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Attenuation of liver normothermic ischemia–reperfusion injury by preservation of mitochondrial functions with S-15176, a potent trimetazidine derivative

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Abstract

We investigated the antiischemic properties of a new compound, S-15176, in an experimental model of rat liver subjected to 120-min normothermic ischemia followed by 30-min reperfusion. Rats were divided into groups, pretreated with different doses of S-15176 (1.25, 2.5, 5 and 10 mg/kg/day by intramuscular injection) or solvent alone, and subjected to the ischemia–reperfusion process. Another group served as the sham-operated controls. Ischemia–reperfusion induced huge alterations of hepatocyte functions, namely, a decrease in ATP content and bile flow, and membrane leakage of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT). These effects were associated with alterations in mitochondrial functions characterized by (1) a decrease in ATP synthesis, (2) a decrease in NAD(P)H levels and mitochondrial membrane potential, and (3) an increase in mitochondrial swelling reflecting the generation of permeability transition. Pretreatment of rats with S-15176 alleviated these deleterious ischemia–reperfusion effects at both the cellular and mitochondrial levels in a dose-dependent manner. The protection of mitochondrial functions was almost complete at a dosage of 10 mg/kg/day. In addition, *in vitro*, S-15176 totally abolished the swelling of isolated mitochondria induced by a calcium overload with an IC_{50} value of 10 μM. These data demonstrate that S-15176 protects mitochondria against the deleterious effects of ischemia–reperfusion and suggest that this protective effect could be related to the inhibition of the mitochondrial permeability transition.

Keywords: ATP; Ischemia–reperfusion; Liver; Mitochondrial permeability transition; Mitochondrial swelling

1. Introduction

Ischemia–reperfusion episodes are encountered in all clinical circumstances where there is a reduction or an interruption of blood flow followed by reoxygenation of the tissue, e.g. organ transplantation, by-pass surgery, and thrombolysis. Ischemia leads to a decrease in oxygen supply and therefore to an impairment of the cellular metabolism. A short-term ischemia is generally well tolerated and the reperfusion phase restores cellular function without damage. With prolonged ischemic periods, however, reperfusion can become deleterious for the cell by causing a massive calcium overload, an oxidative stress that can lead to cell death. In this sequence of events, mitochondria play a pivotal role. A growing body of evidence suggests that a channel formed in the mitochondrial membranes is involved in cell death associated with ischemia–reperfusion [1,2]. This channel, called the permeability transition pore or PTP [3], increases the permeability of the mitochondrial inner membrane to solutes [4,5]. The PTP opening is triggered by the association of calcium overload with an inducer, such as oxidative stress or high phosphate concentration, conditions encountered during ischemia–reperfusion. The opening of this pore leads to the destruction of the mitochondrial membrane potential, mitochondrial swelling, total inhibition of ATP synthesis, and finally to cell death. Recent results

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Abbreviations: ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; PTP, permeability transition pore; RCR, respiratory control ratio; ROS, reactive oxygen species; and $\Delta\Psi$, mitochondrial membrane potential.

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suggest that the viability of the cell would depend on the degree of PTP opening during reperfusion [6].

In a recent work, we showed that the antiischemic drug trimetazidine was able to protect cellular function by preserving mitochondrial function in a hepatic model of ischemia–reperfusion [7]. The mechanism of action of this drug was unknown, but could not be correlated with either an antioxidant effect or a direct inhibition of the PTP. In an attempt to find a more active drug, we selected S-15176 ((*N*-[(3,5-di-tertiobutyl-4-hydroxy-1-thiophenyl)]-3-propyl-*N′*-(2,3,4-trimethoxybenzyl)piperazine) from a series of trimetazidine derivatives on the basis of its good antioxidant properties (IC_{50} = 0.3 μM against lipid peroxidation; [8]). We demonstrated that this drug counteracted the hepatic injuries associated with liver ischemia–reperfusion. This effect included maintenance of ATP levels and a restoration of bile flow during the reperfusion phase [8].

In the present study, we further describe the mechanism of action of this drug and demonstrate that its protective effect is related to the preservation of mitochondrial function which is probably due to a direct inhibition of PTP opening.

2. Material and methods

2.1. Drug administration

Adult male Wistar rats, weighing 250 to 300 g, were used in this study. All animal procedures used were in strict accordance with the French agency's policies (Ministère de l'Agriculture et de la Forêt, authorization no. 00768) on animal experimentation.

Animals were divided into six groups (15 rats each). A non-treated group and four treated groups were subjected to 120 min of normothermic liver ischemia followed by a 30-min reperfusion protocol. Animals in the treated groups were randomly allocated to S-15176 pretreatment of 1.25 mg/kg, 2.5 mg/kg, 5 mg/kg, or 10 mg/kg. S-15176 solution was prepared daily, dissolved in a mixture of water–polyethylene glycol (50/50 V/V) and appropriately warmed to body temperature before injection. The non-treated group received the same quantity of the mixture of water–polyethylene glycol solution. S-15176 was administered by intramuscular injection each day for five days before the induction of ischemia. The sham-operated group (N = 15) received the same surgical procedure as the other groups without being subjected to the ischemia–reperfusion protocol.

2.2. Surgical procedure

The technique of liver ischemia described by Nauta *et al*. [9] was used in this study. The surgical procedure was performed half an hour after the last drug administration under general anesthesia. After section of the ligaments of the liver, hepatic normothermic ischemia was induced for 120 min by hilum clamping of the hepatic pedicles of segments I to V. In order to preclude the vascular congestion of the alimentary tract, the blood supply by the portal pedicles of segments VI and VII was not interrupted. During the period of ischemia, 0.5 mL of saline was given through the dorsal vein of the penis every 30 min to maintain hemodynamic stability and to replace losses due to portal stasis. Bile was collected in plastic vials via the cannulation of the common bile duct with a fine catheter. Reperfusion was established by removal of the clamps. After a 30-min reperfusion, the animals were killed, and the liver lobes suffering ischemic injury were immediately removed; mitochondria were isolated according to the procedure described below.

2.3. Liver function tests

Blood samples for measurement of ASAT and ALAT activities were collected after a 30-min reperfusion. Plasma enzyme activities were determined by an enzymatic technique using a Boehringer Mannheim kit. The hepatic ATP content was determined according to the method described by Jaworec *et al*. [10].

2.4. Isolation of mitochondria

Rat liver mitochondria were isolated as described by Johnson and Lardy [11]. Briefly, after the rats were killed, livers were excised rapidly and placed in medium containing 250 mM sucrose, 10 mM Tris, and 1 mM of the chelator EGTA, pH 7.2 at 4°. The tissue was scissor-minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at 600 X *g* for 10 min (Sorvall RC 28 S). The supernatant was centrifuged for 5 min at 15,000 X *g* to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at 15,000 X *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 X *g* resulting in a final pellet containing approximately 50 mg protein/mL. The protein content was determined by the method of Lowry *et al*. [12]. The mitochondrial suspension was stored on ice before the assay of mitochondrial swelling, membrane potential, mitochondrial respiration, and oxidation of mitochondrial NAD(P)H.

2.5. Optical monitoring of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was evaluated according to Emaus *et al*. [13] from uptake of the fluorescent dye rhodamine 123, which accumulates electrophoretically into energized mitochondria in response to their negative-inside membrane potential. 1.8 mL of the phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, 1 μM rotenone,

pH 7.2 at 25°), 3 mM succinate, and 0.3 μM rhodamine 123 were added to the cuvette, and the fluorescence scanning of the rhodamine 123 was monitored using a Perkin Elmer LS 50B fluorescence spectrometer. The excitation and emission wavelengths were 503 and 527 nm, respectively. After 30 sec, mitochondria (0.5 mg/mL) were added. $\Delta\Psi$ was calculated by the relationship: $\Delta\Psi = -59$ log [rhodamine 123]in/[rhodamine 123]out, assuming that the distribution of rhodamine 123 between mitochondria and medium follows the Nernst equation. A matrix volume of 1 μL/mg protein [14] was considered to estimate [rhodamine 123]in.

2.6. 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. For *ex vivo* experiments, liver mitochondria (4 mg) isolated from sham-operated, ischemia-reperfused, or pretreated ischemia-reperfused animals were added to 4 mL of the phosphate buffer. 1.8 mL of this suspension was added to both sample and reference cuvettes and 6 mM succinate was added to the sample cuvette only. The cuvettes were then scanned at A_{520}. For *in vitro* experiments, liver mitochondria (4 mg) isolated from non-operated animals were added to the same phosphate buffer. A quantity of 1.8 mL of this suspension was added to both sample and reference cuvettes in the presence or absence of different inhibitors. After 4 min of incubation at 25°, 6 mM succinate was added to both cuvettes. One minute later, the swelling was initiated by the introduction of 25 μM of $CaCl_2$ to the sample cuvette only and the A_{520} scanning was started.

2.7. Determination of mitochondrial NAD(P)H level

Mitochondrial pyridine nucleotides (NAD(P)H) were monitored by measuring their autofluorescence at excitation and emission wavelengths of 360 and 450 nm, respectively, in a Perkin Elmer LS 50B fluorescence spectrometer, according to the procedure described by Minezaki *et al*. [15]. Mitochondria (1.8 mg) were added to 1.8 mL of the phosphate buffer containing 6 mM of succinate, and the autofluorescence of NAD(P)H was determined.

2.8. Measurement of mitochondrial respiration

O_2 consumption was measured by a Clark-type oxygen microelectrode in a thermostat (25°)-controlled chamber. Mitochondria (2 mg) were added to 1.8 mL of phosphate buffer. Mitochondrial respiration was initiated by addition of succinate (6 mM final concentration), and oxidative phosphorylation was initiated by addition of ADP to a final concentration of 0.1 mM. O_2 consumption recordings allowed the calculation of the respiratory control ratio (RCR) corresponding to the ratio between the state 3 (ADP-stimulated) respiration rate and the state 4 (resting) respiration rate, and the P/O ratio, which is the ADP consumed divided by O_2 used in state 3 respiration.

2.9. Statistical analysis

All values are given as means ± SEM. Statistical comparisons were made between non-treated rats and sham-operated rats or ischemia-reperfused treated rats by using ANOVA. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Protective effects of increasing doses of S-15176 on cellular functions altered by ischemia–reperfusion

3.1.1. Effects of S-15176 on hepatocyte membrane integrity

At the cellular level, ischemia–reperfusion had a deleterious effect on the cellular membrane integrity as shown by the increase in the plasma ALAT and ASAT activities (Table 1). Indeed, the activities of those enzymes are 27 and 23 times higher in non-treated compared with sham-operated rats, respectively. Treatment of rats for 5 days with a dose of S-15176 as low as 1.25 mg/kg reduced the activities of both ALAT and ASAT. As shown in Table 1, 5 mg/kg seems to be the dose which gave the maximum effect.

3.1.2. Effects of S-15176 on hepatic ATP and bile flow decreased by ischemia–reperfusion

Two other parameters, hepatic ATP content and bile flow, which deserve attention in such injury were investigated. As shown in Table 1, 120-min normothermic ischemia followed by 30-min reperfusion drastically decreased the hepatic ATP content and reduced it to almost 33% of that of sham-operated group. S-15176 in a dose-dependent manner reduced this deleterious effect and at a maximum dose of 10 mg/kg, S-15176 significantly improved the hepatic ATP content and maintained its value to 56% of that of the sham-operated group. Bile flow was also affected by ischemia–reperfusion (Table 1). The bile flow of non-treated rats was 18% of the sham-operated group. Again, S-15176 in a dose-dependent manner reduced the ischemia–reperfusion effect on bile flow, and 5 mg/kg of S-15176 appeared to be the dose which gave the maximum effect.

3.2. Protective effects of increasing doses of S-15176 on mitochondrial functions altered by ischemia–reperfusion

3.2.1. Restoration of mitochondrial RCR and ATP synthesis by S-15176 reduced by ischemia–reperfusion

P/O and RCR are good indices of mitochondrial ATP synthesis and coupling. Both parameters are severely diminished by 120-min normothermic ischemia followed by 30-min reperfusion (Fig. 1). P/O and RCR values of mitochon-

Table 1
Protective effect by S-15176 on plasmatic ALAT and ASAT activities, liver ATP content, and bile flow altered by 120-min normothermic ischemia followed by 30-min reperfusion

	sham operation	ischemia–reperfusion	ischemia–reperfusion + S-15176 (mg/kg)			
			1.25	2.5	5	10
ALAT activity (IU/l)	39.6 ± 6.2	1081 ± 224*	885 ± 90	779 ± 102†	707 ± 120†	730 ± 135†
ASAT activity (IU/l)	50.5 ± 9.2	1140 ± 191*	884 ± 148	788 ± 112†	705 ± 37†	794 ± 157†
ATP level (μmol/g)	2.96 ± 0.02	0.97 ± 0.03*	1.39 ± 0.02†	1.47 ± 0.02†	1.63 ± 0.03†	1.67 ± 0.02†
Bile flow (μl/min/g of tissue)	1.11 ± 0.01	0.20 ± 0.01*	0.23 ± 0.01	0.33 ± 0.01†	0.46 ± 0.01†	0.47 ± 0.05†

Ischemia was induced by hilum clamping of the hepatic pedicles of segments I to V. Rats were pretreated with increasing doses of S-15176. The sham-operated group received the same surgical procedure without being subjected to the ischemia–reperfusion protocol. Values represent mean ± SEM (N = 15).

*$P < 0.001$, statistically different from sham-operation.

† $P < 0.001$, statistically different from ischemia–reperfusion.

dria from the sham-operated rats dropped from 1.43 to 0.92 ($P < 0.002$) and 2.84 to 1.26 ($P < 0.001$), respectively when rats were subjected to ischemia–reperfusion. In a dose-dependent manner, treatment of rats with S-15176 clearly protected mitochondria from the deleterious effects of ischemia–reperfusion on both ATP synthesis and mitochondrial coupling. Five mg/kg of S-15176 seems to be the minimal dose which gave the maximum effect on both parameters; 1.25 mg/kg was ineffective. It should be noted that pretreatment with S-15176 did not improve mitochondrial functions from rat livers that were not subjected to ischemia–reperfusion, ruling out a protective effect of the drug during the mitochondrial preparation procedure (data not shown).

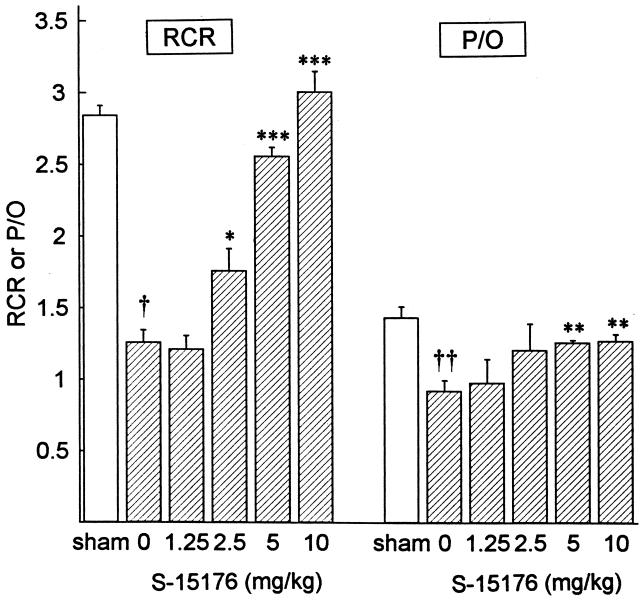

Fig. 1. Effects of S-15176 on RCR and P/O values, in mitochondria of rats with liver ischemia–reperfusion. Rats were treated with S-15176 (1.25, 2.5, 5, or 10 mg/kg/day) for 5 days before the induction of 120-min ischemia followed by 30-min reperfusion. Non-treated rats were subjected to the same ischemia–reperfusion protocol. Sham-operated rats received the same surgical procedure without being subjected to ischemia–reperfusion conditions. After isolation of mitochondria, oxygen consumption was measured polarographically. Values represent means ±SEM (N = 15). †$P < 0.001$, ‡$P < 0.002$, statistically different from sham-operated. *$P < 0.02$, **$P < 0.003$, ***$P < 0.001$ statistically different from non-treated (no S-15176) ischemia-reperfused.

3.2.2. Protective effect of S-15176 on mitochondrial membrane potential dissipated by ischemia–reperfusion

Under our experimental conditions, the membrane potential of mitochondria of sham-operated rats was −208 ± 2.5 mV. This value dropped to −175 ± 3.8 mV ($P < 0.001$) when rats were subjected to 120-min ischemia followed by 30-min reperfusion (Fig. 2). Again in a dose-dependent manner, S-15176 protected mitochondrial membrane potential dissipated by ischemia–reperfusion. At a dose of 5 mg/kg of S-15176, the mitochondrial membrane potential was restored to that of sham-operated rats. A dose of S-15176 of 1.25 mg/kg seemed to increase the membrane potential compared to non-treated rats, but this increase was statistically nonsignificant.

3.2.3. S-15176 preventive effect on NAD(P)H decrease after ischemia–reperfusion

The reductive power of mitochondria can be investigated by measuring their NAD(P)H levels. Fig. 3 showed that this mitochondrial reductive power was strongly affected by ischemia–reperfusion. Mitochondrial NAD(P)H level was reduced from its normal value of 467 ± 18 to 206 ± 26 ($P < 0.001$) when rats were subjected to ischemia–reperfusion. At a dose of S-15176 of 5 mg/kg, the mitochondrial NAD(P)H level was maintained at that of sham-operated animals. As with the other mitochondrial parameters investigated so far, 1.25 mg/kg of S-15176 was ineffective in

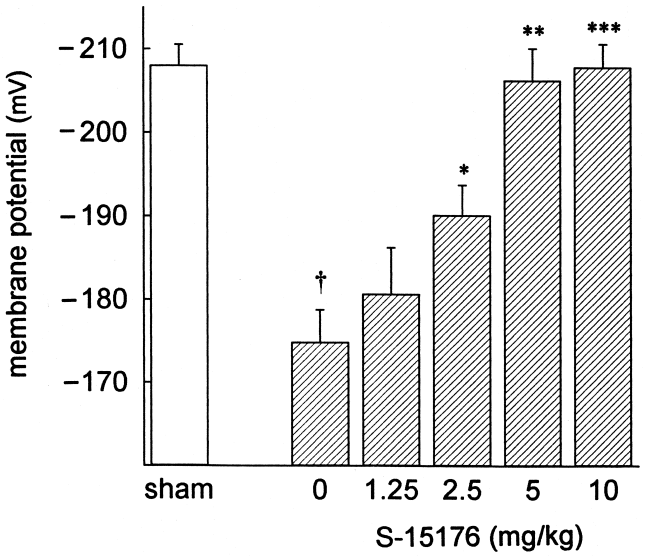

Fig. 2. Prevention by S-15176 treatment of mitochondrial membrane potential dissipation after ischemia-reperfusion. Rats were treated with S-15176 (1.25, 2.5, 5 or 10 mg/kg/day) for 5 days before the induction of 120-min ischemia followed by 30 min reperfusion. Non-treated rats were subjected to the same ischemia–reperfusion protocol. Sham-operated rats received the same surgical procedure without being subjected to ischemia–reperfusion conditions. Liver mitochondria were then isolated and mitochondrial membrane potential was determined using rhodamine 123. Values represent means ±SEM (N = 15). $^{\dagger}P < 0.001$ statistically different from sham-operated. * $P < 0.02$, ** $P < 0.003$, *** $P < 0.001$ statistically different from non-treated (no S-15176) ischemia-reperfused.

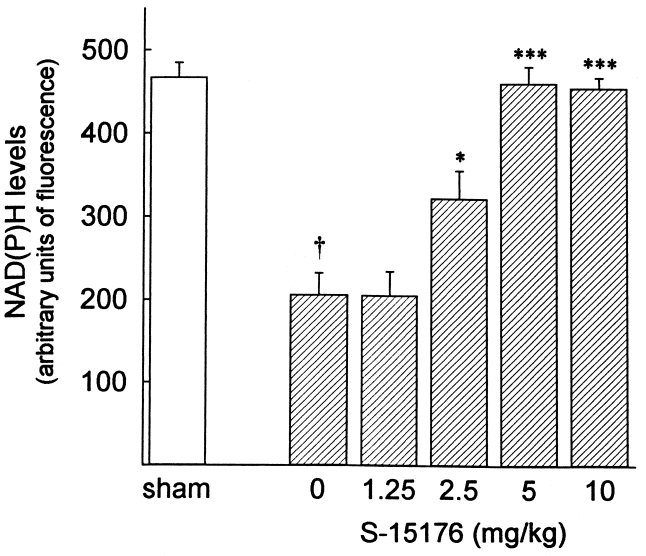

Fig. 3. Effects of S-15176 on NAD(P)H levels, in mitochondria of rats with liver ischemia–reperfusion. Rats were treated with S-15176 (1.25, 2.5, 5 or 10 mg/kg/day) for 5 days before the induction of 120-min ischemia followed by 30-min reperfusion. Non-treated rats were subjected to the same ischemia–reperfusion protocol. Sham-operated rats received the same surgical procedure without being subjected to ischemia–reperfusion conditions. Liver mitochondria were then isolated and NAD(P)H level was determined fluorimetrically. Values represent means ± SEM (N = 15). $^{\dagger}P < 0.001$ statistically different from sham-operated. * $P < 0.02$, *** $P < 0.001$ statistically different from non-treated (no S15176) ischemia–reperfused.

protecting mitochondrial NAD(P)H against oxidation induced by ischemia–reperfusion.

3.2.4. Effect of S-15176 on PTP opening induced by ischemia–reperfusion

PTP opening was assessed by measuring mitochondrial large amplitude swelling. As shown in Fig. 4, the rate of mitochondrial swelling increased in non-treated rats compared to sham-operated rats. In a dose-dependent manner, S-15176 decreased this deleterious ischemia–reperfusion effect on mitochondrial volume. S-15176 at a dose of 1.25 mg/kg was ineffective in preventing PTP opening.

3.2.5. Determination of ED_{50} values

In order to compare the effect of S-15176 and trimetazidine on the different mitochondrial parameters, we determined ED_{50} values from previous experiments (Table 2). Data from Figs. 1, 2, 3, and 4 were analysed by means of non-linear regression using commercially available software (Micropharm, INSERM 1990; [16]). Trimetazidine data were taken from Elimadi *et al*. [7]. To facilitate the comparison, the doses were expressed in μmol/kg. S-15176 ED_{50} values were similar whatever the mitochondrial parameter considered (ED_{50} = 3 μmol/kg), but lower than those of trimetazidine (ED_{50} = 15 μmol/kg).

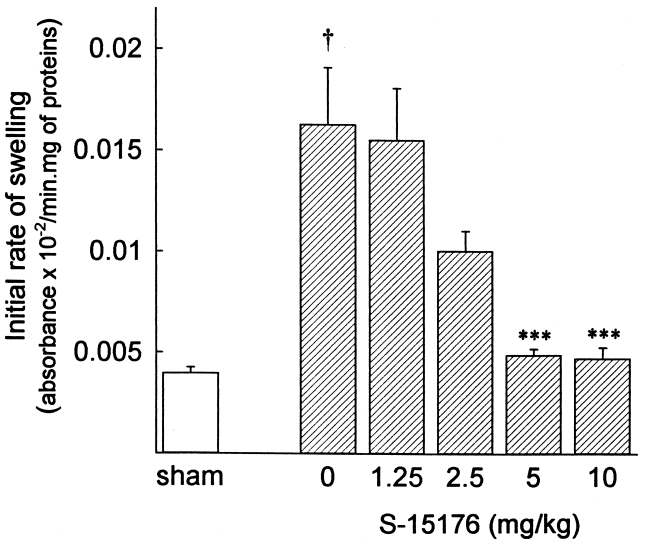

Fig. 4. Effect of S-15176 administration on the rate of mitochondrial swelling increased by ischemia–reperfusion. Ischemia was induced by hilum clamping of the hepatic pedicles of segments I to V. Rats were pretreated with increasing doses of S-15176. The sham-operated group received the same surgical procedure without being subjected to the ischemia–reperfusion protocol. Values represent means ± SEM (N = 15). $^{\dagger}P < 0.001$ statistically different from sham-operated. *** $P < 0.001$ statistically different from non-treated (no S-15176) ischemia-reperfused.

Table 2
ED_{50} values for restoration of mitochondrial functions altered by ischemia–reperfusion

	S-15176		trimetazidine	
	ED_{50} mg/kg	μmol/kg	ED_{50} mg/kg	μmol/kg
RCR	3.31	4.26	5.28	15.5
P/O	1.75	2.25	5.00	14.7
$\Delta\Psi$	2.56	3.29	4.89	14.4
NAD(P)H	2.52	3.24	5.57	16.4
swelling	2.41	3.10	5.05	14.9

For comparison, the results obtained with trimetazidine from a previous study [7] were included in the Table. Values are expressed in mg/kg and in μmol/kg to allow a direct comparison of both molecules.

3.3. Effect of S-15176 on the swelling of isolated liver mitochondria

Rat liver mitochondria energized with succinate were incubated in the phosphate buffer at 25° and the light scattering of the mitochondrial suspension was monitored at 520 nm (Fig. 5). Addition of 25 μM Ca^{2+} in the medium induced mitochondrial swelling (trace a), and this effect was prevented by increasing concentrations of S-15176 (traces b–g) with an IC_{50} value of 10 μM. In the same experiment 1 μM cyclosporin A (trace h) and 150 μM 2,6-di-tert-butyl-4-methylphenol (trace i) counteracted mitochondrial swelling.

4. Discussion

This study demonstrated that a new pharmacological agent, S-15176, protected the liver against ischemia–reperfusion injury by preserving mitochondrial integrity. One hundred and twenty minutes of ischemia followed by 30-min reperfusion induced an extensive damage to liver cells, as attested by the aminotransferase leakage, a good indicator of structural membrane damage [17]. These alterations are associated with the appearance of large areas of necrotic cells [18], but it was recently demonstrated that apoptosis of hepatocytes also occurs during ischemia–reperfusion [19, 20]. This leads to an impairment of liver functions demonstrated in this study by both the decrease in liver ATP content and in bile flow. Identical observations have been reported by others [21–23] using similar experimental protocols.

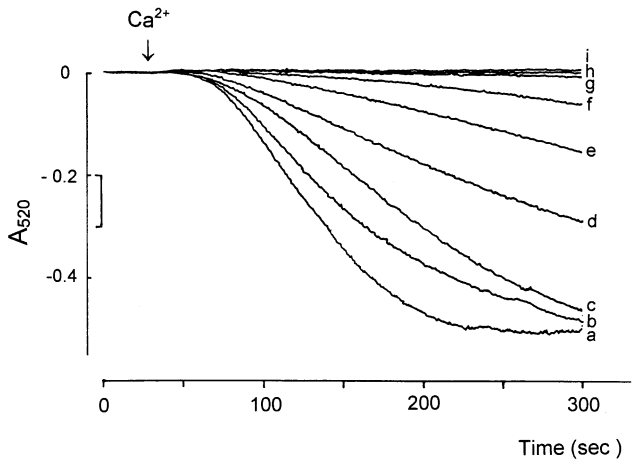

Fig. 5. Effect of S-15176 on mitochondrial swelling induced by Ca^{2+} in the presence of Pi. The incubation medium contained 250 mM sucrose, 6 mM succinate, 5 mM KH_2PO_4, 1 μM rotenone (trace a) and either increasing concentrations of S-15176 (traces b–g), cyclosporin A (1 μM, trace h), or 2,6-di-tert-butyl-4-methylphenol (150 μM, trace i). The experiments were started by the addition of mitochondria. Where indicated, 25 μM Ca^{2+} was added to induce swelling.

Recent studies have provided strong evidence for the involvement of mitochondria in the contribution of cell alterations [7,24]. The present work confirms these observations, showing that the cellular impairment is partly due to a loss of mitochondrial functions. The damage mainly involved mitochondrial uncoupling, resulting in a decrease in ATP synthesis. We also observed that the membrane potential and the NAD(P)H levels of mitochondria from the livers of rats subjected to ischemia–reperfusion were very low compared with that of mitochondria isolated from livers of sham-operated rats. Furthermore, these mitochondria undergo extensive swelling once they are energized with succinate. This swelling probably reflects PTP opening. Indeed, its opening probability is highly increased under ischemia–reperfusion circumstances which favor appropriate conditions (Ca^{2+} overload, ROS production). A likely explanation is that mitochondria submitted to ischemia–reperfusion are highly sensitive to ROS generated by the activation of the respiratory chain.

The pretreatment of rats with S-15176 for five days before the induction of ischemia–reperfusion reduced the leakage of hepatic enzymes, the decrease in ATP content; it also improved bile flow. The cellular protection was partial (maintenance of 56% of ATP at the higher dose, for instance) but correlated well with mitochondrial protection, the cellular effect being optimal with total mitochondrial protection. However, mitochondria being the main sites for ATP production, one could think that a complete protection of mitochondria would normally be followed by a complete restoration of hepatic ATP content. Our results show that at

larger doses, S-15176 did not completely protect the plasma membrane integrity, as attested by plasma aminotransferase activity. This probably caused a leak of ATP through plasma membranes. This may explain why we found a low ATP content even at a concentration of S-15176 which fully preserved mitochondrial functions.

S-15176 pretreatment with 5 or 10 mg/kg/day maintained mitochondrial respiration, membrane potential, NAD(P)H levels, and swelling to the level of that of sham-operated animals. This indicated that mitochondria are probably the main pharmacological targets of S-15176 and that the drug is able to inhibit the opening of the PTP *in vivo*, which is responsible for the permeabilization of mitochondrial membranes and the collapse of mitochondrial potential. This idea was reinforced by the fact that ED_{50} values were identical whatever the mitochondrial parameter tested, indicating that all these protecting effects are linked. Although only a few studies have evaluated the opening of the PTP on mitochondrial functions *in situ* [25], it is now considered to play a major role in necrotic cell death associated with ischemia–reperfusion [26]. Indeed, ischemia–reperfusion is closely associated with a Ca^{2+} overload, an overproduction of ROS, an increase in Pi and a decrease in cellular ATP concentration, conditions favoring the opening of the PTP [4,5].

Strong evidence also incriminates PTP in apoptosis [27], which has been shown to occur after ischemia–reperfusion injury in the liver [20]. Interestingly, S-15176 also inhibited mitochondrial swelling *in vitro*, i.e. when isolated liver mitochondria were submitted to high Ca^{2+} concentrations. This effect was concentration-dependent (IC_{50} = 10 μM) and occurred at low concentrations likely to be obtained *in vivo*. Identical results were found with cyclosporin A, the most potent inhibitor of PTP [28,29] and 2,6-di-tert-butyl-4-methylphenol, a well-known inhibitor of this pore [30]. In light of the above results, it is reasonable to hypothesize that S-15176 protected mitochondria by inhibiting PTP and, thus, we can presume that its mechanism of action differs from its parent drug, trimetazidine, which was not very active towards PTP *in vitro* [31].

The last question is the precise mechanism by which S-15176 inhibits PTP opening. Two working hypotheses that are not mutually exclusive can be considered. The first may involve the inhibition of the production and/or the trapping of ROS. Indeed, numerous studies have shown that PTP is highly sensitive to the oxidative-reduced state of mitochondria and have provided evidence that oxidative stress triggers PTP [32,33]. An argument for this mechanism is the pronounced antioxidant properties displayed by S-15176 towards lipid peroxidation [8]. However, a direct effect of the drug on a site affecting PTP cannot be excluded. Our group has recently described the existence of low-affinity [^{3}H]trimetazidine binding sites involved in the regulation of the PTP [34]. More experiments are needed to validate or to reject these hypotheses.

In conclusion, S-15176 pretreatment protects hepatocytes from the deleterious effect induced by 120-min normothermic ischemia followed by 30-min reperfusion. This effect is dose-dependent and involves an improvement of bile flow and ATP regeneration. This cytoprotective activity is accomplished through a total preservation of mitochondrial function which appears to be due to the closure of the mitochondrial permeability transition pore.

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