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Effect of the mitochondrial transition pore inhibitor, S-15176, on rat liver mitochondria: ATP synthase modulation and mitochondrial uncoupling induction

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CsA, cyclosporin A
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PTP, permeability transition pore
6-KCh, 6-ketocholestanol

ABSTRACT

S-15176 is a new inhibitor of the permeability transition pore (PTP) which has been shown to display anti-ischemic properties. We show here that S-15176 prevented PTP, cytochrome c release and maintained mitochondrial membrane potential when low concentrations of S-15176 were used (not exceeding 50 nmol/mg protein). For higher concentrations S-15176 is able to collapse mitochondrial potential. This effect was reversed by the recoupling agent 6-ketocholestanol (6-KCh) suggesting that S-15176 has uncoupling properties. In addition, S-15176 is able to inhibit ATP synthase activity and to stimulate the hydrolytic activity of the enzyme but none of these effects appears to be related to its PTP inhibiting property. These data demonstrate that S-15176 interacts with several targets in mitochondria and these pharmacological properties should be considered in the examination of its health benefits as well as its potential cytotoxicity.

1. Introduction

Mitochondria are known to be involved in the processes that lead to cell death following reperfusion after a period of ischemia, in both necrotic and apoptotic forms of cell death [1]. It is now well demonstrated that reperfusion leads to an increase in the permeability of the inner membrane which is caused by the opening of the mitochondrial permeability transition pore (PTP), a non-specific complex channel [2]. This leads to mitochondrial swelling by massive entrance of solutes into the matrix, membrane depolarization, uncoupling of oxidative phosphorylation and ATP depletion and ultimately to cell death. For these reasons, molecules which can inhibit PTP opening can provide protection against reperfusion injury and the search for novel PTP inhibitors should be considered with great interest [3].

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This inhibition may be mediated by a direct interaction with PTP and cyclosporin A (CsA) provided the first evidence of this strategy [4] or by an indirect effect by decreasing calcium loading and radical oxygen species generation. The protective effects of such drugs have been demonstrated in different models of ischemia-reperfusion [5–7] and a recent study demonstrated that inhibition of PTP opening at the onset of reoxygenation protected the human myocardium against lethal hypoxia-reoxygenation injury [8].

In the recent years we developed a new compound, S-15176 (*N*-[(3,5-di-tertiobutyl-4-hydroxy-1-thiophenyl)]-3-propyl-*N*′-(2,3,4-trimethoxybenzyl)piperazine) which was able to limit the hepatic injury induced by ischemia-reperfusion [9]. This protective effect was associated with preservation of mitochondrial functions and seemed to be related to the inhibition of PTP opening [10]. More recently, we provided evidence that S-15176 operates through low specificity binding sites located in the inner mitochondrial membrane [11].

In the present study, we confirmed the PTP inhibitory properties of S-15176 on liver mitochondria and demonstrated that this drug was able to modulate the activity of mitochondrial ATP synthase (F1F0-ATPase) and to induce mitochondrial uncoupling when high concentrations were used.

2. Materials and methods

2.1. Drugs

S-15176 was purchased from Sigma. Lipophilic molecules, especially 6-ketocholestanol (6-KCh), were dissolved in dimethylformamid at 100 mM and a small volume was introduced in the reaction medium. In these conditions 6-KCh did not precipitate and the maximal final percentage of dimethylformamid did not alter liver mitochondrial functions.

2.2. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated from Wistar rats as described previously [12]. Liver samples were placed in medium containing 250 mM sucrose, 10 mM Tris and 1 mM of the chelator 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) to remove unbroken tissue and nuclei. 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. The resulting mitochondrial pellet was washed with medium from which the EGTA was omitted and centrifuged for 5 min at 15,000 × *g* resulting in a final pellet containing approximately 70 mg protein/ml. The mitochondrial suspension was stored on ice before use.

2.3. Measurement of mitochondrial swelling and optical monitoring of mitochondrial membrane potential

Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 520 nm (A_{520}) by using a Hitachi model UV-3000 spectrophotometer. Experiments were carried out at 25 °C in 1.8 ml of sucrose-phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, pH 7.2) with addition of 2 μM rotenone and 6 mM succinate. Mitochondria (1 mg/ml) were incubated for 1 min in this buffer and swelling was induced by addition of 25 μM Ca^{2+}. Mitochondrial membrane potential ($\Delta\Psi$) was evaluated by the uptake of rhodamine 123, which accumulates electrophoretically into energized mitochondria in response to their negative-inside membrane potential [13]. The fluorescence scanning of the rhodamine 123 was monitored using a Perkin-Elmer SA LS 50B fluorescence spectrometer. The excitation and emission wavelengths were 503 nm (slit width 3.0) and 527 nm (slit width 2.5), respectively. Experiments were carried out in 1.8 ml of the sucrose-phosphate buffer including 2 μM rotenone and 0.3 μM rhodamine 123. After 30 s of incubation, mitochondria (0.1 mg/ml) were added. This was followed by the addition of succinate. The huge uptake of rhodamine 123 gave rise to self-quenching and to a reduced signal. Hence, when the mitochondrial membrane potential increased, the fluorescence intensity decreased. Relative changes of membrane potential were expressed in arbitrary fluorescence units and were not converted to potential values.

2.4. Measurement of ATP synthase and ATPase activities

ATP synthase activity was measured according to two different protocols. In the first one, we measured the ATP produced in liver mitochondria by means of a well-established bioluminescence technique using the ATP dependancy of the light emitting luciferase catalyzed oxidation of luciferin. This was made by means of a commercial kit (ATP bioluminescence assay kit HS II, Boehringer Mannheim). In a second approach, we determined H^+ flux across ATP synthase. This parameter was evaluated indirectly by measuring the extramitochondrial concentration of H^+. Liver mitochondria (2.5 mg/ml) were suspended in a medium containing 250 mM sucrose, 5 mM $MgCl_2$, 6 mM succinate and 1 μM rotenone at 37 °C. H^+ influx was induced by the addition of 0.5 mM of ADP prepared in 1 M KH_2PO_4 and the change of mitochondrial pH was measured with a radiometer® pH meter (PHM290).

Mitochondrial ATPase activity was determined by measuring the concentration of inorganic phosphates released by ATP hydrolysis. Briefly, 50 μl of mitochondria (0.1 mg/ml) were incubated at 37 °C in 1 ml of a medium containing 50 mM Tris, 5 mM $MgCl_2$, 6 mM succinate, 1 μM rotenone (pH 7.5 at 37 °C) in the absence or in the presence of the drug tested. ATPase activity was started by the addition of 5 mM ATP and the reaction was stopped by the addition of 1% trichloroacetic acid after 10 min of incubation. The resulting precipitate was eliminated by centrifugation and inorganic phosphate concentration was determined in the supernatant according to Fiske and Subbarow method [14]. Results are reported in μM of ATP hydrolysed per 10 min.

2.5. Determination of mitochondrial cytochrome c release

Cytochrome *c* release was determined by Western immunoblot analysis as previously described [15]. Briefly, mitochondria (1 mg/ml) were suspended in 250 μl of phosphate buffer

(250 mM sucrose, 5 mM KH_2PO_4, pH 7.2) including 2 μM rotenone and 6 mM succinate and incubated with 25 μM Ca^{2+} ($CaCl_2$) in the absence or the presence of 1 μM CsA for 20 min. The mitochondrial suspension was centrifuged at 15,000 × *g* for 10 min at 4 °C and 5 μl of the resulting supernatant was added to 5 μl of a buffer containing sucrose (20%), SDS (2.4%), β-mercaptoethanol (5%), and bromophenol blue (5%). Samples were boiled at 100 °C, subjected to electrophoresis on a 4–15% gradient SDS-PAGE gel and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in a Tris buffer (Tris 0.02 M, NaCl 0.14 M, pH 7.6) containing 0.1% Tween 20 and incubated overnight at 4 °C with mouse monoclonal anti-rat cytochrome *c* antibody (5/1000; MAB897, R&D systems, UK). After incubation with sheep anti-mouse horseradish peroxidase (1/1000; Amersham Pharmacia Biotech, Les Ulis, France) 1 h at room temperature, the blots were revealed by enhanced chemiluminescence reaction (Amersham ECL+) using X-ray film (Sigma, Biomax).

3. Results

3.1. Dual effect of S-15176 on membrane potential in liver mitochondria

Energized mitochondria (1 mg/ml) were incubated in phosphate buffer and swelling was obtained by the addition of Ca^{2+} (Fig. 1). Ca^{2+} induced a large amplitude swelling associated with an alteration of mitochondrial membrane potential and a cytochrome *c* release, which were fully sensitive to CsA and therefore mediated by PTP. In these experimental conditions, S-15176 prevented both mitochondrial swelling and cytochrome *c* release and maintained mitochondrial membrane potential confirming that the drug is a PTP inhibitor. However, when high concentrations of S-15176 were used (>50 μM), we noticed that mitochondrial membrane potential tended slightly to decrease and we investigated this point in Fig. 2. This figure reports the effect of S-15176 on membrane potential when the ratio: mitochondrial protein concentration/S-15176 concentration, was lowered. Energized liver mitochondria (0.1 mg/ml) were loaded with rhodamine 123 and, after its accumulation, 30 μM S-15176 were added to the medium. S-15176 induced a release of rhodamine 123 reflecting a sudden membrane depolarization. This effect was not inhibited by CsA ruling out a possible induction of PTP opening at high concentrations. However, the pattern and the extent of the depolarization (Fig. 2) were very similar to that observed with the uncoupler agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). This raised the possibility that S-15176 might act as an uncoupling agent at high concentrations. To check this assumption, we studied the effect of recoupling agents.

3.2. 6-Ketocholestanol (6-KCh) reversed mitochondrial uncoupling induced by S-15176

The same set of experiments was performed in the presence of recoupling agents. Different recouplers were used. Fig. 2 shows that successive addition of 100 μM of 6-KCh restored

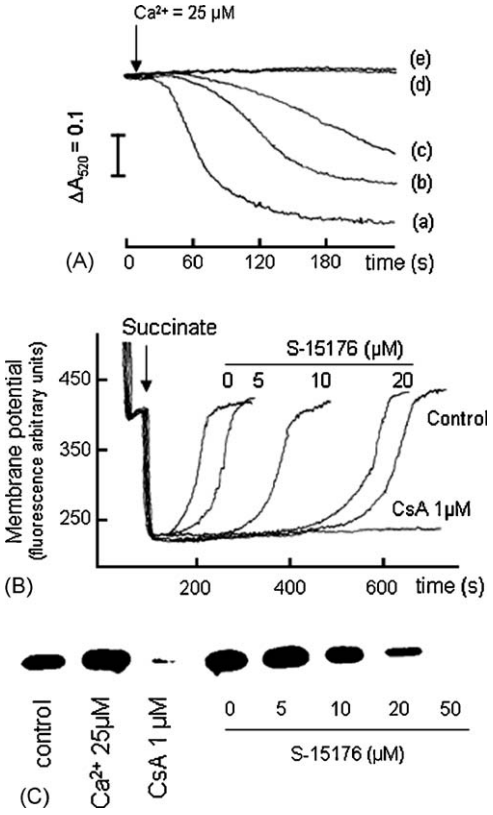

Fig. 1 – S-15176 inhibited PTP opening. Panel (A): Swelling was induced by 25 μM Ca^{2+} in the presence of either increasing concentrations of S-15176 (10 μM (b); 20 μM (c); 40 μM (d)) or 1 μM CsA (e). S-15176 and CsA were added to the medium just before Ca^{2+} addition. Panel (B): S-15176 and CsA restored mitochondrial potential altered by 25 μM Ca^{2+}. Mitochondria (0.5 mg/ml) were incubated in a medium containing 250 mM sucrose, 5 mM KH_2PO_4, 1 μM rotenone, 0.3 μM rhodamine 123, 25 μM Ca^{2+} (except control experiment) in the presence or in the absence of 1 μM CsA or increasing concentrations of S-15176. After 30 s, 6 mM succinate were added to the medium to establish the membrane potential. Panel (C): Isolated liver mitochondria were incubated in a medium containing 250 mM sucrose, 5 mM KH_2PO_4, 1 μM rotenone, 6 mM succinate and submitted or not to 25 μM Ca^{2+} in the presence or in the absence of 1 μM CsA or increasing concentrations of S-15176. At the end of the incubation, the mitochondrial suspension was centrifuged and the supernatant was analysed by western blot using a monoclonal anti-mouse cytochrome *c* antibody. The data shown are typical of three such experiments.

the membrane potential abolished by 30 μM S-15176 while other recouplers, i.e. progesterone, testoterone, atractylate or carboxyatractylate, were ineffective (data not shown). Similar results were obtained with 1 μM CCCP but higher concentrations of 6-KCh were necessary to obtain the same recoupling effect. For example, in the particular experiments depicted in Fig. 2, 200 μM 6-KCh were able to reverse 35% of the uncoupling effect promoted by 30 μM S-15176 whereas 700 μM were needed to obtain the same effect with 1 μM CCCP.

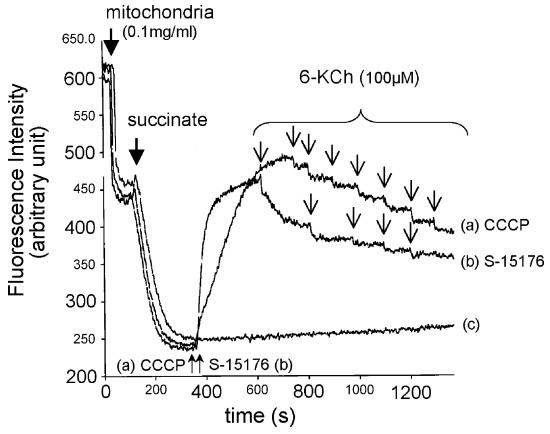

Fig. 2 – Effect of 6-KCh on CCCP and S-15176-induced uncoupling. Isolated liver mitochondria (0.1 mg/ml) were incubated in a medium containing 250 mM sucrose, 5 mM KH_2PO_4, 1 μM rotenone and 0.3 μM rhodamine 123. Membrane potential was measured after the addition of 6 mM succinate, followed by the addition of CCCP (1 μM) or S-15176 (30 μM). Successive additions of 100 μM 6-KCh reversed the effects of CCCP or S-15176. Curve (c): control curve without addition.

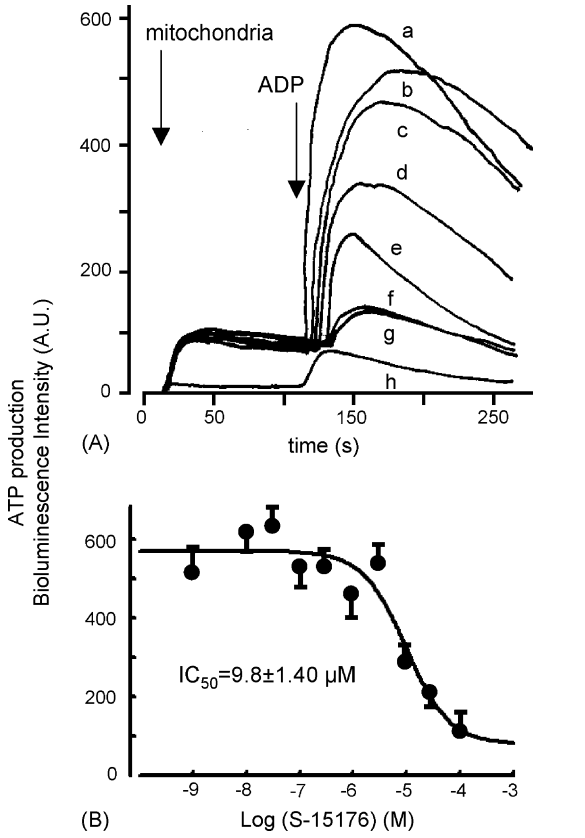

Fig. 3 – Measure of the mitochondrial ATP synthase activity estimated by the generation of ATP in the presence of increasing concentrations of S-15176. (A) Generation of ATP was measured using bioluminescence. Addition of ADP (5 μM) to the mitochondrial suspension induced ATP production (a). It was inhibited by 1 μM oligomycin (g), 1 μM atractyloside (h) and increasing concentrations of S-15176 (0.1 μM (b), 1 μM (c), 10 μM (d), 30 μM (e), and 100 μM (f)). The data shown are typical of three such experiments. (B) Concentration-response curve for S-15176 inhibition of ATP synthase stimulation. The data represent the mean ± S.D. of three-independent experiments.

3.3. *S-15176 modulates ATP synthase activity*

Several data [16] have suggested a possible relationship between ATP synthase activity and PTP induction and the ATP synthase inhibitor oligomycin was shown to prevent PTP opening in isolated mitochondria [17–19]. We confirm these data in our laboratory and find that oligomycin inhibited calcium-induced PTP opening with an IC_{50} of 0.22 μM in liver mitochondria. This leads us to investigate the effect of S-15176 on this enzyme.

Fig. 3A indicates that ATP synthase was active in our mitochondrial preparation. When ADP was added, mitochondria generate ATP as evidenced by the increase in light emission. This was due to ATP synthesis as it was inhibited by 1 μM oligomycin, a specific inhibitor of the enzyme and by 1 μM of the specific inhibitor of the adenyl nucleotide translocase, atractyloside. S-15176 inhibited ATP synthase activity in a concentration-dependent manner with a mean IC_{50} value of 9.8 μM (Fig. 3B). Another approach to measure ATP synthase activity is to estimate the activity of the proton pump. Indeed, ATP synthesis induced a proton flux across the enzyme and the measure of the extramitochondrial pH reflects a change of the activity of the enzyme.

This measure required a high concentration of mitochondria (2.5 mg/ml), otherwise we were unable to see any change of pH. Thus, we tested the effect of S-15176 in this model. Fig. 4A shows a typical experiment analysing the effect of increasing concentrations of ADP on proton flux. Addition of ADP to the medium caused a slight decrease in pH which was independent of ATP synthase activity. Then, the pH increased to reach an equilibrium and the difference between the basal and the equilibrium values reflected the activity of the enzyme. This activity was dependent on the concentration of ADP and in our experimental conditions a maximal effect was observed around 0.5 mM. So, this concentration was used in all our experiments. As expected, proton flux was prevented by oligomycin but was also blocked by S-15176 (Fig. 4A), confirming the inhibiting property of the drug on the ATP synthase. In addition, Fig. 4B demonstrates that these effects were concentration-dependent.

The effect of a drug on ATP synthase activity can also conveniently be measured in the direction of ATP hydrolysis [20]. ATPase activity was monitored on intact mitochondria by measuring the apparition of phosphate ion released by ATP hydrolysis. Surprizingly, S-15176 stimulates the activity of the enzyme in a concentration-dependent manner (Fig. 5A). This effect was ATP concentration-dependent (Fig. 5A) but could not be related to the mitochondrial membrane potential. Indeed, ATPase stimulation was identical in deenergized mitochondria (in absence of substrate) or after uncoupling or electron transfer inhibition and the addition of the recoupling agent 6-KCh did not modify the effect of the drug.

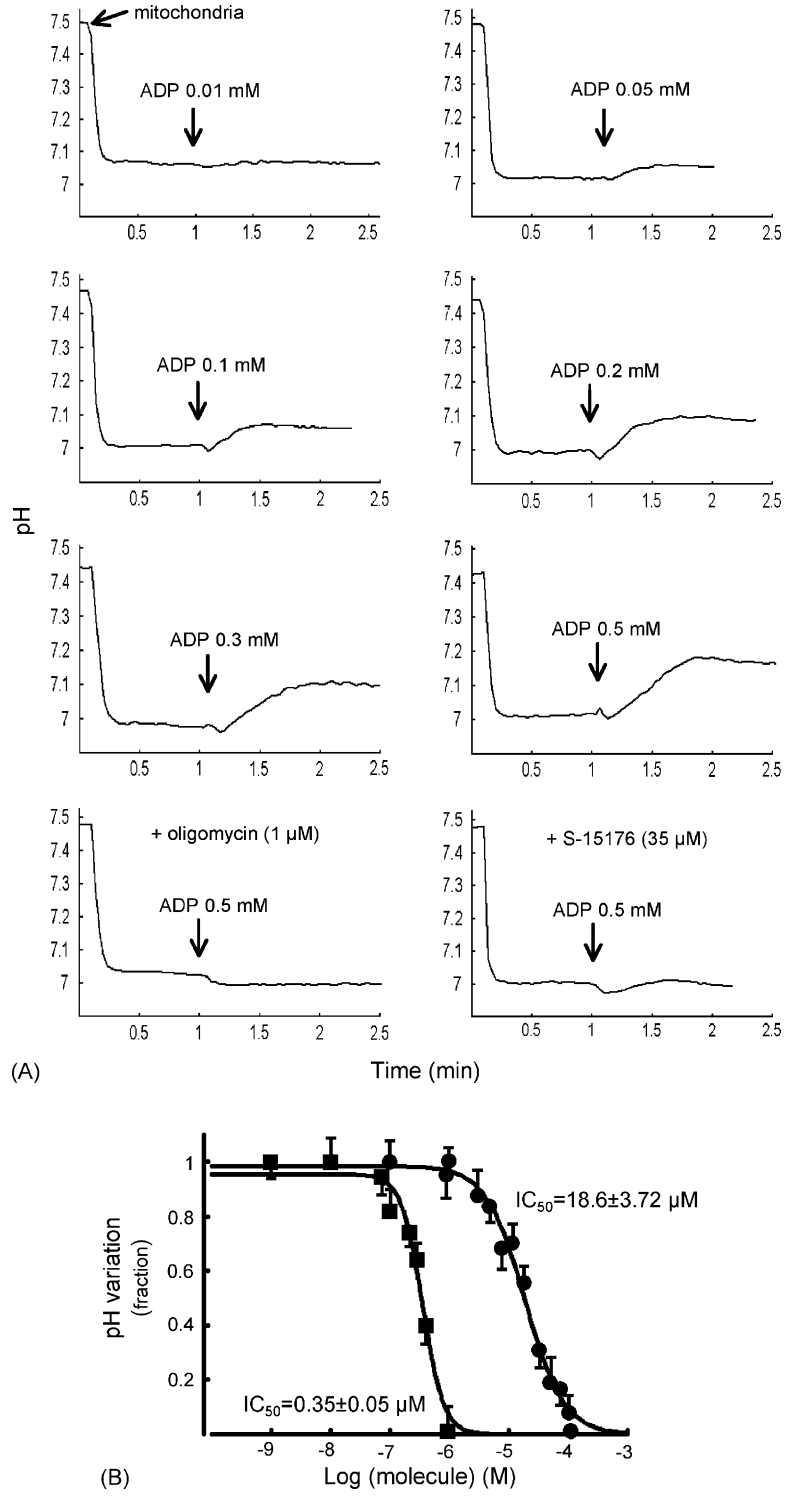

Fig. 4 – ATP synthase activity estimated by the measure of the proton flux across the enzyme. Panel (A): Effect of increasing concentrations of ADP. Addition of mitochondria (2.5 mg/ml) to the medium induced a drop of extramitochondrial pH and after equilibrium increasing concentrations of ADP were added. ADP produced an increase in pH which was due to the activation of ATP synthase. Pretreatment of mitochondria with either 1 μM oligomycin or 35 μM S-15176 eliminated ATP synthase activity (lower curves). A similar result was observed in the presence of atractyloside (not shown). In this particular experiment, maximal pH variation corresponds to 0.167 pH unit. Panel (B): Inhibition of proton flux induced by increasing concentrations of oligomycin (■) or S-15176 (●). Experimental conditions are similar to panel (A) and ADP concentration was 0.5 mM. Data are plotted as fractions of the maximal pH variation obtained in the absence of inhibitor (pH variation = 1) and represent means ± S.D. of four-independent experiments.

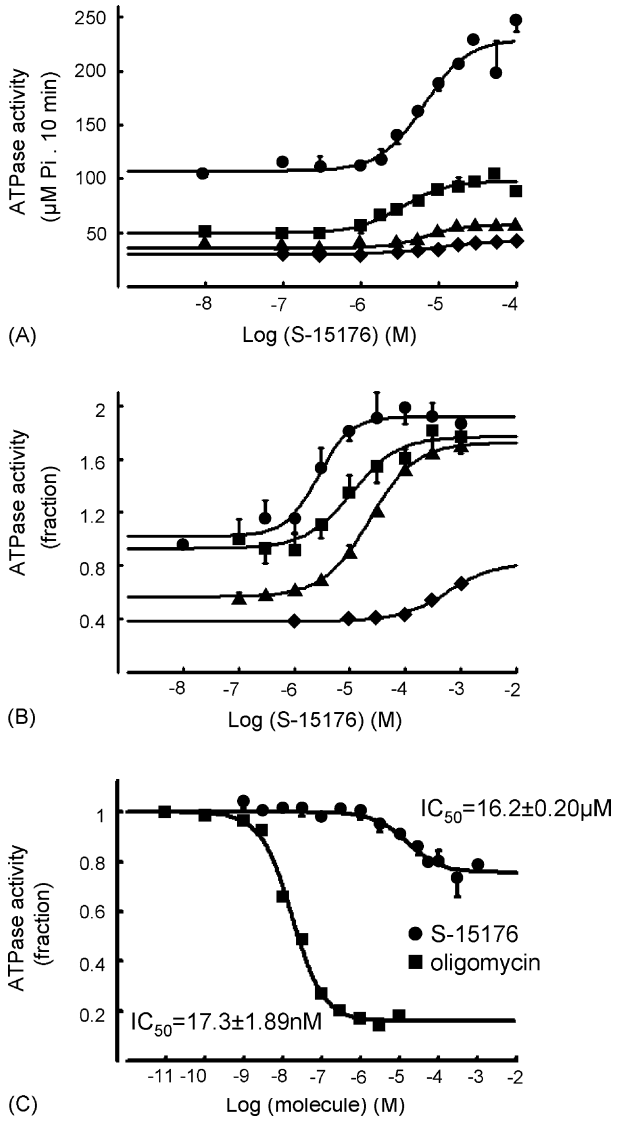

Fig. 5 – Effects of S-15176 on ATPase activity. Panel (A): S-15176 stimulated ATPase activity in intact isolated liver mitochondria. Mitochondria (0.1 mg/ml) energized with succinate were incubated in the presence of increasing concentrations of S-15176 and four different concentrations of ATP (0.5 mM (◆), 0.75 mM (▲), 1 mM (■) and 5 mM (●)). Results are reported in μM of inorganic phosphate (Pi) produced for 10 min during ATP hydrolysis. EC_{50} values were determined by nonlinear regression analysis of the concentration–response function. In the presence of 5 mM ATP, EC_{50} = 6.5 μM. Panel (B): Effect of increasing concentrations of oligomycin on S-15176 induced ATPase activity. Results are expressed as fraction of the maximal concentration of inorganic phosphate produced during ATP hydrolysis. Each point represents the mean ± S.D. from three-independent determinations performed in duplicate. EC_{50} values which were determined by nonlinear regression analysis of the concentration–response function were 2.69, 11, 22.8 and 576 μM for S-15176 alone (●), or in the presence of 0.01 μM (■), 0.05 μM (▲) or 10 μM (◆) oligomycin, respectively. Panel (C): Inhibition of ATPase activity by increasing concentrations of S-15176 and oligomycin in sonicated liver mitochondria. Data are plotted as fraction of the control ATPase activity (in the absence of inhibitor, ATPase activity = 1). Each point represents the mean ± S.D. from three-independent determinations performed in duplicate. EC_{50} values were determined by nonlinear regression analysis of the concentration–response function.

On the other hand, the effect of S-15176 was antagonized by oligomycin (Fig. 5B). In this experiment the concentration of S-15176 was increased from 10^{-8} to 10^{-3} M in the presence of either 10, 50 or 10000 nM oligomycin. In the presence of 50 nM of oligomycin EC_{50} of S-15176 was about eight-fold higher than that observed in the absence of oligomycin whereas the extent of the effect was not statistically modified tending to indicate that the two drugs act in a competitive way. For high oligomycin concentrations (10 μM) the effect of S-15176 was almost abolished.

It should be noted that this effect required the integrity of the organelle. Indeed, if ATPase activity was studied on sonicated mitochondria, an opposite effect was observed. S-15176 inhibited the enzyme with an IC_{50} of 16.2 μM (Fig. 5C) but the extent of the effect was low (≈15%). In the same experimental conditions, a different pattern of inhibition was observed with oligomycin which suppresse 85% of the ATPase activity with an IC_{50} values very close to the value obtained on intact mitochondria (Fig. 5C).

4. Discussion

Our previous studies demonstrated that S-15176 might be a novel potential anti-ischemic drug and that this effect could be associated with its PTP inhibiting properties. In the present work, we demonstrated that the drug interacts with several mitochondrial targets and displays a dual profile of effect towards mitochondrial membrane potential depending on the concentration used. When S-15176/protein concentration ratio was low (not exceeding 50 nmol S-15176/mg mitochondrial protein), S-15176 prevented PTP opening and maintained mitochondrial membrane potential in a concentration-dependent manner. Arguments were provided indicating that the drug operates through low specificity binding sites located in the inner mitochondrial membrane [11]. The present study demonstrated that S-15176 also inhibits ATP synthase activity in the same range of concentrations and this can also contribute to PTP closure and, ultimately, to an anti-ischemic effect. The protective effect promoted by ATP synthase inhibition has been interpreted in different ways: (a) an increase in transmembrane potential, (b) an increase in ADP matrix content [21], and (c) an increase in phosphate uptake [19].

It should be noted that S-15176 was also effective in deenergized conditions [11] where oligomycin was inactive [19]. Therefore, the inhibition of PTP by the drug cannot be entirely related to its effect on ATP synthase.

Whether S-15176 inhibited the synthase activity of the enzyme, it was shown to stimulate the hydrolytic capacity of the enzyme when high concentrations of ATP were provided to the enzyme. ATP hydrolysis occurs by a simple reversal of

the mechanism of synthesis [22], and may be responsible for the consumption of ATP under ischemic conditions [23,24].

This behavior is atypical. Indeed, most of the molecules which were described to inhibit the enzyme inhibited both ATP synthesis and hydrolysis. One explanation could be that S-15176 displaced in a concentration-dependent manner the endogenous inhibiting factor IF1 [20], from its sites which is known to limit the ATPase reaction and thus prevented its inhibition. However, additional experiments are required to verify this assumption.

When higher S-15176/mitochondrial protein concentration ratios were used, uncoupling properties of the drug appeared and at 400 nmol/mg mitochondrial protein, S-15176 completely uncoupled mitochondria. Interestingly, the recoupling agent 6-KCh was able to restore membrane potential. 6-KCh is a 3-keto-derivative of cholesterol which was shown to inhibit the action of uncouplers which cross the membrane in the form of the anion as CCCP. 6-KCh's mechanism of action is not well understood but it was suggested to incorporate into the outer leaflet of the membrane and to promote a deceleration of the protein-mediated movement of the uncoupler agent in the inner membrane [25]. This protein is actually unknown but could be a common target for CCCP and high concentrations of S-15176.

This observation prompted us to critically reconsider the mechanism by which S-15176 inhibited PTP and we asked whether PTP closing properties of the drug could be related to its uncoupling properties at low concentrations. Several previous data argue against this hypothesis: (1) S-15176 inhibited mitochondrial swelling in non-respiring conditions when no membrane potential could be detected, (2) S-15176 was able to retain high calcium concentrations in the mitochondrial matrix by inhibiting pore opening, and (3) S-15176 did not inhibit superoxide production whereas uncoupling agents did [11]. This makes unlikely the occurrence of an uncoupling effect of the drug to explain PTP inhibition.

Taken together, these data demonstrate that S-15176 interact with several targets on liver mitochondrial membranes as was observed for other drugs like local anesthetics or phenothiazines [26–28] and these pharmacological properties should be considered in the examination of its health benefits as well as its potential cytotoxicity.

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