

Mitochondrial Uncoupling Protein 1 Expressed in the Heart of Transgenic Mice Protects Against Ischemic-Reperfusion Damage

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Background—Mitochondrial respiration is the main source of energy in aerobic animal cells and is adapted to the energy demand by respiratory coupling. Uncoupling proteins (UCPs) perturb respiratory coupling by inducing a proton leak through the mitochondrial inner membrane. Although this could lead to deleterious energy waste, it may prevent the production of oxygen radicals when the rate of phosphorylation of ADP into ATP is low, whereas oxygen and substrate availability to mitochondria is high. The latter conditions are encountered during cardiac reperfusion after ischemia and are highly relevant to heart infarction.

Methods and Results—Heart function of 6 transgenic mice expressing high amounts of UCP1 and of 6 littermate controls was compared in isolated perfused hearts in normoxia, after 40-minute global ischemia, and on reperfusion. In normoxia, oxygen consumption, contractility (quantified as the rate-pressure product), and their relationship (energetic yield) were similar in controls and transgenic mice. Although UCP1 expression did not alter the sensitivity to ischemia, it significantly improved functional recovery on reperfusion. After 60 minutes of reperfusion, contractility was 2-fold higher in transgenic mice than in controls. Oxygen consumption remained significantly depressed in controls ($53 \pm 27\%$ of control), whereas it recovered strikingly to preischemic values in transgenic mice, showing uncoupling of respiration by UCP1 activity. Glutathione and aconitase, markers of oxidative damage, indicated lower oxidative stress in transgenic mice.

Conclusions—UCP1 activity is low under normoxia but is induced during ischemia-reperfusion. The presence of UCP1 mitigates reperfusion-induced damage, probably because it lowers mitochondrial hyperpolarization at reperfusion. (*Circulation*. 2004;110:528-533.)

Key Words: mitochondria ■ ischemia ■ reperfusion ■ free radicals

The mitochondrial uncoupling protein 1 (UCP1) is normally found in the brown adipose tissue of mammals, in which it leads to thermogenesis. The mechanism by which it operates is fully explained within the framework of Mitchell's chemiosmotic theory: the UCP1 constitutes a pathway for the protons pumped by the respiratory chain to return to the mitochondrial matrix, bypassing the ATP-producing return pathway through the FoF1 ATP synthase.¹ Hence, activated UCP1 dramatically increases energy expenditure. This has attracted much attention for the control of body weight, and transgenic models overexpressing UCP1 or its close homologue UCP3 showed improved resistance to obesity.^{2,3} However, possible adverse effects of the mitochondrial uncoupling protein expressed in organs of vital importance have not yet been investigated. Another consequence of partial uncoupling is a lower membrane potential, reducing the mitochondrial production of superoxide radicals.⁴ Using

perfused heart and a transgenic model of mice in which a high expression level of UCP1 has been obtained in heart, we addressed the issue of how it affects heart bioenergetics and whether it protects against the damage induced by ischemia-reperfusion.

Methods

Animals

The transgenic lines expressing UCP1 in muscle or heart have been described previously and showed no sign of cardiac hypertrophy.⁵ Four groups of animals were studied: transgenic U13 mice in which the high expression level of UCP1 in heart mitochondria is close to that found in brown adipose tissue;⁵ C13 mice, their littermate controls; transgenic U20 mice, which showed a low expression of UCP1 in heart;⁵ and C20 mice, their littermate controls. The animals used in this study were progeny of the mating between animals hemizygotic for the transgene and wild-type mice (new B6D2F1 hybrid from Iffa Credo, L'Arbresles, France). The investigation was

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conducted in accordance with our institutional guidelines, defined by the European Community guiding principles in the care and use of animals and French decree no. 87/848 of October 19, 1987. Authorizations to perform animal experiments according to this decree were obtained from the French Ministry of Agriculture, Fisheries, and Food (no. 7475, May 27, 1997).

Perfused Heart

Eight- to 11-month-old male mice were anesthetized by intraperitoneal injection of urethane (2 mg/g). The heart was quickly removed and perfused at constant pressure (75 mm Hg) with Krebs-Henseleit solution (95% O₂ and 5% CO₂, pH 7.35, temperature 37±0.2°C) containing calcium (1.8 mmol/L), glucose (11 mmol/L), pyruvate (5 mmol/L), and mannitol (1.1 mmol/L) as described previously.⁶ A latex balloon inserted into the left ventricular chamber was inflated to maximal isovolumic condition of work (end-diastolic pressure of 5 to 8 mm Hg). The online measured parameters were heart rate, left ventricular systolic pressure, end-diastolic pressure (EDP), coronary flow, and oxygen consumption (QO₂), calculated from the difference in oxygen content in incoming (aortic) and outgoing (pulmonary artery) perfusate. Hearts of the U13 and C13 groups were first submitted to a stepwise change in outer calcium concentration (from 0.5 to 1.8 mmol/L), and steady-state contractility and QO₂ were obtained after 5 to 8 minutes. The sensitivity to ischemia was then evaluated in the same U13 and C13 mice by applying 40 minutes of global normothermic ischemia followed by 1 hour of reperfusion. The same ischemia-reperfusion protocol was applied to the U20 and C20 groups. Hearts were frozen in liquid nitrogen for subsequent analysis of total glutathione content (determined according to Griffith⁷) and aconitase-to-fumarase ratio in mitochondria as described previously.⁸

Results

Normoxic Heart

In the perfused normoxic heart, few functional differences were noted between U13 mice (n=6) and their controls (n=6). The rate-pressure product (RPP), which is the product of heart rate and left ventricular systolic pressure used as an index of contractility, was 2.9±0.4 versus 2.6±0.3×10⁴ mm Hg · beat⁻¹ · min⁻¹ in the U13 and C13 groups, respectively. The oxygen consumption (QO₂ in μmol O₂ · min⁻¹ · g frozen weight⁻¹) was as follows: 9.1±1.8 versus 8.1±1.2, and the coronary flow per gram frozen weight was 15±4 versus 11±1. These values are the maximal values obtained in the presence of 1.8 mmol/L calcium. This shows that the presence of UCPI does not impair heart contractile function. To estimate cardiac energetic efficiency, the relationship between QO₂ and RPP was investigated by decreasing the external calcium concentration to produce different states of cardiac activation. Increasing contractile activity increases the rate of ATP hydrolysis in cardiac fibers, and this increase in ATP demand is compensated by an increased mitochondrial phosphorylation of ADP into ATP, which causes an increase in mitochondrial respiration (QO₂). This explains the classic positive correlation between QO₂ and RPP observed in control hearts (Figure 1A shows the mean of the correlations estimated in each individual heart). Complete uncoupling of respiration would result in mitochondrial respiration being independent of the pathways of ATP synthesis and utilization, ie, a maximal QO₂ for any value of RPP. A partial uncoupling in UCPI-expressing heart (U13) would increase QO₂ in U13 in comparison with C13, with this difference increasing as RPP decreases. As a

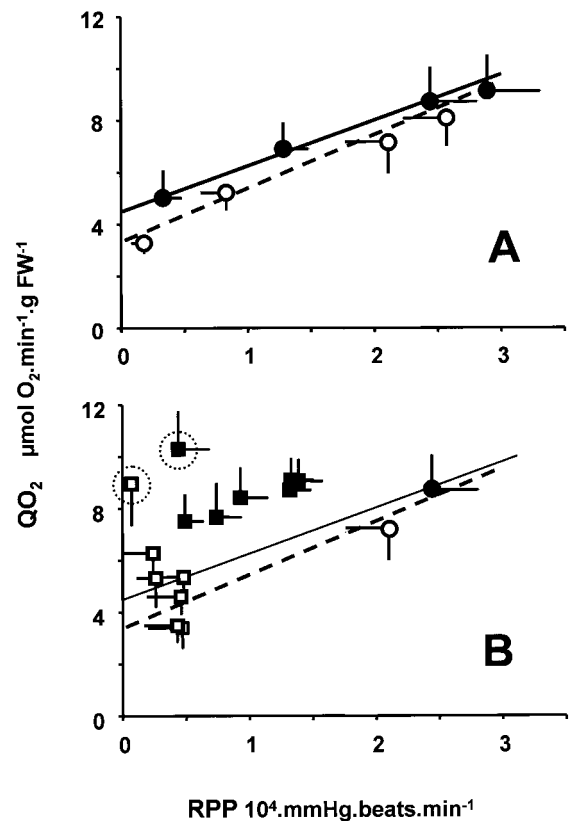


Figure 1. Relationship between contraction (RPP) and oxygen consumption (QO₂). A, Normoxia: variation of contractile activity quantified as RPP and of oxygen consumption (QO₂) on sequential changes in external calcium concentration: first, 1.8 mmol/L (normal perfusate, highest values of RPP and QO₂), then 0.5 mmol/L (lowest RPP and QO₂), followed by 1 mmol/L (intermediate values), and finally return to 1.8 mmol/L, which restored ≈85% of its initial value. Open circles, control hearts, C13 (n=7); closed circles, transgenic mice, U13 (n=6). For each calcium concentration, values of RPP and QO₂ were not significantly different in U13 and C13 hearts. Linear relationship between QO₂ and RPP was analyzed for each heart, which allowed calculation of mean±SEM values of slope ordinate and regression coefficient: C13: QO₂=2.0(±0.3)×RPP+3.4(±0.4), r²=0.97±0.02; and U13: 1.8(±0.3)×RPP+4.5(±0.8), r²=0.97±0.01. Examination of correlation lines suggests a slight difference between C13 and U13. A calculation of β error risk value gave 0.59 for slope and 0.07 for ordinate at origin. (In other words, an increase of <1 unit in slope coefficient and of <2% in difference of ordinates at origin would remain statistically nonsignificant). B, Reperfusion: RPP and QO₂ during reperfusion, open square, control hearts, C13 (n=6); closed square, transgenic hearts, U13 (n=6). These data points correspond to values of RPP and QO₂ shown in Figure 2 at 5, 10, 15, 20, 30, 50, and 60 minutes of reperfusion. Circled data points refer to 5 minutes of reperfusion. To compare with normoxic conditions: correlation lines of Figure 1A are shown, as well as data points (open circles, C13, and closed circles, U13) before onset of ischemia.

result, the regression between QO₂ and RPP would exhibit a lower slope and a higher ordinate at the origin. Although such a tendency exists (Figure 1A), the difference was modest and remained statistically not significant (see figure legend). Given the low expression level of UCPI in U20, no such experiment was performed with the U20 and C20 groups.

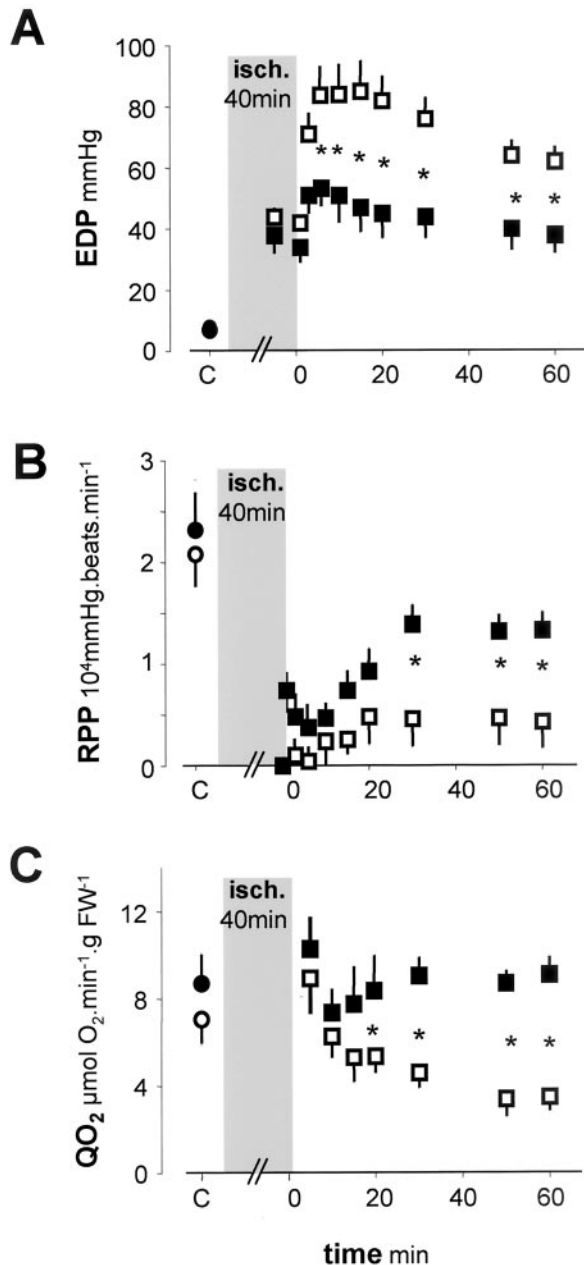


Figure 2. Time course (U13 vs C13). Comparison of heart from transgenic mice overexpressing UCP1 in heart (U13) with their littermate controls (C13). Use of symbols as in Figure 1B. Isch. indicates global normothermic ischemia. Time 0 is start of reperfusion time. Values are shown as mean \pm SEM. EDP (contracture), * $P < 0.05$. RPP, * $P < 0.01$. Respiratory activity (oxygen consumption, QO₂), * $P < 0.05$.

Ischemia-Reperfusion Period

The rigor-type contracture (ie, the rise in EDP) induced by ischemia shows similar kinetics: time at the onset of contracture, time to reach the maximum (not shown), and amplitude (Figure 2A) in C13 and U13 hearts. On reperfusion, this contracture increased further in C13 hearts (as well as in C20 and U20, Figure 3A) but not in U13 hearts (Figure 2A). The difference between C13 and U13 became significant within the first 5 minutes of reperfusion. This increase in EDP participates in the deterioration of contractile properties;

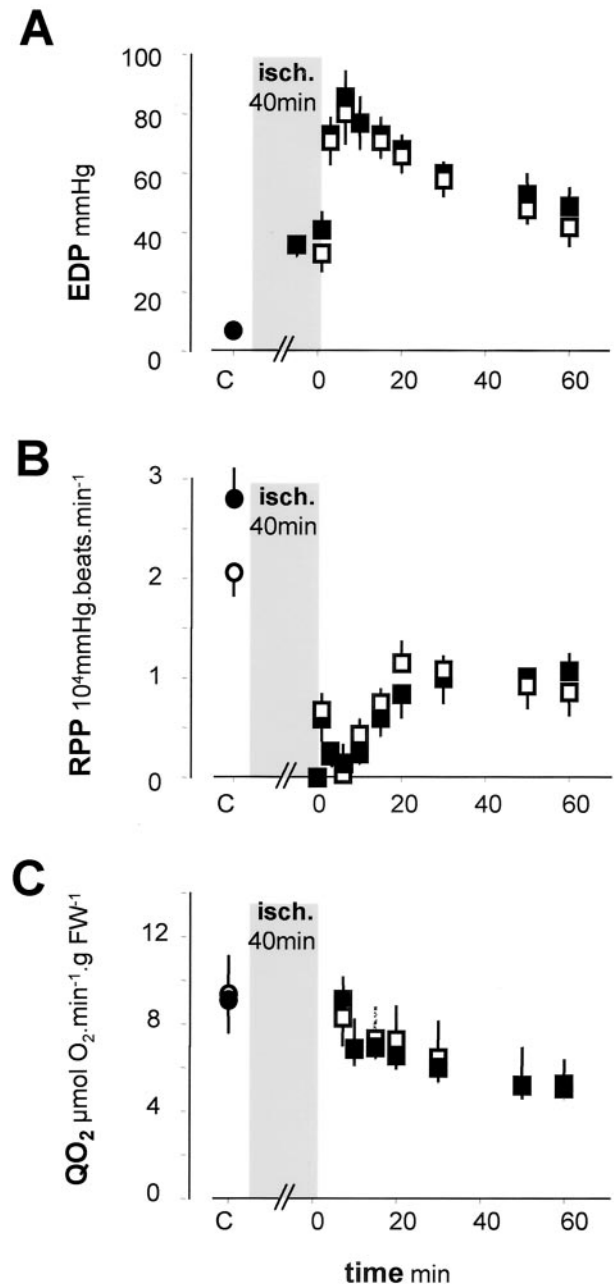


Figure 3. Lack of protection in U20 mice. Comparison of heart from transgenic mice overexpressing UCP1 in heart (U20) with their littermate controls (C20). Symbols and legend as Figure 2.

indeed, the prevention of this second phase of deterioration during reperfusion in U13 hearts contributes to an improved recovery of their systolic activity (RPP, Figure 2B) compared with controls (C13). Enhanced contractile recovery in U13 hearts was not a result of a better perfusion or oxygenation, because the postischemic coronary flows were similar (data not shown). At the onset of reperfusion, although contractility was impaired, oxygen consumption (QO₂) rapidly increased, and maximal respiration rates were observed in both C13 and U13 hearts after 5 minutes of reperfusion (Figure 2C). However, at this time, contractility was impaired, and therefore, this oxygen consumption is not coupled to contraction. Accordingly, in a graphic representation such as Figure 1A,

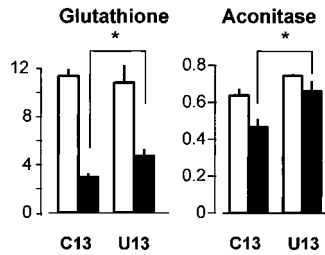


Figure 4. Markers of oxidative stress. Genotype is indicated below histograms; statistical analysis was performed by use of ANOVA. Glutathione content is expressed in nmol/mg protein (glutathione): open bars, preischemic conditions (C13, $n=3$; U13, $n=4$); filled bars, after ischemia-reperfusion (C13, $n=5$; U13, $n=6$), $*P<0.05$. Aconitase activity is related to fumarase activity, resulting in a dimensionless aconitase-to-fumarase ratio (aconitase): open bars, preischemic conditions controls (C13, $n=4$) and transgenic mice (U13, $n=2$ values: 0.741 and 0.747); filled bars, after ischemia-reperfusion, C13 ($n=7$) and U13 ($n=5$), $*P<0.05$. Values are shown as mean \pm SEM except for U13 before ischemia ($n=2 \pm$ min-max). Change in aconitase-to-fumarase ratio ischemia-reperfusion with reference to preischemic conditions was not significant ($P=0.36$) with U13 but was significantly reduced with C13 ($P=0.02$).

the data points at 5 minutes appear at low RPP but high QO_2 , well above the regression lines observed in normoxia (Figure 1B). Oxygen consumption declined with time in C13 hearts, whereas contraction resumed; finally, data points comply with the $RPP \times QO_2$ relationship observed in normoxia. Conversely, the oxygen consumption of U13 hearts remained elevated and similar to its preischemic value for 1 hour of reperfusion: data points remained clustered above the normoxic $QO_2 \times RPP$ relationship. In conclusion, C13 hearts restored a QO_2 coupled to contraction, and U13 hearts did not. This is easily interpreted as the result of mitochondrial uncoupling caused by the induction of UCP1 activity in U13 hearts, which caused a sustained increase in respiration rate that did not lead to ATP synthesis. It can be deduced that the activity of UCP1 amounts to approximately one fourth of the oxygen consumption in U13 hearts. When UCP1 was present at low level (U20), neither increased oxygen consumption nor protection of diastolic and systolic function was observed (Figure 3, A–C).

Markers of Oxidative Stress

At the end of the ischemia-reperfusion experiments, total glutathione content was found to be significantly less reduced in U13 than in C13 hearts (Figure 4), whereas no difference was found between the 2 groups before ischemia. After ischemia-reperfusion, the glutathione content was not different in U20 and C20 hearts (5.07 and 4.92 nmol/mg protein, respectively, mean values; $n=4$, $P=0.92$). The aconitase activity was significantly more affected by ischemia and reperfusion in C13 than in U13 mitochondria, in which it seemed hardly decreased (Figure 4).

Discussion

UCP1 During Normoxia

Physiological regulation of the UCP1 in brown adipose tissue involves a balance between inhibition of proton transport (hence uncoupling) by cytosolic guanine nucleotide diphos-

phate or triphosphate (GDP, GTP, ADP, and ATP, the last 2 being the more relevant inside cells) and activation by free fatty acids.¹ In normoxic heart, high ATP and low free fatty acid levels are likely to ensure nearly complete inhibition of UCP1. Moreover, the mitochondrial membrane potential acts as a regulator of UCP1: when proton pumping by the respiratory chain is challenged by intense ATP production through the ATP synthase, as in a working heart, the membrane potential remains at a value of ≈ 130 mV, and UCP1 activity is much lower than when ATP synthesis is not required and the membrane potential rises to 170 mV⁵; hence the experiment in which ATP usage is reduced by decreasing contractile work to evidence this regulation of UCP1 by membrane potential. According to this experiment, the effect of UCP1, if any, is of reduced amplitude (Figure 1A). Therefore, the normal proton circuit across mitochondrial inner membrane would be almost unchanged in comparison with control heart (Figure 5, top).

Ischemia

During ischemia, ATP level drops, whereas AMP rises,⁹ and an increase in free fatty acids occurs.¹⁰ Consequently, the ischemic period lowers the concentration of inhibiting nucleotides (Σ ATP+ADP) and increases the concentration of UCP1 activators. Because neither oxygen nor substrates are supplied to the respiratory chain, proton pumping is impaired. Therefore, although intracellular conditions would authorize its activity, UCP1 remains inactive because of the lack of proton motive force (Figure 5, middle). Ischemic contracture was unaltered by the presence of UCP1, suggesting a similar ischemic rise in cytosolic calcium and free ADP concentrations. This point is of importance because short periods of ischemia or chemical treatments induce preconditioning¹¹ of the myocardium, which leads to a better resistance to subsequent long-term ischemia. Pretreatment with chemical uncouplers triggers this protective mechanism.¹² The presence of UCP1 is unlikely to induce such a mechanism, because preconditioning alters the time course of ischemic contracture,^{13,14} whereas the ischemic contracture was identical in C13 and U13 hearts.

Reperfusion

On reperfusion, both oxygen and substrates are supplied to mitochondria, which start to respire immediately (Figure 2C) and recreate the proton driving force. Therefore, proton return through UCP1 is made possible, leading to uncoupled respiration. This mitochondrial uncoupling, however, does not impair ATP production, because restoration of contraction takes place (Figure 2B). Therefore, one must assume that during reperfusion, proton return occurred simultaneously through UCP1 and the ATP-producing FoF1 ATPase (Figure 5, bottom). Examination of Figure 1B suggests that UCP1 activity accounts for $\approx 2 \mu\text{mol oxygen} \cdot \text{min}^{-1} \cdot \text{g fresh weight}^{-1}$ in U13 reperused heart. This UCP1 uncoupling activity remained detectable 1 hour after the start of reperfusion. This means that conditions able to lead to inhibition of UCP1 are not restored within 1 hour. Ischemia leads to loss of purine nucleotides, and their rate of resynthesis is slow.¹⁵ Therefore, although the flux of mitochondrial phosphoryla-

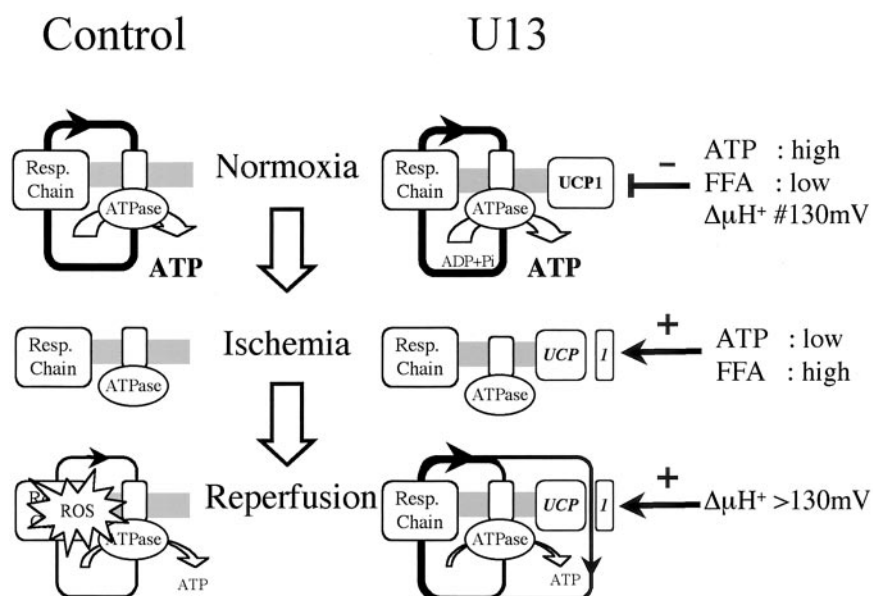


Figure 5. Activation of UCP1 during ischemia reperfusion. Schematic explanation of hypothetical sequence of events during normoxia, ischemia, and reperfusion. Mitochondrial inner membrane, schematized as a gray rectangle with cytosolic side (out) at top and mitochondrial matrix (in) at bottom, shows producer of proton motive force (respiratory [resp.] chain) and consumers (FoF1 ATP synthase [ATPase] and possibly UCP1). Adenine nucleotide translocase, which exports ATP to cytosol and imports ADP inside mitochondria, is not shown. Orientation of flux of protons is symbolized by arrow on circuit. Parameters relevant in explaining UCP1 state of activation/inhibition are indicated on right.

tion of ADP into ATP is restored with the remaining intracellular nucleotides, ATP concentration requires several hours to be restored to preischemic values.¹⁵ It is therefore plausible that intracellular ATP concentration is still not sufficient to inhibit UCP1, although ATP turnover is able to sustain contraction. Another explanation would be that 2 types of cells were present in the reperfused heart: intact cells responsible for contraction, in which UCP1 returned to its inhibited state, and noncontracting damaged cells, in which UCP1 remained activated.

Protection by UCP1

Damage linked to ischemic periods results from the consequences of substrate and oxygen deprivation¹⁶ and also from reactive oxygen species (ROS) production,¹⁷ which is suspected to occur during both ischemia and reperfusion.^{18,19} Many of these ROS are of mitochondrial origin, and their production increases together with the reduction of components of the respiratory chain.^{20,21} This can be a result of a high membrane potential that opposes proton pumping and therefore electron transfer by respiratory chain complexes. This situation is likely to occur during reperfusion, because it takes time for contractile activity to restart (Figure 2B), whereas mitochondrial respiration starts immediately (Figure 2C). The high mitochondrial membrane potential also drives mitochondrial uptake of calcium.²² It is likely that during reperfusion, both superoxide and calcium uptake cooperate to induce opening of the mitochondrial transition pore that leads to cell death. The proton conductance brought by UCP1 authorizes a faster oxidation rate (uncoupling) and lowers membrane potential. Therefore, activity of UCP1 would reduce both ROS production and calcium uptake by mitochondria. Two parameters linked to oxidative damage were estimated (Figure 4). Defense against oxidative stress results in a drop in cellular glutathione that is less marked in U13 hearts. The mitochondrial Krebs cycle enzyme aconitase, which is a known target of mitochondrial ROS,^{23,24} is less affected by ischemia-reperfusion in U13 hearts. Therefore,

although causality is not demonstrated, the protection afforded by UCP1 is associated with a reduced oxidative stress. While this article was undergoing revision, 2 other reports showed that recombinant expression of UCP1²⁵ or UCP2 in cultured cardiomyocytes²⁶ protects against damage induced by oxidative stress²⁶ or hypoxia/reoxygenation.²⁵ Both reports demonstrate lower ROS production in the presence of UCPs and reduced calcium uptake into mitochondria in the presence of UCP2.²⁶ Our work extends these studies to the level of whole heart. It is noticeable that in transgenic (U13) hearts, the respiratory activity (QO_2) during reperfusion remained the same as before ischemia. This suggests that during ischemia, no damage occurred to redox components of the mitochondrial respiratory chain, whereas contractile function was damaged.

Effect of Transgenic UCP1 and Putative Role of UCP2 and UCP3

Two proteins similar to UCP1 have been described: UCP2 and UCP3.^{27,28} An obvious hypothesis is that their purpose is to limit ROS production by mitochondria.²⁹ The expression level of UCP2 or UCP3 in vivo seems closer to the amount of UCP1 found in U20,^{30,31} in which no protection was observed. Therefore, if we consider that they act as UCP1 does,³²⁻³⁴ this study predicts that the protection afforded against reperfusion-induced damage by endogenous levels of UCP2 or UCP3 is negligible. This does not preclude the possibility that their overexpression could be protective, as has recently been reported for UCP2 in cardiomyocytes.²⁶

Conclusions

Uncoupling proteins are usually considered to be deleterious for ATP production. We show here that this is not the case for UCP1 introduced into mouse heart by transgenesis, probably because intracellular conditions in normoxia lead to inhibition of UCP1 uncoupling activity. It is noticeable that ischemia produces conditions leading to activation of UCP1, allowing it to operate during subsequent reperfusion. The

observation that the induction of UCP1 uncoupling activity is accompanied by an improved recovery of heart function is consistent with the fact that a reperfused heart suffers from calcium- and ROS-mediated effects, which are consequences of the hyperpolarization of mitochondria in their normal energy-conservative coupled state.

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