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Reptilian uncoupling protein: functionality and expression in sub-zero temperatures

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SUMMARY

Here we report the partial nucleotide sequence of a reptilian uncoupling protein (repUCP) gene from the European common lizard (*Lacerta vivipara*). Overlapping sequence analysis reveals that the protein shows 55%, 72% and 77% sequence homology with rat UCP1, UCP2 and UCP3, respectively, and 73% with bird and fish UCPs. RepUCP gene expression was ubiquitously detected in 4°C cold-acclimated lizard tissues and upregulated in muscle tissues by a 20 h exposure to sub-zero temperatures in a supercooling state or after thawing. In parallel, we show an increase in the co-activators, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and peroxisome proliferator-activated receptors (PPAR), mRNA expression, suggesting that the mechanisms regulating UCP expression may be conserved between mammals (endotherms) and reptiles (ectotherms). Furthermore, mitochondria extracted from lizard skeletal muscle showed a guanosine diphosphate (GDP)-sensitive non phosphorylating respiration. This last result indicates an inhibition of extra proton leakage mediated by an uncoupling protein, providing arguments that repUCP is functional in lizard tissues. This result is associated with a remarkable GDP-dependent increase in mitochondrial endogenous H_2O_2 production. All together, these data support a physiological role of the repUCP in superoxide limitation by lizard mitochondria in situations of stressful oxidative reperfusion following a re-warming period in winter.

Key words: *Lacerta*, cold hardiness, mitochondria, superoxide, supercooling, freezing.

INTRODUCTION

Although the phenomenon of proton leakage has been considered for a long time to be an artefact of damage to mitochondria, it is now clear that it is a natural process occurring in intact mitochondria in their usual environment: the cytosol (Nobes et al., 1990). The energy devoted to counter this proton leakage represents roughly 25% of the organism's energy budget, as determined across a wide range of ectothermic and endothermic vertebrates (Brand et al., 1991; Brookes et al., 1998). However, important differences occur in mitochondrial functioning between endotherms and ectotherms. Several studies showed (1) a significantly higher mitochondrial O_2 consumption rate in endotherms (5–10 times) compared to ectotherms (Berner, 1999; Brand et al., 1991) and (2) that mitochondria from ectotherms are less leaky to proton than those from endotherms (Brand et al., 1991; Brookes et al., 1998). These considerations suggest differences in the fatty acid composition of mitochondrial membrane phospholipids and/or different degrees of modulation of mitochondrial proton leakage by proteins such as uncoupling proteins (UCPs) (Brookes et al., 1998). UCPs belong to a sub-family of proteins found in the inner mitochondrial membrane. Until 1997, only one UCP, expressed specifically in the brown adipose tissue of mammals, was known. However, the discovery of other proteins sharing about 60% sequence similarity has led to the classification of supplementary types of UCPs: UCP2 found in various mammalian tissues and UCP3 mainly expressed in the skeletal muscle (Ricquier and Bouillaud, 2000). The thermogenic function of mammalian UCP1 is well established (Cannon and Nedergaard, 2004), but the biological roles of UCP2 and UCP3 are still controversial. Homologues of mammalian UCP1 have also been detected in a great variety of organisms such as birds, plants, unicellular organisms and a few ectothermic vertebrates (Sluse et al., 2006), suggesting that these proteins have been strongly conserved through evolution. Despite this probable conservation, evidence of the presence of UCP in ectothermic vertebrates is scarce and only restricted to fish (Jastroch et al., 2005; Liang et al., 2003; Mark et al., 2006; Stuart et al., 1999) and amphibians (*Xenopus laevis*) (Keller et al., 2005).

Many hypotheses on the physiological role of UCPs in mammals have been raised, including the regulation of energy metabolism, the control of body mass and the attenuation of mitochondrial reactive oxygen species (ROS) production (Brand and Esteves, 2005; Negre-Salvayre et al., 1997). In other taxa, the main function of UCP1 homologues also remains uncertain, however *in vitro* experiments have shown that they can modulate the mitochondrial proton motive force, which in turn is a key factor influencing ROS production at complex I and III of the mitochondrial respiratory chain (Boveris and Chance, 1973; Papa and Skulachev, 1997). For instance, avian UCP has been shown to protect yeast mitochondria against the effect of ROS (Criscuolo et al., 2005). Furthermore, exogenous superoxide has been reported to activate UCP-mediated uncoupling *in vitro* (Echtay et al., 2002; Krauss et al., 2003). Therefore, the uncoupling activity of UCPs *in vivo* may play a crucial role in protecting tissues from oxidative stress during periods

when overgeneration of ROS is expected (Papa and Skulachev, 1997).

Low temperatures followed by re-warming are probably one of the most commonly found ROS-generating stress in nature. Ectotherms survive these thanks to the contribution of cellular adaptive mechanisms that include, plasmic and mitochondrial membranes alteration, increase in mitochondrial volume density, specific isoenzymes and stress proteins synthesis (Johnston and Bennett, 1996). At subzero temperatures, freeze tolerance and increasing supercooling capacities constitute the two means of ectotherm survival. These two physiological states (frozen and supercooled) induced by sub-zero temperatures limit oxygen availability to tissues, compelling ectotherms to cope with potential oxidative stress generated by the transition between ischemic/anoxic conditions and the reperfusion of oxygenated blood during recovery (Hermes-Lima and Zenteno-Savin, 2002; Storey, 1996). Both increase in temperature and thawing are then concomitant with the oxygen reperfusion into tissues restoring organ aerobic metabolism, and potentially generate oxidative stress. However, data on reptiles submitted to supercooling, freezing and thawing conditions showed no significant oxidative damage on DNA and only slight increase of antioxidant defences (Voituron et al., 2006). These observations suggest the existence of other adaptive mechanisms such as ROS limitation within the mitochondria, since mitochondria are a significant source of ROS in cells, and/or the existence of powerful DNA repair mechanisms. In the present study, we investigated the potential existence of a UCP that would reduce ROS production in reptile mitochondria under subzero temperatures. We report for the first time the detection and the localization of a UCP homologue in a reptile (*Lacerta vivipara*). Its functionality was assessed by measurements of respiration and H_2O_2 production of muscle mitochondria. Furthermore, we also demonstrate the influence of subzero temperatures on its pattern of expression together with its co-activators peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and peroxisome proliferator-activated receptors (PPAR).

MATERIALS AND METHODS

Animals

The present investigation was carried out according to the ethical principles of the French Ministry of agriculture and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Council of Europe, no. 123, Strasbourg, 1985).

Lacerta vivipara (von Jacquin 1787) individuals, mean body mass 3.27±0.57 g, were captured in late September from a highland population (1450 m) in the Cévennes mountains in France. They were held in boxes with sand and wet moss and cold acclimated at 4°C for 6–7 weeks in the dark with no feeding before being split into four groups. Subsequently, the experiments were performed in November and December.

One group was kept at 4°C as the control group and the remaining lizards where divided among the three groups as described below.

Preparation of supercooled, frozen and thawed animals

Freezing exposures were performed as previously reported (Voituron et al., 2002a). Briefly, the lizards in the 'frozen' group were placed on a pad of wet absorbent paper, itself placed in a 50 ml tube. The animals were then progressively cooled at a constant rate of 0.2°C min^{-1} in a low temperature incubator 815 PRECISION (Grenoble, France), from 4°C (initial temperature) down to the crystallization temperature (T_c, –2.5°C). We considered that the freezing exposure of each lizard began immediately after its exotherm and ended when the individual was removed from its tube 20 h later. During the freezing period all the individual tubes were placed in an incubation chamber set at –2.5°C (±0.1°C). A 20-h freezing period was chosen because it induces an ice content of about 50% and recovery of the frozen lizards strongly decreased with longer periods of freezing (Voituron et al., 2002a).

Lizards in the 'thawing' group were primarily frozen for 20 h as described for the frozen group but were then thawed in an incubator at +3°C for 24 h.

Lizards in the 'supercooling' group were placed on a pad of dry absorbent paper and progressively cooled at a constant rate of 0.2°C min^{-1} down to –2.5°C. They were then maintained at this temperature for 20 h before being sacrificed.

Lizards from all groups were killed by decapitation and organs were quickly dissected out, frozen in liquid nitrogen and stored at –80°C to await molecular analysis. For the controls and supercooled lizards, blood was collected in heparin, and plasma fatty acid content was determined using the enzymatic NEFA C kit (Wako Chemicals, Neuss, Germany) according to the manufacturer's instructions.

Relative mRNA abundance of repUCP, PGC-1α and PPARγ in lizard muscles

Total RNA was extracted from tail muscle samples (80 mg) using Trizol® (Invitrogen, Cergy Pontoise, France). Concentration and purity were checked by measuring optimal density at 260 and 280 nm and their integrity was confirmed by 1% agarose gel electrophoresis (Eurobio, Les Ulis, France). The relative expression of mRNAs were measured by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) using β-actin as standard. For each sample, a reverse transcription was performed from 1 μg of total RNA with 100 i.u. of M-MLV reverse transcriptase (Promega, Charbonnières-les-Bains, France), 5 μl of M-MLV RT 5× buffer, 20 i.u. of RNasin ribonuclease inhibitor, 12 pmol of deoxynucleoside triphosphate and 1 μg of oligo(dT), in a final volume of 25 μl. The reaction was carried out for 5 min at 70°C [RNA and oligo(dT)], then 60 min at 42°C (all mix) followed by 15 min at 70°C. After chilling, 2.5 μl were used for a PCR reaction. Primer sequences are shown in Table 1. The 2.5 μl of RT medium were added to 47.5 μl of PCR mix containing 5 μl 10× REDTaq PCR buffer, 6 pmol $MgCl_2$, 8 pmol of deoxynucleoside triphosphate, 2.5 i.u. of REDTaq DNA polymerase (Sigma), 22.5 pmoles of corresponding antisense and sense primers. The PCR conditions were: 2 min at 94°C followed by 31, 39, 40 or 22 cycles for UCPshort, PPARα, PGC-1α and β-actin, respectively (1 cycle=1 min at 94°C, 1 min at 60°C, 1 min at 72°C). PCR was ended by 10 min at 72°C. Products were separated on 2% agarose gel prestained with ethidium bromide. For quantification of relative band intensities, pictures were taken with a DC120 camera (Kodak) and the ratio of each target to β-actin was determined for each sample with Kodak Digital Science 1D 2.0 (Kodak Scientific Imaging System, Les Ulis, France).

RepUCP expression was also investigated following the same protocol, on a panel of six tissues from control lizards (liver, muscles, lungs, heart, brain and adipose tissue; N=5) to determine if its expression was ubiquitous or tissue specific.

The PCR products obtained with UCPlong primers (Table 1) contained a 780 bp fragment of repUCP and were purified using a Cleamix kit (Talent, Trieste, Italy) and sequenced (Genoscreen, Lille, France).

All the primers used were taken from *Gallus gallus* sequences (avUCP accession no. AF287144, β-actin accession no. L08165).

Isolation of muscle mitochondria

Leg and tail muscles from four control lizards (4°C for 6–7 weeks) were dissected, pooled and placed in ice-cold isolation buffer (100 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EGTA and 50 mmol l^{-1} Tris–HCl, pH 7.4). The muscles were chopped with scissors and incubated with 1 mg g^{-1} muscle wet weight of protease (Subtilisin A, Sigma, St Quentin Fallavier, France) for 1 min and then homogenized with a Teflon glass homogenizer. The mixture was diluted 1:2 (v:v) with isolation buffer without protease and centrifuged at 800 ***g*** for 10 min. The resulting supernatant was filtered through a cheesecloth and centrifuged at 8000 ***g*** for 10 min to obtain the mitochondrial pellet. The pellet was washed twice with isolation buffer and then centrifuged at 8000 ***g*** for 10 min. Finally, the pellet was resuspended in ice-cold storage medium (250 mmol l^{-1} sucrose, 20 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EGTA at pH 7.4). After protein quantification by the Biuret method using BSA as standard, the mitochondria were diluted to 20 mg ml^{-1} with storage medium. All centrifugations were performed at 4°C.

Measurement of mitochondrial O_2 consumption

Oxygen consumption was measured with a Clark oxygen electrode (Rank Brothers Dual Digital, Bioblock, Illkirch, France) in a 0.75 ml glass cell, thermostatically controlled at 25°C, with constant stirring. The mitochondria (0.5 mg/ml) were placed in a respiratory medium saturated with room air, containing 200 mmol l^{-1} sucrose, 10 mmol l^{-1} potassium phosphate and 20 mmol l^{-1} Tris-HCl, pH 7.4, with 0.3% fatty-acid-free bovine serum albumin (BSA). The phosphorylating respiration rate (state 3) was measured in the presence of 5 mmol l^{-1} succinate (current substrate used to study mitochondrial bioenergetics parameters providing $FADH_2$ to complex II) and 5 μmol l^{-1} of rotenone after addition of 1 mmol l^{-1} ADP. The control state of respiration (state 4+) was obtained by the addition of 1 μg/ml oligomycin to inhibit F0-F1 ATPase. The contribution of UCP to mitochondrial respiration was then assessed by measuring inhibition of state 4 respiration rate (5 mmol l^{-1} succinate and 5 μmol l^{-1} of rotenone) by addition of 2 mmol l^{-1} guanosine diphosphate (GDP), a known inhibitor of UCP (Echtay et al., 2002; Stuart et al., 2001). The effect of 30 μmol l^{-1} atractylate on state 4 mitochondrial respirations was also measured in an independent set of experiments in order to detect the potential impact of the adenine nucleotide transporter.

Measurement of mitochondrial H_2O_2 production

The rate of mitochondrial H_2O_2 release was measured at 25°C on mitochondria energized with succinate (state 4), following the linear increase in fluorescence (λ_{ex} 312 nm and λ_{em} 420 nm) due to oxidation of homovanillic acid (HVA) by H_2O_2 in the presence of horseradish (HRP), on a SFM-25 fluorometer (Kontron Instruments, Dardilly, France), as described previously (Servais et al., 2003). Reaction conditions were 0.1 mg of mitochondrial protein per ml, HRP (6 i.u. ml^{-1}), HVA (0.1 mmol l^{-1}) and succinate (5 mmol l^{-1}) as substrates, in the same incubation buffer as was used for oxygen consumption measurements. Known concentrations of H_2O_2 were used to establish a standard concentration curve. Addition of catalase showed a dose-dependent drop of fluorescence (data not shown). There was no increase in fluorescence in the absence of substrates or mitochondria. Measurements of oxygen consumption and H_2O_2 release were performed at the same temperature with the same concentration of substrates in absence or in presence of 1 mmol l^{-1} GDP.

Statistical analysis

The data are presented as mean ± s.e.m. The statistical analysis was performed with the Statview computer statistical package. Data on mRNA expression (comparing tissues and comparing conditions) was analysed using an ANOVA followed by a *post-hoc* Tukey's test, and means values of mitochondrial respiration were compared using a paired *t*-test. Blood fatty acid levels in control and supercooled lizards were compared using a *t*-test. A 5% (P<0.05) level of significance was used in all tests.

RESULTS

Partial sequencing and localisation of lizard repUCP

The selected primers led to the PCR amplification of a DNA fragment of the expected size with cDNAs from lizard tissues (GenBank accession no. EU571947). The amplified fragment (UCPlong; Table 1) was closely related to those of other UCPs since the predicted amino acid sequence was 73% homologous to avian UCP (*Gallus gallus*), fish UCP (*Dano rerio*) and amphibian UCP (*Xenopus laevis*), and 76%, 72% and 55% homologous to mammalian UCP3, UCP2 and UCP1 (*Rattus norvegicus*), respectively (Fig. 1).

RepUCP expression was then investigated on a panel of six tissues from control lizards (N=5; liver, muscles, lungs, heart, brain and adipose tissue). The data showed expression in every tissue tested with an equivalent expression in liver, muscles, brain and adipose tissue (Fig. 2; Tukey's test d.f. 22; P<0.3). By contrast, a higher expression was observed in heart tissue (d.f. 22; P<001), and the lung showed a weaker expression (d.f. 22; P<0.001) of repUCP (Fig. 2).

Expression of PGC-1α, PPARγ and repUCP mRNA in lizard muscle

The expression of PGC-1α and PPARγ mRNAs was significantly increased (P=0.0006 and P=0.01, respectively) with sub-zero temperatures whatever the physiological states (supercooled state: 1.9- and 2.4-fold, respectively; frozen state: 2.3- and 2.4-fold, respectively) compared with control lizards (Fig. 3). In lizards thawed for 24 h, the expression of these mRNAs was still significantly higher than in control lizards (1.7 and 3-fold respectively), but whereas PPARγ reached its highest expression, PGC-1α expression was significantly lower than its expression under supercooled and frozen states.

RepUCP mRNA expression followed a similar pattern, with the exception of the freezing treatment, which induced a non significant increase (P=0.2) of about 1.5-fold whereas supercooling and

Table 1. Primer sequences used for RT-PCR

	Sense (5′→3′)	Antisense (5′→3′)	Size (bp)
β-actin (gi11420427)	GACGAGGCCCAGAGCAAGAGA	GGGTGTTGAAGGTCTCAAACA	225
UCPshort (gi12083897)	GTGGATGCCTACAGGACCAT	ATGAACATCACCACGTTCCA	390
UCPlong (gi12083897)	GCGGTCGACATCACCTTCCCGCTGGACA	GCGACTCGGATCCTGGAACGTCGACATG	870
PPARγ (gi47825368)	TACATAAAGTCCTTCCCGCTGACC	TCCAGTGCGTTGAACTTCACAGC	480
PGC-1α (gi50749696)	GACTCAGGTGTCAATGGAAGTG	ATCAGAACAAGCCCTGTGGT	240

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RepUCP          ------------------------------------KYRLHIQGEGK--ASRAMKNVKY 21
Rattus UCP1     MVSSTTSEVQPTMGVKIFSAGVSACLADIITFPLDTAKVRLQIQGEGQ-----ASSTIRY 55
Rattus UCP2     MVGFKATDLPPTATVKFLGAGTAACIADLITFPLDTAKVRLQIQGESQGLARTAAS-AQY 59
Rattus UCP3     ----------PTTVVKFLGAGTAACFADLLTFPLDTAKVRLQIQGE-----NPGVQSVQY 45
Gallus gallus   MVGLKPPEVPPTAAVKFFSAGTAACIADLCTFPLDTAKVRLQIQGEVR--IPRSTNTVEY 58
Danio rerio     MVGFRAGDVPPTATVKFIGAGTAACIADLFTFPLDTAKVRLQIQGENKASTNMGRGPVKY 60
Xenopus laevis  MVGLKPSDIPPTPAVKFIGAGTAACIADLFTFPLDTAKVRLQIQGETTG--SAAVNGIRY 58
                                                 * ** ****          *

RepUCP          KGALGTITTMVRTEGPKSLYNGLVAGLQRQMSFASIRIGLYDSVKQFYTPKGSESASIPT 81
Rattus UCP1     KGVLGTITTLAKTEGLPKLYSGLPAGIQRQISFASLRIGLYDTVQEYFSSGRETPASLGS 115
Rattus UCP2     RGVLGTILTMVRTEGPRSLYNGLVAGLQRQMSFASVRIGLYDSVKQFYT-KGSEHAGIGS 118
Rattus UCP3     RGVLGTILTMVRTEGPRSPYSGLVAGLHRQMSFASIRIGLYDSVKQFYTPKGTDHSSVAI 105
Gallus gallus   RGVLGTLSTMVRTEGPRSLYSGLVAGLQRQMSFASIRIGLYDSVKQLYTPKGAESTGLLA 118
Danio rerio     RGVFGTISTMVRVEGPRSLYSGLVAGLQRQMSFASVRIGLYDSVKQFYT-KGSDHAGIGS 119
Xenopus laevis  KGVFGTLSTIVKTEGPKSLYNGLVAGLQRQMSFASIRIGLYDTVKLFYT-NGKEKAGIGS 117
                 *  **  *    **    * ** **  ** **** ****** *

RepUCP          RLLAGCTTGAMAVTCAQPTDVVKVRFQAHIRL-VGGPKKYNGTVDAYKTIAREEGVRGLW 140
Rattus UCP1     KISAGLMTGGVAVFIGQPTEVVKVRMQAQSHL-HGIKPRYTGTYNAYRVIATTESLSTLW 174
Rattus UCP2     RLLAGSTTGALAVAVAQPTDVVKVRFQAQARA--GGGRRYQSTVEAYKTIAREEGIRGLW 176
Rattus UCP3     RILAGCTTGAMAVTCAQPTDVVKVRFQAMIRLGTGGERKYRGTMDAYRTIAREEGVRGLW 165
Gallus gallus   RLLAGCTTGAVAVTCAQPTDVVKVRFQALGAL-PESNRRYSGTVDAYRTIAREEGVRGLW 177
Danio rerio     RLMAGCTTGAMAVAVAQPTDVVKVRFQAQVSA--GSSKRYHSTMDAYRTIAKEEGFRGLW 177
Xenopus laevis  RILAGCTTGALAVTVAQPTDVVKVRFQAQANL-HGVKKRYNGTMDAYKTIAKKEGIKGLW 176
                   **  **  **   *** ***** **           *  *  **  **  *    **

RepUCP          KGTLPNIIRNSIVNCGEMVTYDLVKETLLRYHLMTDNFPCHFVAAFGAGFCATIVASPVD 200
Rattus UCP1     KGTTPNLMRNVIINCTELVTYDLMKGALVNHHILADDVPCHLLSALVAGFCTTLLASPVD 234
Rattus UCP2     KGTSPNVARNAIVNCTELVTYDLIKDTLLKANLMTDDLPCHFTSAFGAGFCTTVIASPVD 236
Rattus UCP3     KGTWPNITRNAIVNCAEMVTYDIIKEKLLDSHLFTDNFPCHFVSAFGAGFCATVVASPVD 225
Gallus gallus   RGTLPNIARNSIINCGELVTYDLIKDTLLRAQLMTDNVPCHFVAAFGAGFCATVVASPVD 237
Danio rerio     KGTGPNITRNAIVNCTELVTYDLIKDALLKSSLMTDDLPCHFTSAFGAGFCTTIIASPVD 237
Xenopus laevis  KGTFPNVTRNAIVNCTELVTYDLIKENLLHHKLMTDNLPCHFVSAFGAGFCTTVIASPVD 236
                 ** **  ** * ** * **** *  *  *       *  ***   *  **** *  *****

RepUCP          VVKTRYMNSIPGQYKNALNCMFTMVVKEGPTAFYKGFIPSFLRLGS-------------- 246
Rattus UCP1     VVKTRFINSLPGQYPSVPSCAMTMYTKEGPAAFFKGFAPSFLRLGSWNVIMFVCFEQLKK 294
Rattus UCP2     VVKTRYMNSALGQYHSAGHCALTMLRKEGPRAFYKGFMPSFLRLGSWNVVMFVTYEQLKR 296
Rattus UCP3     VVKTRYMNAPPGRYRSPLHCMLRMVAQEGPTAFYKGFMPSFLRLGSWNVMMFVTYEQLKR 285
Gallus gallus   VVKTRYMNASPGQYRNVPSCLLALLLQDGIAGLYKGFVPSFLRLGSWNVVMFISYDQLQR 297
Danio rerio     VVKTRYMNSAQGQYSSALNCAVAMLTKEGPKAFYKGFMPSFLRLGSWNVVMFVTYEQLKR 297
Xenopus laevis  VVKTRYMNSPPGQYKSALNCAWTMITKEGPTAFYKGFVPSFLRLGSWNVVMFVSYEQLKR 296
                *****  *   * *     *        *     *** ********
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Fig. 1. Partial predicted amino acid sequence and alignment of repUCP with other homologues of mammalian UCPs. The asterisks indicate amino acid preserved in all presented UCPs. A black background indicates the nucleotides that are essential for GDP binding (Arg^{83}, Arg^{182} and $Hist^{214}$) and that they are present in all the mammalian UCP homologues.

thawing increased significantly repUCP mRNA (2.6- and 2.1-fold increase, respectively).

O_2 consumption of muscle mitochondria from control lizards

We first examined the functioning of the lizard muscle mitochondria using succinate as substrate (state 4). Addition of ADP to the respiratory medium leads to oxidative phosphorylation respiration (active state 3=24.4±2.4 natomO min^{-1} mg^{-1} protein). Addition of oligomycin, a specific inhibitor of F0-F1 ATPase, allowed the measurement of basal O_2 consumption (state 4+=14.9± 0.6 natomO min^{-1} mg^{-1} protein), a respiration not coupled to ATP synthesis. This non-phosphorylating state reflects energy wastage that is the consequence of proton leakage through the inner mitochondrial membrane. Proton leakage, which partially uncouples phosphorylation from oxidation, can be regulated through inducible UCP-dependent processes (Echtay et al., 2002; Stuart et al., 2001; Talbot et al., 2004). The presence and activity of such a protein in the membrane of lizard muscle mitochondria were tested using its potent inhibition by purine nucleoside diphosphates (GDP). Indeed, despite differences between lower vertebrates and mammals, the response of proton conductance to GDP might have been conserved during evolution thanks to three specific nucleotides [Arg^{83}, Arg^{182} and His^{214} (Modriansky et al., 1997)]. It appears that these three nucleotides are conserved in all the UCP isoforms shown in Fig. 1, providing arguments that the site of GDP binding is conserved in a number of taxa including the reptiles.

Fig. 4 and Table 2 show that state 4 respiration was inhibited by addition of 2 mmol l^{-1} GDP to the mitochondrial suspension (–24%), which suggests the presence and the functionality of an uncoupling protein (repUCP) in lizard muscle mitochondria. However, a recent study showed that GDP could also interact with the adenine nucletide transporter (ANT) (Brand et al., 2005). We therefore tested the effect of an ANT inhibitor (30 μmol l^{-1} atractylate) and checked that it had no significant effect on state 4 respiration rate (N=4; paired t-test, t=–0.64; d.f.=3.1; P>0.05). Therefore, the reduction of state 4 after addition of GDP presumably results totally from repUCP inhibition.

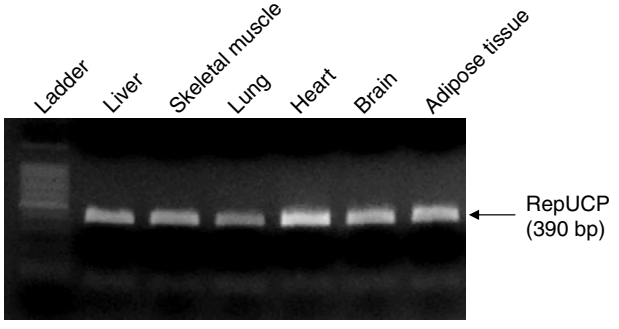

Fig. 2. Tissue distribution of repUCP mRNA (390 bp) in *Lacerta vivipara* after 32 cycles of PCR. Lane 1 is a 100 pb ladder, lanes 2–7 show expression of reptilian UCP in liver, skeletal muscle, lung, heart, brain and adipose tissue, respectively, from one representative individual.

H_2O_2 production of muscle mitochondria from control lizard

The mitochondrion is known to be an important source of ROS production (Boveris and Chance, 1973; Nohl, 1994). This production depends on many factors such as respiratory rate, redox status of the respiratory complexes (Herrero and Barja, 1998) and the mitochondrial membrane potential (Papa and Skulachev, 1997).

The rate of H_2O_2 production by mitochondria, energized with succinate, was clearly dependent upon a reverse electron flow from complex II toward complex I, since it was dramatically inhibited by rotenone (–67%; data not shown). Addition of GDP on state 4 respiration was followed by a significant increase in mitochondrial H_2O_2 production (+22%; expressed as pmol H_2O_2 min^{-1} mg^{-1} mitochondrial proteins; Table 2). This suggests that inhibition of repUCP by GDP might enhances the mitochondrial membrane

potential, amplify the reverse electron flow through complex I and thus increase mitochondrial H_2O_2 production. As H_2O_2 production and O_2 consumption were measured in the same conditions (buffer, substrate concentration, temperature), we calculated the fraction of O_2 turned into H_2O_2 instead of being reduced to produce water (H_2O_2 production/O_2 consumption), with the result that H_2O_2 released per unit of oxygen consumed was significantly higher in the presence of GDP (+36%; Table 2), indicating that the uncoupling effect of repUCP contributes to limiting mitochondrial ROS production.

DISCUSSION

Early studies demonstrated the occurrence of UCP in a number of taxa but the data presented here provides (1) the first cDNA sequence of a UCP homologue protein from a reptile exhibiting high homology with UCP sequences (see Fig. 1), (2) the relative expression of this sequence in several tissues of *Lacerta vivipara*, (3) evidence that the expression of this sequence is influenced by environmental temperature and its resulting physiological states and (4) indications that this protein should be operative in lizard mitochondria.

Even if mRNA levels do not correspond perfectly with protein activity because of transcriptional regulation (Pecqueur et al., 2001), the discovery of a new uncoupling protein in a reptile provides new elements to the debate regarding the physiological role of these proteins. Indeed, if initial studies argued for a general function in thermogenesis (Enerback et al., 1997; Gong et al., 1997; Vidal-Puig et al., 2000), recent work renders the situation less clear, even in mammals (Harper et al., 2001; Krauss et al., 2003; Trenker et al., 2007).

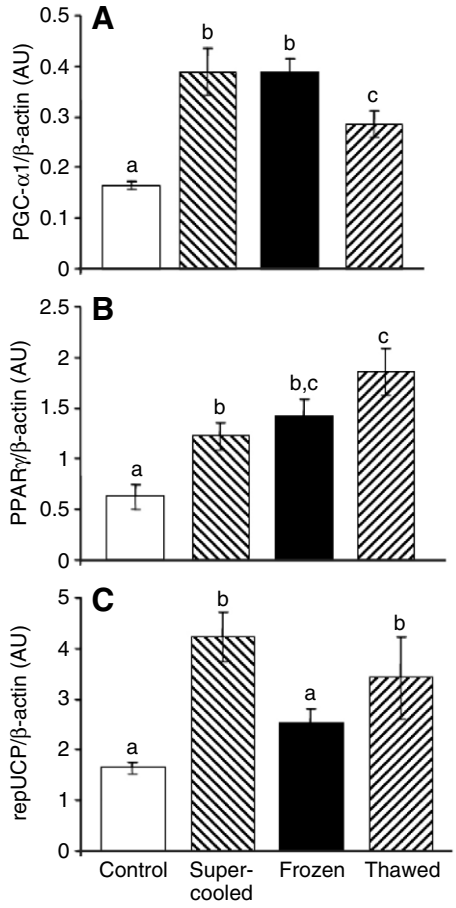

Fig. 3. RT-PCR analysis of (A) PGC-1α, (B) PPARγ and (C) reptilian UCP mRNA levels in muscle from controls, supercooled, frozen and thawed lizards (*Lacerta vivipara*). For details, see Materials and methods. β-actin was used as an invariant gene. Values are means ± s.e.m. for eight lizards in each group. Different letters indicate a significant difference (P<0.05).

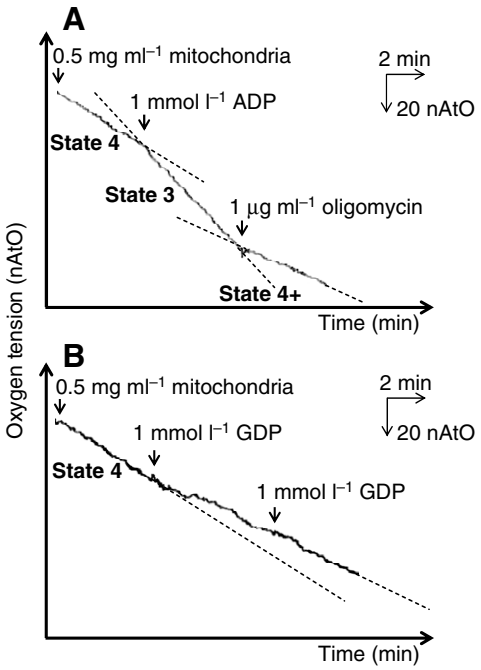

Fig. 4. Representative experimental traces of oxygen consumption by muscle mitochondria from control lizard energised with succinate (for details, see Materials and methods). (A) Mitochondrial state 4, state 3 (addition of 1 mmol l^{-1} ADP) and state 4+ (addition of 1 μg ml^{-1} oligomycin). (B) Effect of two sequential additions of 1 mmol l^{-1} GDP on state 4 mitochondrial oxygen consumption.

Based on data from literature, we first compared the activation pathway of UCP expression in reptiles and other animals, and secondly considered two possible physiological functions.

FFAs, PGC-1 and PPARs in reptiles at subzero temperatures

Several reports have proposed that, *in vivo*, fatty acids induce UCP gene expression in skeletal muscles. In rodents, situations resulting in high circulating free fatty acid (FFA) levels (such as starvation and cold exposure) are associated with upregulation of UCP3 mRNA expression (Schrauwen et al., 2006), but its expression is low when the FFA levels decrease, as in lactation (Pedraza et al., 2000).

Accumulating evidence also indicates that fatty acids may act through peroxisome proliferator-activated receptors (PPAR) to induce UCP gene expression given that in mammals the UCP promoter contains PPAR-responsive elements (Solanes et al., 2003; Son et al., 2001). Since FFAs are potent ligands for PPAR, it has been suggested that the upregulation of UCP caused by fasting and other stress conditions could be explained by increased FFA levels. Once activated by their respective ligands, PPARs control the transcriptional rate of a large panel of genes controlling notably lipid and glucose metabolism (Luquet et al., 2004; Son et al., 2001).

PPAR activity is regulated by a coactivator (PGC-1; peroxisome proliferator-activated receptor gamma coactivator-1), originally identified as a transcriptional coactivator of the nuclear receptor PPARγ, which regulates the activity of several nuclear receptors

(Puigserver and Spiegelman, 2003). PGC-1α mRNA expression is dramatically induced in both brown fat and skeletal muscle by exposure of animals to cold, and regulates biogenesis and several key mitochondrial genes, such as UCPs, which contribute to energy metabolism regulation and the program of adaptive thermogenesis (Puigserver et al., 1998). The data presented here, correlating cold-induced increase of blood FFAs levels (8.0±2.5 and 16.4±4.8 mmol l^{-1} in control and supercooled lizards, respectively; data not shown), PGC-1α and PPARγ expression with repUCP mRNA expression in lizards, are thus totally congruent with other results from the literature on mammals and suggest a strong conservation through evolution of this activation pathway.

Table 2. Effect of guanosine diphosphate on state 4 mitochondrial oxygen consumption and H_2O_2 production in lizard muscle mitochondria

State 4	*N*	–GDP	+GDP	% Variation	*P*
J_{O_2} (natomO min^{-1} mg^{-1} protein)	8	13.7±0.7	10.4±0.9	–23.9±1.9	0.02
H_2O_2 production					
(pmol H_2O_2 min^{-1} mg^{-1} protein)	12	390.1±81.9	452.1±88.2	+21.7±7.3	0.01
(pmol H_2O_2 natomO^{-1})	8	23.0±3.9	30.4±5.1	+35.9±10.5	0.002

GDP, guanosine diphosphate

Oxygen consumption (J_{O_2}) and H_2O_2 production were measured in same respiratory medium at 25°C as described in the Material and methods. Values are means ± s.e.m.; *N*, number of independent mitochondrial preparations.

RepUCP as a fatty acid oxidation inducer?

Since overwintering lizards are exposed to the cold and are aphagic for a few months, the increased FFA present in the blood and the subsequent PGC-1, PPAR and repUCP overexpression observed in the present study are clearly a physiological response to sub-zero temperatures. It is generally acknowledged that mammalian UCPs play some role in energy metabolism during situations in which fatty acid oxidation is high. Several studies show a correlation between overexpression of UCP and stimulation of fatty acid oxidation (Bezaire et al., 2001). In this context, the physiological role of UCP might be to export fatty acid anions from the matrix, thereby preventing the accumulation of fatty acid anions inside the matrix (Schrauwen et al., 2001) and protect mitochondria against the detrimental effects of high fatty acid accumulation (lipotoxicity).

Furthermore, some studies have shown that UCP overexpression in cultured human muscle cells enhanced fatty acid-dependent inhibition of glucose oxidation and therefore it has been proposed that UCP could be involved in a nutrient partitioning process favouring the use of fatty acid over that of glucose. Moreover, the switching of muscle substrate metabolism to a state of enhanced lipid utilization during starvation (when glucose is limiting) suggests UCP could play a role in glucose preservation (Garcia-Martinez et al., 2001). In view of these findings and in light of the cold hardiness framework, we can hypothesize that under sub-zero temperatures *Lacerta vivipara* improve their use of lipids as a substrate that will enable them to preserve glucose for its cryoprotective properties. This hypothesis is strengthened by the increased level of plasma glucose detected in overwintering European common lizards during supercooling and freezing (Voituron et al., 2002a), but clearly needs further investigation.

RepUCP as mitochondrial free radical regulator?

The increased expression of repUCP can also suggest that these proteins play an antioxidant role. Indeed, the majority of ROS are generated by the mitochondrial electron transport chain (Nohl, 1994; St-Pierre et al., 2002) and lead to oxidative stress when an imbalance is observed between the ROS generation and the detoxification processes. Re-warming tissues after a period of cold exposure corresponds to a physiological situation comparable with stressful oxidative situations of tissue ischemia-reperfusion (Hermes-Lima and Zenteno-Savin, 2002). For instance, freezing exposure stimulates antioxidant defences against the overproduction of ROS in muscle and lung in the grater snake (*Thamnophis sirtalis*) (for a review, see Storey, 1996). The wood frog (*Rana sylvatica*), a model for vertebrate freeze tolerance, displays considerably higher antioxidant defences in its tissues than the freeze-intolerant leopard frog (*Rana pipiens*) (Storey, 1996). Surprisingly, data from the literature show low ROS-related damage in frozen–thawed lizards despite low variations in antioxidant defences (Voituron et al., 2006). The possible antioxidant function of the reptilian UCP might thus play significant role in the biology of reptiles in cold conditions.

Even if the main function of repUCP remains to be established, its catalytic activity may causes a 'mild uncoupling' and may lower ROS generation by decreasing the reduced state of the mitochondrial respiratory chain as proposed for mammalian UCP2 and UCP3 (Lambert and Brand, 2004; Papa and Skulachev, 1997). At this early point, we demonstrate an inhibition of mitochondrial non phosphorylating respiration (state 4) by GDP, providing arguments that a mild uncoupling requiring repUCP occurs in reptile mitochondria and presumably lowers the proton gradient limiting the ROS generation of mitochondria. This hypothesis is supported by the fact that inhibition of repUCP by GDP increased the endogenous mitochondrial production of H_2O_2.

Towards a 'global' cold hardiness strategy in *Lacerta vivipara*

The two hypotheses proposed above for the role of repUCP (fatty acid oxidation inducer and antioxidant role) appear complementary rather than antagonistic. Indeed, *Laceta vivipara* under sub-zero temperatures probably (1) preserves glucose for its cryoprotective functions by using fatty acids as substrate and (2) avoids oxidative damage by preventing generation of large amounts of ROS.

These data (antioxidant defences and increase in repUCP expression, especially during supercooling and not freezing) together with those focused on the aerobic metabolism in both supercooling and freezing states suggest a 'global' strategy in *L. vivipara* under sub-zero temperatures. Indeed, previous studies on this species suggested an activation of aerobic metabolic pathways between 0.5 and –1.5°C (Voituron et al., 2002b) allowing the synthesis of different metabolites that probably play a role in the two cold-hardiness strategies of *L. vivipara* (freeze tolerance and freeze avoidance) (Costanzo et al., 1995). Thus it seems that an adequate cryoprotective system is activated before the animal reaches its crystallization temperature. The fact that freezing by itself does induce antioxidant defences (Voituron et al., 2006) together with the fact that after 20 h of freezing UCP expression is not different from that measured in controls, suggests that a freezing without supercooling will not lead to optimal cryoprotection. If these speculations are accurate, such a mechanism would have evolved in *Lacerta vivipara* in order to cope with the extreme conditions present in their environment, and needs to be further investigated in future studies.

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