In: "Life in the Cold" (2000) Eds. G. Heldmaier, M. Klingenspor,
Springer Berlin Heidelberg New York, pp.431-442.

Mitochondria in the Cold

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Abstract. Development of hibernation strategies for cold preservation of human organs represents a far-reaching goal in transplantation surgery. Short cold storage times of <6 h tolerated by the human heart remain a major clinical problem. Mitochondrial cold storage-reperfusion injury is becoming recognized as a limiting factor in preservation of organs from non-hibernating mammals. Damaged mitochondria lead to cellular injury by reduction of ATP supply, oxidative stress, disturbance of ion balance, cytochrome *c* release and induction of apoptosis and necrosis. Profiles of mitochondrial injuries differed after (1) cold preservation of isolated rat heart mitochondria, (2) cold preservation of the rat heart, and (3) after transplantation and rewarming/reperfusion. Importantly, a specific defect of complex I of the electron transport chain, uncoupling of oxidative phosphorylation and the pronounced release of cytochrome *c* from mitochondria were absent after cold storage but developed during reperfusion, in proportion to the loss of heart function. Cold preservation of isolated heart mitochondria could be significantly prolonged by a mitochondrial preservation solution containing antioxidants, mitochondrial substrates, ATP, histidine, and oncotic agents. Successful cold storage of heart mitochondria demonstrates a large scope for improvement of heart preservation solutions. In this context, comparison of intracellular conditions and cold ischemia-reperfusion injury in hibernating and non-hibernating mammals may provide a rationale for improvement of clinical organ hibernation strategies.

Introduction

Hypothermia and hypoxia are among the most important strategies for and consequences of metabolic depression. Down-regulation of tissue temperature is known in a wide range of ecophysiological settings including hibernation and torpor, and is applied clinically in critical operations and particularly for organ transplant preservation at a temperature around 4 °C. Whereas cold ischemic preservation times of 36 h are tolerated by the kidney, the human heart is rather intolerant to cold ischemia, limiting cardiac cold preservation to 4-6 h. Organ preservation solutions have been developed for clinical applications. Nevertheless, protection of the heart from cold ischemia-reperfusion injury is not yet successful after longer preservation times. Prolongation and improvement of organ preservation, therefore, are among the most important issues in modern experimental heart transplantation.

The physiological and molecular stress of cold preservation in organ storage might be compared to hibernation strategies where body temperature drops to <5 °C, heart rate is reduced to a couple of beats per minute, and oxygen uptake and metabolic energy requirement are down-regulated up to 100-fold. For clarification it may be noted that cardiac pathologists use the term "hibernating myocardium" without reference to low heart temperature (Opie, 1997), although hypothermia is critical in the classical definition of hibernation in comparative physiology.

In the present study the problem of cardiac mitochondrial injury after cold exposure is addressed with novel methodological, clinical and comparative physiological perspectives: *(1)* Recognizing that essentially all studies of isolated mitochondria involve a period of cold storage on ice, we introduce a new mitochondrial preservation solution which improved mitochondrial function significantly after short cold storage up to 1 hour. *(2)* After long-term cold storage of isolated rat heart mitochondria for 4 days in preservation solution, respiration was maintained at >50 % of control and mitochondria remained coupled. By comparison, a similar depression of respiratory capacity is observed in

mitochondria isolated from hibernators after several days of torpor relative to normothermic animals (Brustovetsky et al, 1993; Martin et al 1999). *(3)* No change of mitochondrial respiratory capacity was measured in permeabilized cardiac fibers after cold ischemic (CI) storage of the rat heart for 11 hours, whereas the same CI time resulted in significant cold ischemia-reperfusion injury. Respiratory capacity declined by 50 % when measured 24 h after reperfusion. *(4)* Presently available data on the depression of mitochondrial respiratory capacity in deep hibernation of endotherms have been interpreted as an adaptation to reduce metabolism during torpor. We propose an alternative hypothetical interpretation in terms of cold preservation injury which requires repair during periodic interbout arousals.

Materials and Methods

Animals and heart transplantation. Hearts of adult male Louis rats were used as a source of cardiac mitochondria and muscle fibers, and in a syngeneic heterotopic heart transplant protocol. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital. Before transplantation, donor hearts were flushed with 10 ml ice cold preservation solution (0 °C) and stored on ice for 10 h of cold ischemia (CI) in 100 ml preservation solution. Another cold flush with 10 ml storage solution preceded implantation. The second CI time with moderate rewarming was standardized at 1 h, during which time grafts were wrapped in moist gauze and intermittently cooled with preservation solution. After normothermic reperfusion for 24 h and relaparatomy, approximately 50 mg of endocardiac tissue were dissected from the left ventricle for preparation of muscle fibers. Two clinically established organ preservation solutions were used, histidine-tryptophan-ketoglutarate solution (HTK; Custodiol; Dr. F. Köhler Chemie) and University of Wisconsin solution (UW; ViaSpan; Du Pont; Table 1).

Permeabilized fibers and isolated mitochondria. After rapid mechanical dissection of myocardial tissue, bundles of fibers were permeabilized in relaxing, low calcium solution by treatment with saponin (50 µg/ml for 30 min on ice; Saks et al, 1998). The permeabilized fibers were washed in ice cold respiration medium (Table 1), and kept in this medium on ice for 1 to 6 h until respirometric measurements. Mitochondria were isolated from left ventricles in isotonic sucrose medium (S; 310 mosmol/l) containing 300 mM sucrose, 0.2 mM EDTA and 10 mM HEPES, adjusted to pH 7.4 with KOH at 25 °C. After the last centrifugation, mitochondrial pellets were resuspended in parallel in sucrose medium or mitochondrial preservation solution (Table 1). Mitochondrial suspensions (7 - 10 mg protein/ml) were stored on ice and used for respirometric experiments within 1 h during day 0, and at intervals of up to 4 days.

High-resolution respirometry. Respirometric experiments were performed in 2 ml glass chambers of the OROBOROS *Oxygraph,* using the DATLAB software (OROBOROS Instruments, Innsbruck, Austria) for on-line display, acquisition and data analysis (Gnaiger et al 1995). Complex I respiration (10 mM glutamate, 5 mM malate) was measured in isolated mitochondria (c. 0.05 mg protein/ml) and permeabilized fibers (2-4 mg wet weight/ml) in respiration medium (30 °C; Table 1). 1 mM ATP was added to isolated mitochondria to observe the transition between resting state 2 (no adenylates) and state 4 (no ADP, but high ATP). ATP was not added to fibers owing to the presence of high ATPase activity. Active respiration at state 3 was established after titration of 1 mM ADP. State 3*c* is defined here as the respiratory state after additional stimulation of state 3 by 10 µM cytochrome *c*. The respirometric protocol was continued by uncoupling by 2 µM FCCP (state 3*c*u), addition of 0.5 µM rotenone and 10 mM succinate (complex II respiration), and finally 5 µM antimycin A, 2 mM ascorbate and 0.5 mM TMPD for cytochrome *c* oxidase (complex IV) respiration. Respiratory control ratios were calculated as ratios of flux in different states as indicated by the subscript, $RCR_{n/m}$. Instrumental background and oxygen flux due to autoxidation of TMPD and ascorbate were determined as a function of oxygen concentration and subtracted from total volume-specific oxygen flux (Gnaiger et al., 1995; 1998). For each preparation, 2 (mitochondria) or 2-6 (permeabilized fibers) replicate measurements were averaged. Means (±SD) were then calculated for independent preparations. Significance levels were based on *t*-tests.

Results

Cold preservation of isolated mitochondria. The effect of cold storage time on respiratory capacity of isolated rat heart mitochondria is shown in Figure 1. Following conventional procedures as a baseline control, mitochondria were kept in isotonic sucrose medium (MitoMed S) on ice after isolation, and respirometric measurements were initiated within 1 h (day 0). As expected, heart mitochondria were not stable when stored under these conditions for long periods of time (Fig. 1; S). To test whether this instability is an intrinsic property of rat heart mitochondria or a function of storage solution (Scholte et al, 1997), we developed a mitochondrial medium for preservation (MitoMed P; Table 1).

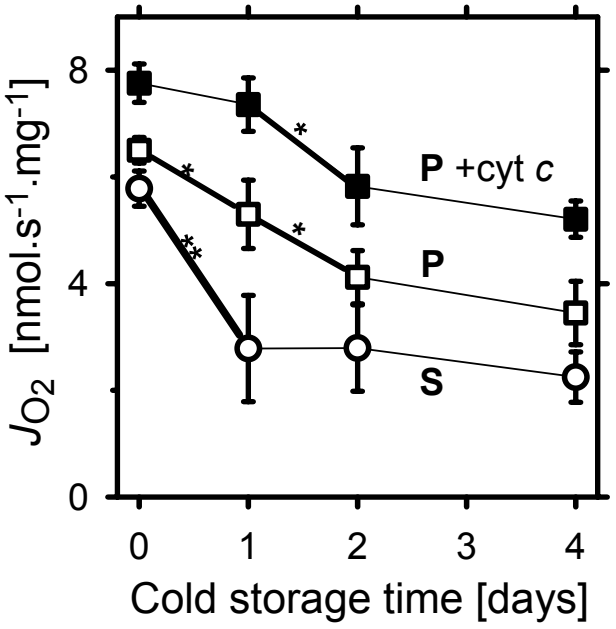

Figure 1. Respiration of isolated rat heart mitochondria, J_{O_2} [nmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ protein], as a function of cold storage time [days]. For complex I respiration at state 3, respiration medium (Table 1) contained [mM]: 10 glutamate, 5 malate, 1 ATP, 1 ADP. Error bars are ±SD (*N*=3 mitochondrial preparations for days 0 and 1; 4 for day 2; 2 for day 4). Cold storage in sucrose medium (S; open circles) caused immediate loss of respiratory capacity over 24 h (*P*<0.01), and resulted in significantly lower respiration at all times compared to mitochondria stored in preservation medium (P; open squares). Respiration declined significantly within 24 h even in mitochondria stored in preservation medium (* *P*<0.05). After addition of 10 µM cytochrome *c*, however, complex I respiration was stimulated (P +cyt *c*; closed squares). Under these conditions, no significant loss of respiratory capacity was observed for 24 h cold storage in preservation medium, indicating cytochrome *c* release from mitochondria as the earliest event in cold injury of well preserved mitochondria.**

Parallel respiratory tests revealed significant improvements of mitochondrial function after cold storage in MitoMed P versus S. *(1)* After only 0.5 to 1 h cold storage, respiratory capacity (state 3, glutamate+malate) increased by 12 % (P<0.05). *(2)* The respiratory control ratio was 18 to 19 in both groups, when expressed as state 3/state 2 respiration ($RCR_{3/2}$). In the transition from state 2 to 4, however, respiration was stimulated by ATP to a significantly larger extent in group S. Consequently, the $RCR_{3/4}$ declined to 3.6 ± 0.3, much lower than 7.0 ± 0.8 in group P. Taken together, these RCR values indicate excellent coupling in both groups of mitochondria, but ATPase activity was higher in group S compared to P. *(3)* The decline of state 3 respiration was 52 % after 24 h cold storage in sucrose medium, whereas this defect was reduced to 18 % by use of MitoMed P (Fig. 1). Heart mitochondria, therefore, display a significant potential of enduring cold storage under appropriate conditions. *(4)* Addition of cytochrome *c* stimulated respiration from state 3 to 3*c* by 19 % at day 0 in both mitochondrial groups ($RCR_{3c/3}$ = 1.19 ± 0.01). Cytochrome *c* stimulation increased significantly at 24 h cold storage, and the $RCR_{3c/3}$ levelled off at about 1.5 after 1 to 4 days of cold storage (corresponding fluxes are shown for group P in Fig. 1). MitoMed P did not improve cytochrome *c* conservation. *(5)* Respiratory rate of heart mitochondria in state 3*c* was not different from control after 1 day cold storage in MitoMed P (Fig. 1 for complex I). Thus the functional properties of mitochondria were fully preserved, except for a significant increase of cytochrome *c* release. Even the high $RCR_{3c/2}$ of 24 remained constant for 1 day of cold storage. In mitochondria stored for 2 and 4 days in the cold, however, addition of cytochrome *c* could not restore control rates of respiration, indicating multiple defects after cold preservation. *(6)* The phosphorylation system including the ATP/ADP translocase was not rate limiting under any conditions of Fig. 1, as tested by the lack of respiratory stimulation when uncoupler was added after cytochrome *c*. This contrasts with a report on limitation by decreasing ATP/ADP translocase activity after a few hours storage of liver mitochondria at 18 °C (Parce et al, 1980). *(7)* Cytochrome *c* release and enzymes or transport systems upstream of

respiratory complex II were the most sensitive targets of mitochondrial cold preservation injury. In contrast, cytochrome *c* oxidase activity was maintained at or near control levels for up to 4 days of cold storage in sucrose medium and mitochondrial preservation solution. The high stability of cytochrome *c* oxidase agrees with observations reported by Scholte et al (1997).

Table 1. Composition of organ preservation solutions, HTK (Custodiol; Dr. Köhler Chemie) and UW (Southard and Belzer, 1995), and media for mitochondrial preservation and respiratory measurement.

	Organ preservation solutions		**Mitochondrial media**		**Comments**
	HTK	**UW**	**Preservation**	**Respiration**	
Sucrose	-	-	110 mM	110 mM	Impermeant, ROS scavanger.
Mannitol	30 mM	-	-	-	Impermeant, ROS scavanger.
Raffinose	-	30 mM	-	-	Impermeant.
Hydroxyethyl starch	-	50 g/l	-	-	Impermeant colloid.
EGTA	-	-	0.5 mM*	0.5 mM*	High affinity for Ca^{2+}, low affinity for Mg^{2+}; * 0.46 mM free EGTA.
$MgCl_2$	4.0 mM	5.0 mM[a]	3.0 mM[b]	3.0 mM[b]	[a] $MgSO_4$; [b] 2.1 mM free Mg^{2+}.
$CaCl_2$	15 µM	-	-	-	Extracellular Ca^{2+} stabilizes membranes.
NaCl	15 mM	25 mM*	-	-	* Total Na^+.
KCl	9.0 mM	125 mM*	80 mM*	80 mM*	* Total K^+, not KCl.
K-lactobionate	-	100 mM	60 mM	60 mM	Impermeant anion; chelator of Ca^{2+}.
KH_2P0_4	-	25 mM	10 mM	10 mM	pK_2 close to 7; not α-stat.
Taurine	-	-	20 mM	20 mM	Membrane stabilizer, antioxidant.
Histidine	198 mM	-	20 mM	-	Imidazol-based (α-stat) pH buffer; anti-oxidant; chelator of transition metal ions.
Tryptophan	2.0 mM	-	-	-	
Ketoglutarate	1.0 mM	-	-	-	Mitochondrial substrate.
HEPES	-	-	20 mM	20 mM	p*K* close to 7.
BSA	-	-	1.0 g/l	1.0 g/l	Free of free fatty acids; membrane stabilizer, antioxidant, chelator of Ca^{2+} and fatty acids.
Vitamin E	-	-	20 µM	-	Lipophilic antioxidant.
Glutathione	-	3.0 mM[a]	3.0 mM[b]	-	Lipophobic antioxidant; [a] oxidized in ViaSpan; [b] freshly added, reduced.
Glutamate	-	-	2.0 mM	(10 mM)*	Mitochondrial substrate, potential for anaerobic substrate level phosphorylation; * added for complex I respiration.
Malate	-	-	2.0 mM	(5 mM)*	Mitochondrial substrate; * added for complex I respiration.
Mg-ATP	-	-	2.0 mM	(1 mM)*	* Titrated to isolated mitochondria, state 4.
Adenosine	-	5.0 mM	-	-	Precursor of ATP; K_{ATP} channel opening; reduces endothelial cell permeability.
Insulin	-	100 IU/l	-	-	Glucose transport.
Penicillin	-	200,000 IU/l	-	-	Antibiotic.
Dexamethasone	-	16 mg/l	-	-	Antiinflammatory, synthetic glucocorticoid.
Allopurinol	-	1.0 mM	-	-	Inhibitor of xanthine oxidase.
Leupeptine	-	-	1.0 µM	-	Inhibitor of neutral Ca activated protease.
pH	7..0-7.2	7.4	7.1*	7.1*	* 30 °C.
Osmolarity	310 mosmol/kg	320 mosmol/l	340 mosmol/l	320 mosmol/l*	* Increases in titration regimes.
Ionic strength	80-90 mM	80-90 mM	108 mM	97 mM*	* Increases in titration regimes.

Cold preservation of the heart. The above results with mitochondrial preservation solution indicate a high potential for cold preservation of mitochondrial function under carefully controlled incubation conditions. While some critical components may not be fully optimized in MitoMed P (Table 1) when compared to the intracellular milieu in vivo, the latter is subject to pathological changes during ischemia and reperfusion. Consequently, mitochondrial function becomes compromized, leading to reversible and eventually irreversible CIR injury (Kay et al 1997; Steinlechner et al 1997). To obtain a pathophysiological perspective for our results on isolated heart mitochondria, we studied mitochondrial defects after cardiac transplant preservation (Kuznetsov et al 1999).

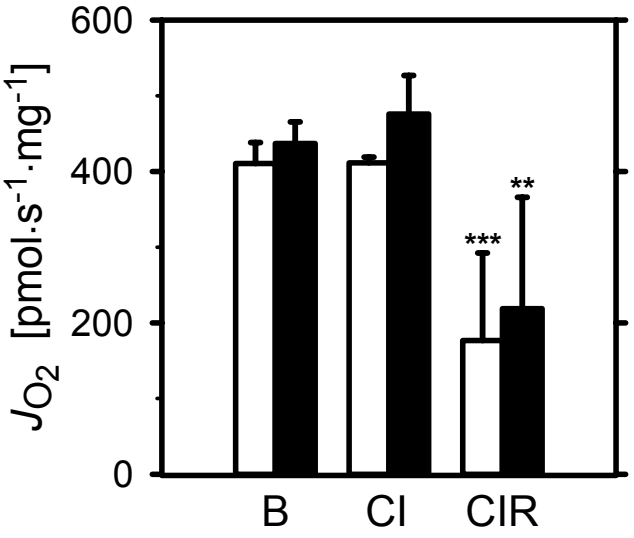

Figure 2. Respiration of permeabilized rat cardiac muscle fibers, J_{O_2} [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ dry tissue weight], in hearts serving as baseline controls without cold ischemia (B; *N*=10), after cold ischemia (CI; 10 h at 0 °C in UW preservation solution plus 1 h of rewarming; *N*=3), and in hearts after cold ischemia and reperfusion (CIR; 10 h at 0 °C and 1 h moderate rewarming to c. 15 °C in UW or HTK preservation solution, and 24 h reperfusion after transplantation; *N*=15). Measurements were made in mitochondrial respiration medium (Table 1), containing [mM]: 10 glutamate, 5 malate, 1 ADP. Open and full bars show respiration before and after addition of 10 µM cytochrome *c*. Error bars are ±SD; significant differences versus baseline controls are ** *P*<0.01; * *P*<0.001. Paired *t*-tests indicate a significant stimulation of respiration by cytochrome *c* in all cases (*P*<0.05 in B; *P*<0.01 in CI and CIR). Cold ischemia for 11 h does not lead to mitochondrial injuries which, however, become significant after reperfusion as a decline of complex I respiration. This defect cannot be compensated by addition of cytochrome *c*.**

Respiration of permeabilized cardiac fibers remained at baseline control levels after 11 h preservation of the heart under cold ischemia (CI), when measured at state 3 and 3*c* immediately after CI without reperfusion (Fig. 2). When an identical CI regime was followed by 24 h reperfusion, however, the capacity for complex I respiration declined by approximately 50 % (Fig. 2). This depression was independent of HTK or UW solution used for organ preservation. Importantly, addition of cytochrome *c* did not restore complex I respiration, indicating severe mitochondrial defects other than cytochrome *c* release. Indeed, various respirometric titration regimes and enzymatic analyses demonstrated a specific decrease of complex I activity, a large increase of cytochrome *c* release, and a relatively small but significant decline of COX activity (Kuznetsov et al, in preparation). These mitochondrial injuries were caused by adverse intracellular conditions faced during reperfusion, but were undetectable immediately after cold ischemia.

Discussion

Optimization of mitochondrial cold storage. Hypothermia represents the most important strategy for myocardial protection from ischemia-reperfusion injury (Tseng and Cameron, 1999), yet optimization of organ preservation solutions greatly increases the cold preservation time of grafts for transplantation (Menasché et al 1993; Southard and Belzer, 1995; Ku et al., 1997). We adopted, therefore, several components from high-quality preservation solutions (HTK and UW; Table 1) and combined these with specific intracellular compounds, to develop the mitochondrial preservation solution (Table 1). Like mitochondrial respiration medium, MitoMed P contains a high potassium concentration, approaching the intracellular K^+ of >100 mM. In contrast to KCl media, a correspondingly high Cl^- concentration is avoided by choosing lactobionate as an impermeant anion, like in UW. In combination with other cell membrane impermeant agents, lactobionate prevents the cells from swelling during cold ischemic storage and appears to be similarly beneficial for isolated mitochondria. A high intracellular Cl^- concentration is unphysiological and inhibitory to mitochondrial

enzymes such as creatine kinase. Sucrose medium, by comparison, has a similar osmolarity but very low ionic strength, artificially stabilizing membrane-bound cytochrome *c*. The antioxidant taurine is beneficial for mitochondrial storage (Scholte et al, 1997) and is used at the intracellular concentration of cardiomyocytes (Table 1). Histidine is the major component of HTK and acts as an imidazol-based pH buffer in the α-stat mode, i.e. the H^+/OH^- ratio is maintained independent of temperature, since the p*K* increases with a drop in temperature at a slope identical to that of water. In addition, histidine acts as an antioxidant, in combination with several other antioxidant compounds used in MitoMed P, particularly reduced glutathione and α-tocopherol.

Mitochondrial cold ischemia-reperfusion injury and transplantation. Short storage times of up to 6 h limit the geographic area from which a heart can be procured for a transplant center. Modern organ preservation solutions applied for cold storage of the heart might be sufficiently effective to push cold ischemia times to the intrinsic limit of the human heart. Alternatively, there remains further scope for optimization of CIR strategies in the heart, by which the problem of organ shortage for transplantation might be addressed. If mitochondrial defects are indeed critical for irreversible CIR injury in endothelial cells (Gnaiger et al, 1999; Steinlechner et al, 1997) and heart (Kay et al., 1997; Kuznetsov et al, 2000), then a shift of intracellular conditions to a favourable state for stabilization of mitochondrial function will lead to the desired prolongation of save CI times (Fig. 1). Since cardiac cold ischemia did not immediately cause mitochondrial CI injury (Fig. 2), special focus is required on *(1)* improvement of cold storage to minimize CIR injury in the course of reperfusion, and *(2)* optimization of initial reperfusion conditions (Lemasters, 1993). *(3)* Ischemic preconditioning (Sumeray and Yellon, 1999) may primarily involve opening of the mitochondrial ATP-sensitive K^+ channel (Garlid et al., 1997), which provides further evidence for the key role of mitochondria in cardiac CIR injury and suggests a promising target for improved heart preservation. It is interesting to note that while mitochondrial K^+ transport decreases 3 times in a hibernator during hypothermia, it increases 1.5-fold during arousal in comparison with the active animal (Fedotcheva et al, 1985). *(4)* Hibernation induction triggers exert beneficial effects on cardiac CI preservation in rabbits (Bolling et al, 1997), pointing to protective mechanisms in hibernators as a model for long-term preservation strategies.

Mitochondrial cold preservation injury and hibernation. Metabolic depression in hibernators is thought to be the cause rather than the mere result of hypothermia, suggested by the observation that metabolic down-regulation occurs to an extent beyond the temperature effect (Q_{10}) on enzyme reaction rates (Heldmaier and Ruf, 1992; Heldmaier et al, 1999; Storey, 1997). Similarly, down-regulation of energy requirements in the hypothermic heart is amplified by cardioplegia, and the two beneficial effects are additive (Tseng and Cameron, 1999). Attempts to elucidate the molecular basis of down-regulation in hibernators beyond the temperature effect have naturally focussed on mitochondrial respiratory control mechanisms. Besides uncoupling of oxidative phosphorylation, a main issue concerns changes in mitochondrial respiratory capacity in the hibernating state of hypothermic torpor versus normothermia, and during transitions between deep hypothermia and normothermia. There is growing evidence for a decrease of respiratory capacity of mitochondria isolated from hibernating animals in their torpid phase and measured in ADP-stimulated or uncoupled states at higher temperatures (Brustovetsky et al 1990; 1993; Martin et al 1999). The decline of maximum mitochondrial respiration has generally been explained in terms of a physiological adaptation to the hibernating state, yet the presence and action of a postulated inhibitor and the full reversibility of the inhibitory effect remain to be demonstrated. Based on the available evidence, an alternative interpretation requires consideration, namely mitochondrial cold preservation injury. The latter would not constitute an adaptive response but confront hibernators with mitochondrial defects caused by hypothermic conditions. These defects would require repair during periodic arousals, to counteract the progressive loss of mitochondrial function below the minimum level required for arousal and beyond the critical limit for induction of necrosis or apoptosis.

Cold preservation injury and reversible down-regulation of mitochondrial energy transformation are not mutually exclusive. For example, covalent modification of enzymes by reversible phosphorylation is a regulatory mechanism described in hibernators, and a shift in the percentage of enzyme to the inactive form can co-occur with a decrease in total enzyme activity (Storey, 1997). Reversible inhibition of mitochondrial respiratory capacity has been reported (Brustovetsky et al,

1990). Quantitatively, however, the uncoupled rate of liver mitochondria isolated from hibernating ground squirrels was reduced to 20 - 30 % relative to mitochondria isolated from active animals. Reversibility of the inhibition was restricted to a 1.9 to 2.5-fold activation of oxygen consumption with β-oxybutyrate, succinate or palmitoyl-L-carnitine as substrates (Brustovetsky et al, 1990). Combining average values for respiratory depression and reversibility, re-activation of mitochondrial respiration resulted in only 55 % of respiratory capacity of mitochondria from active animals. This degree of cold preservation injury is comparable with our results in isolated rat heart mitochondria (Fig. 1).

When core temperature of hibernating Siberian ground squirrels increases from 15 to 37 °C during arousal, mitochondrial respiratory capacity recovers only gradually to normothermic control levels (Brustovetsky et al, 1993). Similarly, the mitochondrial capacity measured during arousal of American ground squirrels (body temperature of 18-25 °C) is not significantly increased above the depressed level of state 3 respiration after 7 d of deep torpor (5-6 °C; Martin et al, 1999). These results do not support a primary role of increased mitochondrial capacity in up-regulation of metabolic heat flux. They are consistent with cold preservation injury which cannot be reversed under hypothermia but requires protein synthesis and cell repair at higher temperature (Hochachka and Guppy, 1987). Whereas a high mitochondrial capacity is restored during arousals (2-8 h after body temperature reaches 35-37 °C), it does not drop significantly during entrance into hibernation (Martin et al 1999). Again, this does not support a primary role of mitochondrial capacity to achieve initial down-regulation of metabolic rate, but is consistent with the hypothesis of mitochondrial cold preservation injury. Defects are not expected to reach significance before body temperature remains severely hypothermic for a critical period of time.

In general terms, the paradigm of cold hibernation injury has been suggested as an explanation for the periodic arousals with rewarming to normothermic temperature: *"The animal then remains at this temperature for 8-10 hr, presumably correcting the imbalances that limit the torpor period"* (Hochachka and Guppy, 1987). As a hypothesis, we extend this concept to mitochondrial cold preservation injury incurred during deep hibernation. Prevention of cytochrome *c* release (Fig. 1) might be of vital importance in hibernators, to avoid induction of the signal transduction pathway leading to apoptosis (Di Lisa et al, 1998; Ghafourifar et al, 1999). Similarly, stabilization of respiratory complexes and improved retention of cytochrome *c* present potential targets for further progress in organ transplant preservation.

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