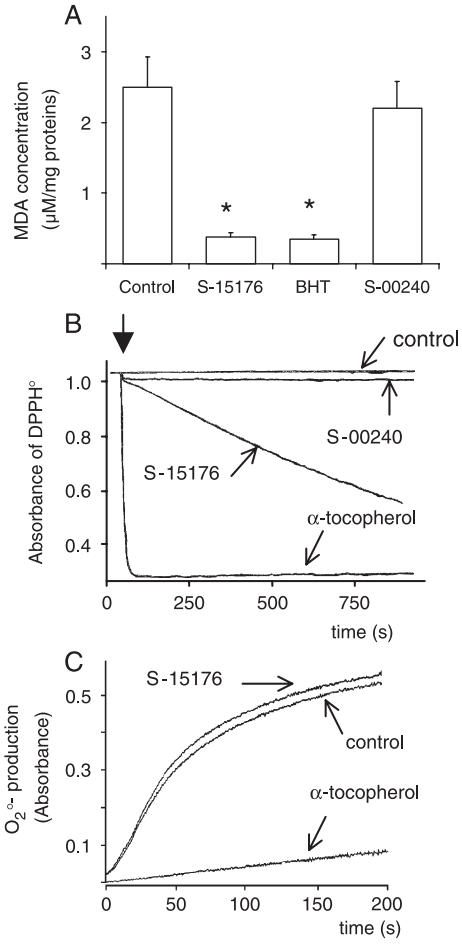

Fig. 7. Effect of S-15176 on lipid peroxidation, diphenyl-2-picryl-hydrazyl radical and O_2^{O-} production. (panel A) After preparation of liver membranes by means of a standard procedure, samples (0.2 mg protein/ml) were incubated in a total volume of 1 ml with a mixture of Fe^{2+}/Fe^{3+} (50μM/150μM) in the absence or in the presence of either 50 μM S-15176, 50 μM S-00240 or 100 μM BHT for 30 min. The reaction was stopped by adding 1 ml of 3% trichloroacetic acid. Then, 1.5 ml of 1% thiobarbituric acid was added. The mixture was heated to 100 °C for 30 min and cooled on ice for 10 min before centrifugation in an Eppendorf centrifuge. The supernatant was read at 530 nm. MDA: malondialdehyde. $p < 0.05$ versus control value. (panel B) When indicated (arrow), either 50 μM S-15176, 50 μM S-00240 or 100 μM α-tocopherol was added to an ethanol solution of 100 μM diphenyl-2-picryl-hydrazyl radical ($DPPH^{O}$), and the decrease in absorbance was monitored at 515 nm. α-Tocopherol was used as a reference inhibiting compound. (panel C) The reaction mixture (25 °C) contained mitochondria (0.25 mg protein/ml), 1 μM cyclosporin A and 100 μM nitroblue tetrazolium in the absence or in the presence of either 50 μM S-15176 or 100 μM α-tocopherol in a final volume of 1 ml. O_2^{O-} production was started by adding 6 mM succinate and was followed by measuring the rate of nitroblue tetrazolium reduction at 560 nm. α-Tocopherol was used as a reference inhibiting compound.

tion reinforced this idea. Indeed, up to 50 μM, S-15176 did not inhibit $O_2^{\cdot -}$ production (Fig. 7, panel C). Therefore, we focused on a possible mitochondrial membrane target.

3.5. Binding studies

Binding studies were undertaken to search for a possible mitochondrial membrane target for S-15176. A low concentration of [^{3}H]S-15176 (3–4 nM) was incubated with purified liver mitochondrial membrane. Fig. 8A shows that increasing concentrations of S-15176 were able to displace bound [^{3}H]S-15176, indicating the presence of specific binding sites of low affinity (IC_{50} = 0.98 μM; Table 2). This low affinity did not allow us to determine the equilibrium binding parameters (dissociation constant and number of binding sites) of the radioligand, but we could study the submitochondrial localization of [^{3}H]S-15176 binding sites using a digitonin solubilization protocol to separate the outer from the inner mitochondrial membrane (Anholt et al., 1986; Morin et al., 1998). Results suggest that [^{3}H]S-15176 binding sites are mainly located on the inner mitochondrial membrane (Fig. 8B). Interestingly, the other trimetazidine derivative S-00240 and the free radical scavenger BHT inhibited [^{3}H]S-15176 binding (Fig. 8A). In the same way, [^{3}H]S-15176 binding was displaced by other drugs known to modulate pore opening, such as trifluoperazine, prenylamine or amiodarone. Conversely, cyclosporin A, dibucaine and Ca^{2+}, even at high concentrations, were ineffective (Table 2).

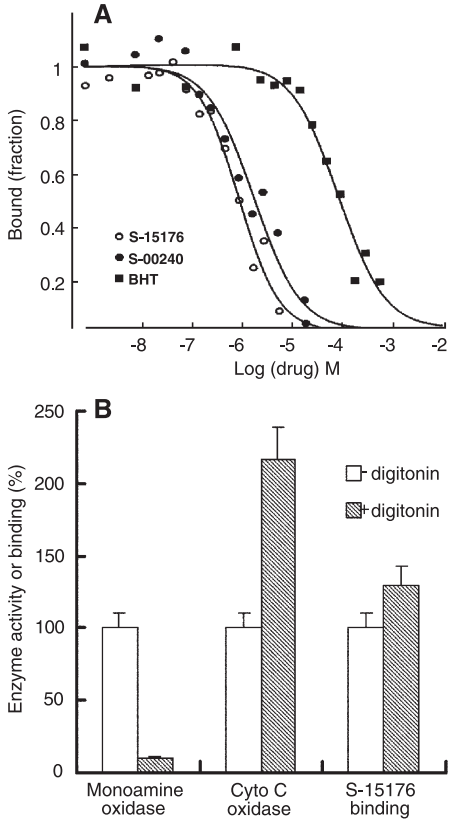

Fig. 8. Binding of [^{3}H]S-15176 to rat liver mitochondria. (panel A) Representative curves for the inhibition of [^{3}H]S-15176 binding to rat liver mitochondria (2 mg/ml). Data are plotted as fractions of the control [^{3}H]S-15176 binding value (in the absence of inhibitor: [^{3}H]S-15176 bound = 1). [^{3}H]S-15176 concentration was 3–4 nM. Control binding values varied between 108 and 150 fmol/mg protein and nonspecific binding between 30% and 40%. (panel B) [^{3}H]S-15176 binding sites are mainly located on the inner mitochondrial membranes. The maximal activity of the markers (100% values) was 0.86 nmol/min/mg protein for cytochrome C oxidase, 1.4 nmol/min/mg protein for monoamine oxidase, and 0.59 pmol/mg protein for 8 nM [^{3}H]S-15176 binding. Each data point is the mean ± S.D. of duplicate measurements obtained from three to four experiments.

Table 2
IC_{50} values for the inhibition of [^{3}H]S-15176 binding to rat liver mitochondria

	IC_{50} (μM)
S-15176	0.98 ± 0.27
S-00240	1.96 ± 0.36
Amiodarone	2.31 ± 1.27
Trifluoperazine	58.9 ± 18.3
Prenylamine	62.3 ± 20
BHT	91.7 ± 14.3
Cyclosporin A	>1 mM
Dibucaine	>1 mM

Values are expressed as means ± S.D. of three independent experiments done in duplicate.

4. Discussion

We have already shown that the trimetazidine derivative S-15176 protects rat liver from the deleterious effect of ischemia–reperfusion injury by preserving mitochondrial functions (Elimadi et al., 2001). However, the mechanism of this protection has not been elucidated yet. In this study, we investigated in depth the mechanism of the mitochondrial protective effect of S-15176, using an experimental model of isolated mitochondria. This study clearly showed that the protective effect of S-15176 on mitochondrial function in the in vivo experimental model of rat liver subjected to 120 min of normothermic ischemia followed by 30 min of reperfusion is directly related to its inhibition of PTP pore opening. Indeed, S-15176 inhibited mitochondrial swelling induced by several agents, i.e., *tert*-butylhydroperoxide, an oxidant of thiol groups, phenylarsine oxide, an hydrophobic thiol cross-linker, inorganic phosphate or arachidonic acid. S-15176 inhibited the swelling induced by phenylarsine oxide and *tert*-butylhydroperoxide with the same potency (EC_{50}), suggesting that the mechanisms by which these two oxidant agents induce mitochondrial swelling are similar. This is in agreement with the finding of Kowaltowski et al. (1997), who showed that both phenylarsine oxide and *tert*-butylhydroperoxide induce PTP via the same process, i.e., oxidation of thiol groups possessing the same reactivity (Kowaltowski et al., 1997). It is likely that both the hydrophobic compounds *tert*-butylhydroperoxide and phenylarsine oxide oxidize preferentially the thiol

groups buried in the inner mitochondrial membrane. Accordingly, S-15176, which is hydrophobic, could inhibit PTP opening by preventing the oxidation of thiol groups buried in the inner mitochondrial membrane.

However, the antioxidant properties of this molecule (scavenger of HO^{O}) did not seem to account for the inhibition of swelling since another trimetazidine derivative, S-00240, which is devoid of such antioxidant effect, possessed the same inhibitory effects as S-15176 on PTP opening induced by different agents. This prompted us to investigate the binding of S-15176 to mitochondria. Indeed, a growing body of evidence suggests the existence of a new structural class of pore inhibitors possessing mitochondrial binding sites that regulate PTP (Fontaine et al., 1998; Gudz et al., 1997; Morin et al., 1998; Plemper van Balen et al., 2002). [^{3}H]S-15176 binds to mitochondrial binding sites, localized in the inner membrane. This molecule displayed a low affinity for these sites but the IC_{50} value was close to that at which S-15176 inhibited mitochondrial swelling. In addition, several well-characterized swelling inhibitors, such as trifluoperazine (Pereira et al., 1992; Elimadi et al., 1997b), amiodarone (Zoratti and Szabo', 1994) or prenylamine (Broekemeier et al., 1985; Morin et al., 1998), which are structurally unrelated, also displaced S-15176 from its sites. This reveals a low structural specificity of these sites, which can account for the low affinity of the drug binding interaction. Taken together, these findings suggest that S-15176 might regulate PTP through its inner mitochondrial binding sites. These binding sites are different from the Ca^{2+} binding sites which have been described to regulate PTP opening (Bernardi et al., 1993; Kowaltowski et al., 1998) since neither Ca^{2+} nor dibucaine, which has anti-swelling properties attributed to its ability to reduce Ca^{2+} binding to the mitochondrial membrane (Kowaltowski et al., 1998), inhibit S-15176 binding. Thus, a common S-15176–Ca^{2+} binding site cannot explain the relation observed between the two agents. Moreover, the binding studies indicated that S-15176 and cyclosporin A do not share the same binding sites, since cyclosporin A was unable to displace S-15176 from its mitochondrial sites. These results suggest that this effect on PTP could be additive or even synergistic if the two drugs were combined.

The hypothesis that S-15176 mitochondrial binding sites might regulate PTP was further strengthened by the fact that, in addition to its antioxidant effect, BHT also possesses binding sites within the hydrophobic zone of the mitochondrial membrane through which it modulates the PTP (Gudz et al., 1997). Our binding studies showed that BHT and S-00240 displaced [^{3}H]S-15176 from its mitochondrial binding sites, which suggests that these molecules might possess a common binding site through which they regulate PTP. It should be noted that the chemical structure of S-15176 contains a di-tertiobutyl-4-hydroxy-1-thiophenyl group similar to BHT, which might partly explain the similar behavior of the two drugs toward mitochondrial membranes.

Prolonged PTP activation is always associated with the dissipation of mitochondrial membrane potential and the oxidation of NAD(P)H. Our results show that, like BHT, S-15176 prevented the dissipation of the mitochondrial potential and the oxidation of NAD(P)H, confirming the protective role of S-15176 on mitochondrial function. It should be noticed that S-00240, which is devoid of an antioxidant effect, also inhibited the oxidation of NAD(P)H, indicating that the oxidation of NAD(P)H occurs downstream of PTP opening, as suggested by Brunner et al. (2001).

In conclusion, the trimetazidine derivative S-15176 is a strong inhibitor of PTP activation. The inhibition of PTP is associated with the prevention of both the dissipation of the mitochondrial membrane potential and the oxidation of mitochondrial NAD(P)H. Comparison of the data obtained with S-15176 and S-00240, which is devoid of antioxidant properties, indicates that these drugs inhibit PTP independently of their reactive oxygen species scavenging activity. They are likely to operate through binding sites with low structural specificity located in the inner mitochondrial membrane, sites which can accept a variety of pore inhibitors and modulate PTP. However, the antioxidant characteristics of S-15176 may also play an additional role in the protective effect of this molecule against the ischemia–reperfusion injury of rat liver seen in vivo experimental models.

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