# Mitochondrial permeability transition during hypothermic to normothermic reperfusion in rat liver demonstrated by the protective effect of cyclosporin A

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The purpose of this study was to test the hypothesis that mitochondrial permeability transition might be implicated in mitochondrial and intact organ dysfunctions associated with damage induced by reperfusion after cold ischaemia. Energetic metabolism was assessed continuously by $^{31}$P-NMR on a model system of isolated perfused rat liver; mitochondria were extracted from the livers and studied by using top-down control analysis. During the temperature transition from hypothermic to normothermic perfusion (from 4 to 37 °C) the ATP content of the per-

fused organ fell rapidly, and top-down metabolic control analysis of damaged mitochondria revealed a specific control pattern characterized by a dysfunction of the phosphorylation subsystem leading to a decreased response to cellular ATP demand. Both dysfunctions were fully prevented by cyclosporin A, a specific inhibitor of the mitochondrial transition pore (MTP). These results strongly suggest the involvement of the opening of MTP *in vivo* during the transition to normothermia on rat liver mitochondrial function and organ energetics.

## INTRODUCTION

Liver transplantation is the accepted treatment for irreversible acute and chronic liver disease. Even though the success rate is high, primary non-function of liver still occurs in approx. 10 % of transplantations [1], and preventing the dysfunctions from occurring during the different phases of the transplantation process (harvesting, preservation and implantation) is of fundamental interest. ATP levels have been shown to be well correlated with organ viability [2,3] and studies of liver energetics by $^{31}$P-NMR have revealed distinct deleterious effects linked to hypothermic ischaemia and normothermic reperfusion [4–6]. Mitochondrial damage during ischaemia–reperfusion have been also reported in isolated liver, resulting in a decrease in oxidation rate, respiratory control ratio [7] and ADP-to-O ratio [8]. Nevertheless the exact mitochondrial cause of damage is still unknown, as are the consequences for organ recovery.

Besides a possible direct effect of free radicals on mitochondrial membranes [9,10], we considered the hypothesis that significant opening of the mitochondrial permeability transition pore (MTP) [11] might occur during post-ischaemic reperfusion [12] after mitochondrial calcium overload [13]. MTP opening is potentiated by free radicals and by the return to normal pH [14,15], both of which occur during organ reperfusion [16]. The study of the MTP is of interest because pore opening occurs as an early irreversible consequence of apoptosis in some cellular models [17–19]. To our knowledge, no direct study of the effect of pore opening *in situ* on mitochondrial function and energetic metabolism in perfused organs has yet been described.

The role of MPT in reperfusion damage was tested by using cyclosporin A (CsA), a highly specific inhibitor of MTP ($K_i = 3.6$ nM) [20], acting by a mechanism distinct from the immunosuppressive effect via calcineurin [21–23]. Energetic metabolism of the liver was continuously assessed by $^{31}$P-NMR analysis of the isolated perfused organ. Mitochondrial damage and protection by CsA during cold ischaemia, reperfusion and transition

to normothermia in isolated rat liver were characterized by using top-down control analysis [24].

## EXPERIMENTAL

### Liver perfusion

Young male Wistar rats (2–3 weeks old; 90–110 g body weight) were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The liver was perfused at 4 °C at a flow rate of 2 ml/min per g of liver (wet weight) with Krebs–Henseleit medium [120 mM NaCl/4.7 mM KCl/2 mM CaCl$_2$/1.2 mM MgSO$_4$/1.2 mM KH$_2$PO$_4$/25 mM NaHCO$_3$ (pH 7.4)] gassed with O$_2$/CO$_2$ (19:1) [25]; this medium was used for all experiments. The perfusion sequence was the following: (i) 20 min of perfusion at 4 °C (flushing phase); (ii) 0 or 24 h of cold ischaemia at 4 °C (preservation phase); (iii) 20 min of reperfusion at 4 °C [required to assess the effect of phase (ii) on ATP recovery of the organ]; (iv) when indicated, 1 h of normothermic reperfusion (implantation phase). The transition from 4 to 37 °C was obtained by shifting between two thermostatic devices set at 4 and 37 °C respectively, and warming the medium. The perfusion flow rate was simultaneously increased from 2 to 3 ml/min per g of liver (wet weight). No differences were observed when a constant rate of 3 ml/min was used (results not shown). Where indicated, CsA (0.2 $\mu$M) was added to the perfusion medium during phase (iii) and was present during phase (iv).

### $^{31}$P-NMR spectroscopy

NMR studies were performed with a Bruker DPX 400 spectrometer, equipped with a superconducting magnet operating at 9.4 T. $^{31}$P-NMR spectra were recorded at 161.9 MHz without proton decoupling. Spectra of isolated liver were obtained in a
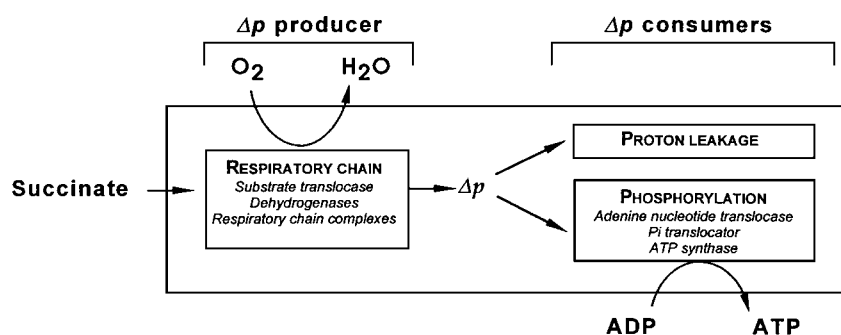
**Scheme 1    Oxidative phosphorylation in rat liver mitochondria**

The system consists of all steps within the box and is divided into three subsystems of reactions (inner boxes) connected by the common thermodynamic intermediate ($\Delta p$) [27,29].

20 mm diameter NMR cell (240 free induction decays; 2 min), as described previously [6,26]. To assess ATP contents in the perfused rat liver under hypothermic (4 °C) and normothermic (37 °C) conditions, the respective areas of $NTP_\beta$ (mainly $ATP_\beta$) NMR signals were corrected for the effect of temperature on relaxation time constants ($T_1$) by the method of Dufour et al. [26].

### Preparation of rat liver mitochondria

Mitochondria were extracted from the livers at key phases of the perfusion sequence, immediately after $^{31}$P-NMR data acquisition. Livers were homogenized in ice-cold sucrose buffer [0.3 M sucrose/5 mM Tes/0.2 mM EGTA/0.1 % BSA (pH 7.2)]. Mitochondria were isolated as described in [27].

### Simultaneous monitoring of oxidative phosphorylation parameters

Mitochondrial respiration rate, phosphorylation rate and membrane potential were measured simultaneously at 25 °C in a 3 ml glass vessel with a Clark-type oxygen electrode (Rank Brothers, Cambridge, U.K.), a high-sensitivity pH electrode and a tetraphenylphosphonium ion-sensitive electrode respectively [27]. The incubation buffer contained 100 mM KCl, 40 mM sucrose, 10 mM $KH_2PO_4$, 5 mM $MgCl_2$, 1 mM EGTA, 2 $\mu$g/ml rotenone and 0.1 % BSA. Mitochondrial protein concentration in the vessel was 350 $\mu$g/ml, except that for proton-leakage measurements 700 $\mu$g/ml was used to improve sensitivity. Succinate (5 mM) was used as substrate as it has been demonstrated to be the most appropriate substrate for control analysis of oxidative phosphorylation [28].

### Top-down analysis

Following the principles of top-down metabolic control analysis [24] and the methodology of previous applications [27,29], we divided the system of oxidative phosphorylation into three subsystems linked by the common intermediate protonmotive force, $\Delta p$: the substrate oxidation subsystem producing $\Delta p$ and the two subsystems that consume the $\Delta p$, these being either coupled or not to ATP synthesis (Scheme 1).

Complete top-down control analysis requires the measurement of the fluxes through the three subsystems, and the determination of corresponding elasticity coefficients over $\Delta p$ for every condition of phosphorylation studied. The fluxes through substrate oxidation ($J_c$) and phosphorylation ($J_p$) subsystems were measured simultaneously with the determination of the

membrane potential. Proton leakage flux ($J_l$) was calculated for the same value of membrane potential from independent titration curves (see below).

### Determination of overall elasticity coefficients to membrane potential, $\Delta\psi$

Determination of the elasticity of a subsystem to the proton-motive force consists of modifying the value of the intermediate ($\Delta p$) by an adequate titration of a subsystem that differs from the subsystem under consideration. This analysis was performed for different ATP synthesis rates set up by using various concentrations of atractyloside in the presence of excess ADP [27].

The elasticity of the respiratory chain ($\Delta p$-producing subsystem) to $\Delta p$ was obtained from the titration of the phosphorylation system ($\Delta p$-consuming subsystem) with atractyloside: the addition of increased concentrations of inhibitor induces increased steady-state values of $\Delta p$ that cause readjustments of the flux through the respiratory chain. The elasticity of the respiratory chain in any phosphorylating condition is defined as the ratio between the fractional change in the respiration flux ($dJ_c/J_c$) and the fractional change in $\Delta p$ ($d\Delta p/\Delta p$), approximated to the fractional change in $\Delta\psi$ ($d\Delta\psi/\Delta\psi$). Elasticity coefficients can be determined from the plot of $J_c$ against $\Delta\psi$ obtained during atractyloside titration and corresponds to the normalized slope of the titration curve multiplied by $\Delta\psi/J_c$:

$$\epsilon_{\Delta\psi}^{J_c} = (dJ_c/d\Delta\psi)(\Delta\psi/J_c) \tag{1}$$

The elasticity of the proton leakage to $\Delta\psi$ was obtained from the titration of the $\Delta\psi$-producing subsystem with malonate in absence of phosphorylation (with 0.25 $\mu$g of oligomycin per mg of protein). Under these conditions, the flux through the respiratory chain under steady-state conditions exactly matches proton leakage flux (the only $\Delta p$-consumer). The elasticity of proton leakage to $\Delta\psi$ corresponds to the ratio between the fractional change in the proton leakage flux (expressed as equivalent oxygen consumption) and the fractional change in $\Delta\psi$ (i.e. to the normalized slope of the malonate titration curve multiplied by $\Delta\psi/J_l$) from the plot of $J_l$ against $\Delta\psi$:

$$\epsilon_{\Delta\psi}^{J_l} = (dJ_l/d\Delta\psi)(\Delta\psi/J_l) \tag{2}$$

In comparison with other reports, the main improvement in the study presented here is in the direct determination of the elasticity of phosphorylation subsystem to $\Delta p$. Data are obtained from the titration of the $\Delta p$-producing subsystem with malonate under each phosphorylating condition. The fractional change in the flux of phosphorylation ($dJ_p/J_p$) divided by the fractional change

in $\Delta\psi$ allows the calculation of the overall elasticity to $\Delta p$ (without any correction for proton leakage), because $J_p$ was measured simultaneously with a pH-sensitive electrode. At a given $\Delta\psi$ the elasticity coefficient corresponds to the normalized slope of the phosphorylation rate (at a given concentration of atractyloside) during malonate titration, multiplied by $\Delta\psi/J_p$:

$$\epsilon_{\Delta\psi}{}^{J_p} = (\mathrm{d}J_p/\mathrm{d}\Delta\psi)(\Delta\psi/J_p) \tag{3}$$

In the foregoing equations, $\Delta\psi$ is the membrane potential, $J_c$ is the flux through the respiratory chain, $J_l$ is the flux through the proton leakage branch (both expressed as rates of oxygen consumption) and $J_p$ is the flux through the phosphorylation branch (expressed as the rate of ATP synthesis).

### Determination of overall control coefficients

Overall control coefficients are defined as the fractional change in flux (flux control coefficient) or in $\Delta\psi$ (concentration control coefficient) in response to an infinitesimal change in the activity of the subsystem under consideration. The control coefficients obtained describe a control pattern that gives an overview of the integrated system, and of its modification in response to a change in activity, e.g. a change in cellular ATP demand. Overall flux control coefficients can be calculated from the overall elasticities by using the equations given in Hafner et al. [24].

## RESULTS

### NMR study of organ bioenergetics

Changes in the ATP content of perfused rat livers during ischaemia and reperfusion have previously been reported in detail [6]. Figure 1 presents a typical experiment illustrating the effect of CsA on the decrease in ATP content of the liver after thermic transition. During perfusion at 4 °C, in the absence or presence of CsA, it was first verified that the ATP content was stable for at least 30 min before the onset of the transition in temperature. Under both conditions, the increase in temperature induced an increase in ATP content, probably due to the
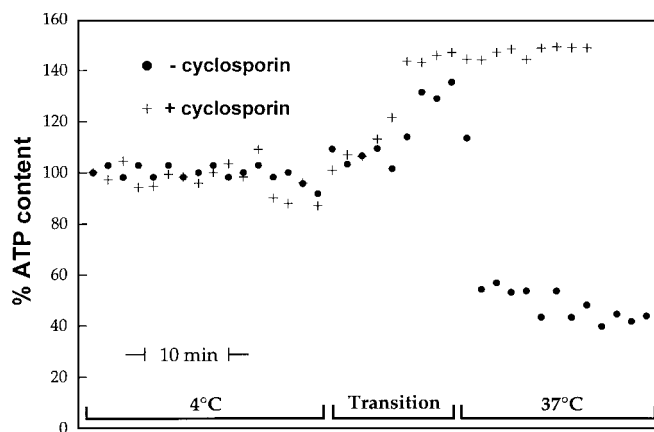


**Figure 1    Evolution of perfused rat liver ATP content during the transition to normothermia; effect of CsA**

Relative ATP content was calculated as described in the Experimental section. In these typical experiments, livers were subjected to 20 min of hypothermic perfusion followed by a transition to normothermia and approx. 1 h of normothermic perfusion in the absence (●; Expt. D in Table 1) or the presence (+; Expt. F) of 0.2 $\mu$M CsA. The final ATP contents of the livers were 46 ± 9% (●, $n = 3$) and 139 ± 4% (+, $n = 3$) of the initial values.

activation of mitochondrial oxidative phosphorylation between 4 and 37 °C [27]. In the absence of CsA a rapid decrease in ATP was observed shortly after normothermic conditions were reached, which stabilized at a value of approx. 50% of the initial ATP content. This decline was completely prevented in the presence of 0.2 $\mu$M CsA; the ATP content reached a plateau at approx. 140% of the initial value (Figure 1). This high ATP level is surprising, as we previously reported that ATP content of a perfused rat liver was consistently higher at 4 °C than at 37 °C, even when liver was first perfused at 37 °C before 4 °C [26]. The time course of the increase and decrease in ATP was similar for all livers studied.

ATP recovery was lower when the livers were subjected to 24 h of cold ischaemia before temperature transition [6], probably due to a loss of nucleotides [30]. The presence of CsA during ischaemia did not modify ATP recovery after cold reperfusion. However, CsA offered similar protection during the temperature transition and ATP was increased 2–3-fold compared with the control (results not shown).

### Oxidative phosphorylation parameters

The classical study of oxidative phosphorylation parameters of mitochondria isolated from livers subjected to different perfusion sequences were assessed by using simultaneous measurements of oxygen consumption, phosphorylation rate and membrane potential [27].

The results are presented in Table 1. True state 3 (excess ADP) and state 4 (excess oligomycin) conditions were set up to measure steady-state oxidation and phosphorylation rates, as well as membrane potential values. Unperfused livers (Expt. A) were removed from the rat and homogenized immediately for mitochondrial isolation. The results are included here to show that perfusion at low temperature (4 °C; Expt. B) did not affect mitochondrial properties (no significant difference between Expts. A and B, Table 1). Similarly, when the organs were subjected to a 24 h cold ischaemic period before being reperfused at 4 °C (Expt. C), only a non-significant decrease in the oxidation rates and no changes in respiratory control ratio, phosphorylation rate or ADP-to-O ratio were observed.

In contrast, significant changes in oxidative phosphorylation parameters were always observed when the organs were subjected to a transition to normothermia (1 h perfusion at 37 °C; Expts. D and E). Qualitatively similar changes were observed with (Expt. E) or without (Expt. D) a 24 h cold ischaemic period. In all cases a transition to normothermia induced a significant decrease in respiratory control ratio, entirely due to a fall in the state 3 oxidation rate as the state 4 oxidation rate did not increase. This absence of uncoupling is further confirmed by the stability of the ADP-to-O ratio and state 4 membrane potential values. The decrease in phosphorylating oxidation rate was correlated with an increase in membrane potential value under state 3 conditions and a 20% decrease in membrane depolarization in response to ADP addition. As previously reported, this change in depolarization can only be associated with the inhibition of one component of the phosphorylation subsystem (see Scheme 1), not to an inhibition of the respiratory chain subsystem [31].

The last set of experiments summarized in Table 1 presents the protective effect of CsA on mitochondrial injury (Expt. F). Mitochondrial function was not affected by ischaemia (compare Expts. C and B), in contrast with the capacity of the organ to regenerate ATP (see below), indicating that non-mitochondrial damage of the liver occurs after 24 h of ischaemia under our experimental conditions. Therefore to understand fully the effect

**Table 1     Measurement of oxidative phosphorylation parameters of isolated mitochondria**

True state 4 conditions were obtained after the addition of oligomycin (250 ng per mg of protein) and state 3 conditions in separate experiments after the addition of 2 mM ADP. Respiratory control ratio (RCR) is the ratio of state 3 and state 4 oxidation rates obtained under these conditions. Depolarization reflects the effect of phosphorylation on membrane potential [31] and was calculated from the difference between the values of membrane potential under state 4 and state 3 as previously described. Oxidation and phosphorylation rates were expressed as nmol of $O_2$ or ATP/min per mg of protein respectively. Membrane potential and depolarization values are given in mV. Expt. F was equivalent to Expt. D except that reperfusion was performed in the presence of 0.2 $\mu$M CsA. All values are given as means $\pm$ S.D.; the number of independent mitochondrial preparations is given in parenthesis. *Significant difference ($P < 0.05$, ANOVA) with reference to the corresponding control for normothermic reperfusion (Expt. D compared with Expt. B, and Expt. E compared with Expt. C) and CsA (Expt. F compared with Expt. D). No significant modifications were noticed for Expt. B and Expt. C compared with Expt. A, or Expt. C compared with Expt. B.

| Expt. | Description | Oxidation rate (nmol of $O_2$/min per mg) | | | Phosphorylation rate (nmol of ATP/min per mg) | ADP-to-O ratio | Membrane potential (mV) | | Depolarization (mV) |
|---|---|---|---|---|---|---|---|---|---|
| | | State 4 | State 3 | RCR | | | State 4 | State 3 | |
| A | Unperfused liver (8) | $10.0 \pm 1.0$ | $70.0 \pm 9.0$ | $7.5 \pm 1.4$ | $189.4 \pm 18.9$ | $1.63 \pm 0.08$ | $173.0 \pm 4.4$ | $122.4 \pm 5.8$ | $50.7 \pm 2.5$ |
| B | Perfusion for 20 min at 4 °C (3) | $10.5 \pm 1.9$ | $72.3 \pm 12.0$ | $6.9 \pm 0.2$ | $182.6 \pm 17.0$ | $1.52 \pm 0.14$ | $176.4 \pm 3.1$ | $127.2 \pm 3.0$ | $49.3 \pm 0.5$ |
| C | B + 24 h of ischaemia 4 °C + B (3) | $8.9 \pm 0.6$ | $58.2 \pm 1.0$ | $6.5 \pm 0.4$ | $180.5 \pm 12.6$ | $1.61 \pm 0.05$ | $172.0 \pm 1.7$ | $122.0 \pm 3.1$ | $50.0 \pm 3.2$ |
| D | B + 1 h of perfusion at 37 °C (3) | $11.7 \pm 0.4$ | $62.4 \pm 5.0$ | $5.3 \pm 0.3$* | $165.2 \pm 0.4$ | $1.50 \pm 0.26$ | $176.7 \pm 3.8$ | $136.5 \pm 3.0$* | $40.2 \pm 0.9$* |
| E | C + 1 h of perfusion at 37 °C (3) | $10.3 \pm 0.9$ | $56.4 \pm 4.1$ | $5.5 \pm 0.1$* | $130.3 \pm 21.8$* | $1.55 \pm 0.18$ | $171.3 \pm 1.5$ | $132.5 \pm 2.2$* | $38.8 \pm 0.8$* |
| F | D + CsA (3) | $10.8 \pm 0.6$ | $68.1 \pm 2.2$ | $6.3 \pm 0.3$* | $220.3 \pm 17.1$* | $1.63 \pm 0.22$ | $177.7 \pm 5.5$ | $131.7 \pm 11.0$ | $46.0 \pm 5.5$ |

of CsA on mitochondrial dysfunctions and ATP recovery, the study was restricted here to the transition to normothermia (compare Expts. D and F). The presence of 0.2 $\mu$M CsA prevented most of the deleterious effects of the transition to normothermia on mitochondrial oxidative phosphorylation: respiratory control ratio and state 3 membrane potential were similar to control values. Moreover, the phosphorylation rate measured after treatment with CsA was consistently much higher than the control value (compare Expts. F and A).

## Proton leakage

We previously demonstrated that proton leakage is dependent only on the protonmotive force and not on mitochondrial activity [32]. Therefore the activity of proton leakage is represented by the relationship between oxygen consumption and $\Delta\psi$ obtained under conditions of progressive inhibition of respiratory chain in the absence of phosphorylation (excess oligomycin), when proton leakage is the only subsystem that consumes $\Delta\psi$. This analysis was performed to examine further a possible modification of the regulation of the proton leakage by membrane potential. Proton leakage short-circuit phosphorylation and its behaviour are of fundamental importance to the effective oxidative phosphorylation efficiency *in situ*. The compilation of the results obtained under the different experimental conditions is presented in Figure 2: no differences could be seen.

## Top-down control analysis of oxidative phosphorylation

Proton leakage analysis (Figure 2) is part of top-down control analysis and allows the calculation of the elasticity of proton leakage subsystem to $\Delta\psi$, as well as the flux through this system for any value of membrane potential [32]. Equivalent data are required for the two other subsystems, namely the respiratory chain and phosphorylation. Similarly, in a given phosphorylating condition, the flux and elasticity of each subsystem were obtained by modulating the common intermediate $\Delta\psi$; this was performed by using an inhibitor or activator of one of the other subsystems. The study was performed over the total range of phosphorylation rate, from state 4 to state 3, modulated by adding variable atractyloside concentrations in the presence of excess ADP [27]. These results were used to calculate the overall control coefficients of the three subsystems over the three different fluxes ($J_c$, $J_p$ and
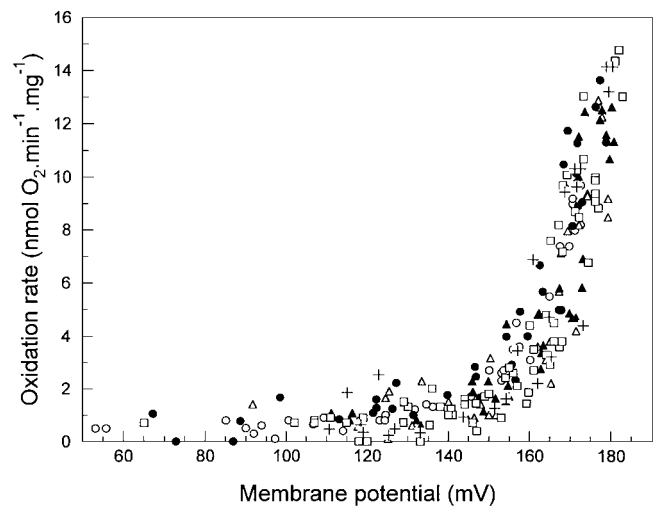


**Figure 2     Mitochondrial proton leakage**

Membrane potential and oxidation rates were simultaneously measured during progressive inhibition by malonate (0–15 mM) of the respiratory chain oxidizing succinate (5 mM) in the presence of excess oligomycin (250 ng per mg of protein). Experiments were performed on mitochondria isolated from livers treated as described in Table 1 (eight mitochondrial preparations for Expt. A and three for Expts. B–F): □, Expt. A; △, Expt. B; ○, Expt. C; ▲, Expt. D; ●, Expt. E; +, Expt. F.

$J_l$) and over $\Delta\psi$ as a function of phosphorylation activity (see the Experimental section for details). The only modifications observed in this study dealt with the control coefficients of the phosphorylation subsystem and are presented in Figure 3.

Results were distributed in two different control patterns. Over the entire range of phosphorylating activity, from state 4 (0 %) to state 3 (100 %), no significant difference in the control pattern was found between mitochondria from livers that did not undergo a transition to normothermia (Figure 3, open symbols). In contrast, mitochondria from livers subjected to a transition to normothermia presented a modified control pattern (Figure 3, filled symbols). The modifications in control pattern were independent of whether or not the livers were subjected to hypothermic ischaemia, and were mainly observed for inter-
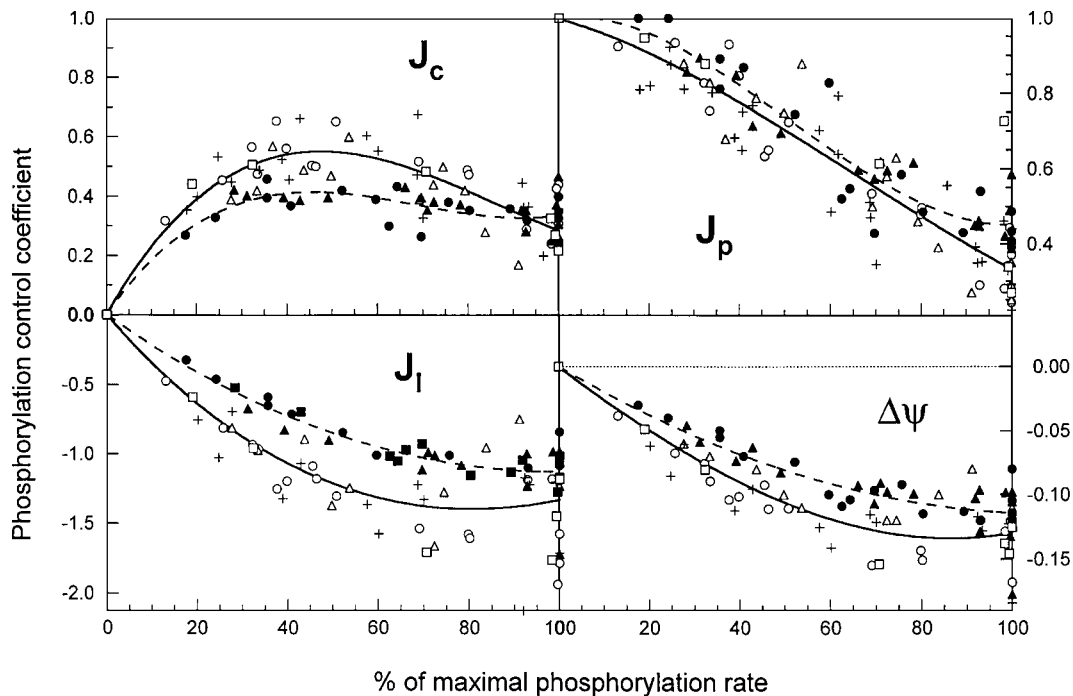
**Figure 3    Effect of CsA on the changes in control coefficients induced by normothermic reperfusion**

Control coefficients exerted by the phosphorylation system over the oxidation rate ($J_c$), the phosphorylation rate ($J_p$), the proton leakage ($J_l$), and membrane potential ($\Delta\psi$) are expressed as percentages of the maximal phosphorylation rate (state 3; see Table 1). Symbols are the same as in Figure 2 and Table 1 (three mitochondrial preparations per condition): $\square$, Expt. A; $\triangle$, Expt. B; $\bigcirc$, Expt. C; $\blacktriangle$, Expt. D; $\bullet$, Expt. E; $+$, Expt. F. Curves represent a third-order polynomial fitting of the data. Results were compared by ANOVA with the mean value of the control coefficients, for each perfusion condition, between 10% and 90% maximal phosphorylation rate. Except for $J_p$, the curves were significantly different ($P \leqslant 0.003$).

mediate phosphorylation rates. This modified pattern was characterized by a general decrease in the control exerted by the phosphorylation subsystem over oxidation rate ($J_c$), proton leakage ($J_l$) and membrane potential ($\Delta\psi$). A significant (approx. 40%) change in the control coefficients was observed at half-maximal phosphorylation rate (Figure 3). It is interesting to note that under these conditions the variability was lower.

A 'normal' control pattern was recovered when CsA was present in the perfusion medium during the transition to normothermia. The values of all control coefficients returned to those obtained from the control livers (Figure 3).

## DISCUSSION

The main purpose of this study was to test the hypothesis of MTP opening during the transition from hypothermic to normothermic perfusion. The experiments were designed to evaluate the possible involvement of the MTP on both the integrated mitochondrial activity and on the capacity of the organ to maintain a high ATP level. Mitochondria were prepared at key phases during a basic protocol (mimicking the conditions occurring in the transplantation process) applied to isolated liver. Mitochondria mainly originate from hepatocytes, which represent up to 70% of the liver cellular volume [33], and can therefore be correlated with energetic state of the whole organ.

The results present evidence that, during organ reperfusion after conservation, the temperature rise from 4 to 37 °C in itself is critical for both mitochondrial and organ energetics. Organ protection demonstrated by the recovery of a high ATP content was observed in the presence of low concentrations of CsA, a highly specific inhibitor of MTP [34,35] (Figure 1). Mitochondrial

activity and particularly control pattern were also fully recovered, and even markedly improved, in the presence of CsA, strongly suggesting that the change in control pattern of mitochondrial activity was correlated with the capacity of the organ to regenerate ATP content.

The classical energetic study of isolated mitochondria showed several differences in activities but mitochondria were, under all conditions, relatively well coupled and presented normal values of membrane potential. The capacity for isolated mitochondria to generate high values of membrane potential was confirmed by the proton leakage analysis. No difference in proton leakage curves was observed between control mitochondria and mitochondria from livers subjected to the different protocols, indicating that membrane properties were affected neither by cold ischaemia nor by temperature transition. In contrast, some modifications in oxidative phosphorylation activity could be noticed, e.g. in the phosphorylation rate after transition to normothermia. However, full analysis of these results was difficult and required the use of control analysis.

The most interesting results concerning mitochondria presented here were collected from the top-down analysis of mitochondrial function [24,36]. Top-down analysis has already been used by different groups to study the effect of complex effectors on oxidative phosphorylation, as it gives an overview of the functioning of the integrated system and helps in the detection of dysfunctions [27,29,37–40].

In contrast with the study of classical parameters of oxidative phosphorylation, top-down control analysis showed two clearly different control patterns: 'normal' and 'abnormal'. The main differences in the control pattern were observed under intermediate phosphorylating conditions, which are not accessible via

classical parameters analysis but are likely to be close to conditions *in situ*. Although no change in control pattern was observed during cold ischaemia or cold reperfusion, a shift from 'normal' to 'abnormal' control pattern was observed only for livers that were subjected to the transition to normothermia and did not occur when CsA was present. The change was characterized by a decrease in the control exerted by the phosphorylation subsystem over the fluxes through the two other subsystems (respiratory chain and proton leakage) and over the value of the intermediate (mitochondrial membrane potential). In other words, the oxidative phosphorylation system had a lower response to the changes in phosphorylation subsystem, e.g. when cellular ATP demand increases. A decline in ATP content of the liver was effectively associated with this mitochondrial dysfunction (Figure 1).

The deviation of the mitochondrial control pattern, from 'normal' to 'abnormal', is a consequence of a specific dysfunction in the system of oxidative phosphorylation, representative of a mitochondrial dysfunction that remains to be precisely characterized.

Protection by CsA from the mitochondrial damage observed after transition to normothermia implies MTP opening. It is now well established that MTP is responsible for mitochondrial disruption [16,41]. This has been demonstrated in heart subjected to normothermic ischaemia/reperfusion in which a part of the total mitochondrial fraction was destroyed and only surviving mitochondria were extracted [12]. Numerous conditions have been reported to induce MTP opening; the exact conditions occurring in perfused livers undergoing a transition to normothermia that induce MTP opening have still to be investigated. However, in the present study the mitochondrial fraction that did not undergo such an irreversible process was also damaged, with this damage being inhibited by CsA. Thus our results suggest that a 'mild' opening of the MTP might be responsible for mitochondrial damage to the phosphorylation system, leading to impaired organ energetics. Because a classical inhibition of enzymes would not modify the control pattern [27], these results suggest a more fundamental change in the phosphorylation system. Halestrap's group recently reported the observation of a thiol modification of adenine nucleotide translocase (a putative component of the pore [42–44]) by oxidative stress in rat heart [41]. Because CsA does not prevent oxidative stress but did protect mitochondria, it seems unlikely that adenine nucleotide translocase was involved in our experiments. A regulation of ATPase activity by its inhibitor protein IF1 in response to the decrease in membrane potential after transient MTP opening seems to be an interesting possibility. We previously studied ATPase deactivation in different systems [45,46].

In conclusion, this paper presents the evidence for an 'abnormal' control pattern of oxidative phosphorylation induced by temperature transition that is correlated with the occurrence of 'mild' MTP opening and a loss of ATP content in perfused liver. The main consequences of such a moderate MTP opening (total opening would cause mitochondrial membrane disruption) on rat liver mitochondrial function and organ energetics were characterized by using top-down control analysis and $^{31}$P-NMR spectroscopy of the intact isolated perfused organ. Preventing the opening of MTP with CsA protected the organ from the damage induced by the transition to normothermia by greatly improving mitochondrial activities and hence ATP recovery.

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