The lipid peroxidation product 4-hydroxynonenal (HNE) is an important mediator of free radical damage [1]. HNE specifically induces uncoupling of mitochondria through the uncoupling proteins (UCPs) and the adenine nucleotide translocase (ANT) although the relative contribution of the two carriers to these effects is unclear [2,3]. To clarify this we studied the sensitivity of HNE-activated proton conductance to GDP (UCPs inhibitor) and carboxyatractylate (ANT inhibitor) in skeletal-muscle and heart mitochondria from mice expressing different amounts of UCP3. Mitochondria were isolated from wild-type and *Ucp3* knockout mice. To increase UCP3 expression, some mice were i.p. injected with LPS (12 mg/kg body weight). HNE activated proton conductance in skeletal-muscle and heart mitochondria. In skeletal muscle, this increase correlated with UCP3 expression levels: it was lower in *Ucp3* knockout mice and higher in LPS-treated wild-type mice. GDP partially abolished HNE effects whereas carboxyatractylate or addition of both inhibitors completely abolished it. In contrast, GDP had no effect on HNE-induced proton conductance in heart mitochondria, but carboxyatractylate or administration of both inhibitors had a partial effect. In skeletal muscle mitochondria, GDP-mediated inhibition of HNE-activated proton conductance was specific for UCP3 since it was not observed in *Ucp3* knockout mice. Carboxyatractylate was able to inhibit UCP3 through an unknown mechanism. We conclude that, in skeletal muscle, HNE-induced increase in proton conductance is mediated by UCP3 (30%) and ANT, whereas in the heart the increase is mediated by ANT and other carriers, possibly including UCP3.

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10P.3 Regulation of H_2O_2 generation by uncoupling protein 1 in thymus mitochondria

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It has been proposed that uncoupling proteins can attenuate mitochondrial production of free radicals and therefore protect against oxidative damage, degenerative diseases and aging. Recently it has been established that uncoupling protein 1 (UCP1) is located in the thymus. We tested a hypothesis that UCP1 can regulate ROS production in rat mitochondria from thymus using an Amplex red/H_2O_2 assay. Our data show that inhibition of UCP1 by GDP caused an increase in ROS production by non-phosphorylating thymus mitochondria respiring on a) succinate and rotenone (1.5 fold increase) and b) glycerol-3-phosphate and rotenone (1.2 fold increase). In parallel with H_2O_2 production measurements, the effect of GDP on membrane potential was monitored by uptake of the fluorescent probe safranine, while the inhibitory effect of GDP on oxygen consumption was measured using an oxygraph respirometer. The observed increase in ROS production upon GDP addition was accompanied by a relative decrease in oxygen consumption. We are currently performing equivalent experiments using thymus mitochondria isolated from *UCP1*$^{-/-}$ mice and their control littermates, to establish that our observations are indeed due to UCP1 activity in the thymus.

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10P.4 Are the novel uncoupling proteins acutely regulated by fatty acids and nucleotides?

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In brown adipose tissue (BAT) mitochondria, uncoupling protein 1 (UCP1) dissipates the protonmotive force to generate heat. UCP1 is activated by fatty acids and inhibited by nucleotides such as GDP, but the precise mechanisms involved remain controversial. Even less is known about the physiological role and regulation of the novel uncoupling proteins, UCP2 and UCP3. Here we present the first demonstration of a conformational change induced by fatty acids for both UCP1 and UCP3 in rat BAT mitochondria. Conformational changes were inferred from the kinetics of proteolysis when isolated mitochondria were treated with exogenous trypsin. Palmitate increased the rate of proteolysis for both proteins, showing that palmitate binds and affects their conformation. Trypsinolysis of UCP1 could be fully rescued by GDP, consistent with its ability to compete functionally with fatty acids. UCP3 degradation, however, was GDP-independent, suggesting that GDP interacts differently (or not at all) with UCP3. Experiments to determine the acute regulation of UCP2 as diagnosed by trypsinolysis are currently being conducted.

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10P.5 *In vivo* gene silencing of uncoupling protein-2 in kidney cortex of diabetic rats results in increased uncoupling, decreased oxidative stress and reduced membrane potential Implications for the adenine nucleotide transporter

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Mitochondria uncoupling *via* uncoupling protein (UCP) 2 may be an important mechanism to reduce oxidative stress and preserve mitochondria function in the diabetic kidney. Short interference (si) RNA to knockdown UCP2 or a non-functional scrambled equivalent (100 μg/rat) was administered to healthy and diabetic (streptozotocin; 60 mg/kg b.w.) Sprague–Dawley rats and the mitochondria isolated 48 h thereafter. Glutamate-stimulated QO_2 in the presence of ATP-synthase inhibitor oligomycin was used to estimate mitochondria uncoupling since proton release across over the inner membrane with subsequent increase in QO_2 will not occur in coupled mitochondria. Diabetes increased UCP2 (192 ± 34% of control corrected for β-actin) and siRNA decreased UCP2 (control + siRNA 70 ± 11 and diabetes + siRNA 88 ± 8% of control). Glutamate-stimulated QO_2 was significantly higher in control + siRNA, untreated diabetics and diabetes + siRNA compared to control (1.6 ± 0.5, 1.5 ± 0.5 and 3.8 ± 0.5 vs. 0.0 ± 0.1 nmol O_2/s/mg protein). The UCP2 inhibitor guanosine diphosphate (GDP) inhibited QO_2 in diabetics (0.9 ± 0.5 vs. 1.6 ± 0.5 nmol O_2/s/mg protein) but not in diabetes + siRNA (4.6 ± 0.6 vs. 3.8 ± 0.5 nmol O_2/s/mg protein). ADP in the presence of oligomycin reduced QO_2 in diabetes + siRNA (2.0 ± 0.5 vs. 3.8 ± 0.5 nmol O_2/s/mg protein) but had no effect in any of the other

groups. Uptake of tetramethylrhodamine methyl ester (TMRM), measurement of membrane potential, was decreased in administration of siRNA in both groups (control − 14% and diabetes − 36% compared to corresponding untreated group). GDP only increased the membrane potential in untreated diabetics. Thiobarbituric acid reactive substances (TBARS), marker of oxidative stress, were increased in kidney cortex of control + siRNA and untreated diabetics compared to controls (both + 220%), and siRNA given to diabetics completely normalized the TBARS levels. Paradoxically, *in vivo* gene silencing of UCP-2 increases the mitochondria uncoupling in both control and diabetic animals, measured both as glutamate-stimulated QO_2 and altered membrane potential in the absence of a functional ATP production. However, this uncoupling is not inhibited by GDP, excluding a role of UCP2. Excessive amounts of ADP in the absence of a functional ATP production (inhibited by oligomycin) results in reduced uncoupling, indicating an uncoupling role of the adenine nucleotide transporter in the absence UCP2 uncoupling in both control and diabetic kidneys.

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10P.6 Cardioprotective activity of dehydrosilybin is linked to its uncoupler-like behavior

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Uncoupling protein 2 (UCP2) is a carrier protein located in the inner mitochondrial membrane, which is thought to confer tolerance of cardiac myocytes to oxidative stress *via* diminished mitochondrial Ca^{2+} overload, reduced generation of ROS by mild uncoupling of mitochondrial respiration, and perhaps other means. Silibinin, also known as silybin (SB), is the major active constituent of silymarin, the mixture of flavonolignans extracted from seeds of milk thistle (*Silybum marianum*). It is used in the treatment and prevention of liver diseases because of its hepatoprotective (antihepatotoxic) properties. We used silybin and its derivative, 2,3-dehydrosilybin (DHSB), to evaluate their effect on UCP2 expression and mitochondrial bioenergetics in neonatal rat cardiomyocytes. Both of the compounds affected thyroid hormone-mediated UCP2 up-regulation possibly attenuating the cardioprotective effects of UCP2. Investigating the effect on UCP2 level, we noted the ability of DHSB to de-energize the cells by monitoring JC-1 fluorescence. The same phenomenon was confirmed in isolated rat heart mitochondria. Further experiments evaluating oxygen consumption revealed that DHSB uncouples the respiration of isolated rat heart mitochondria albeit with much lower potency than FCCP. Furthermore, DHSB showed very high potency to suppress ROS formation in isolated rat heart mitochondria with $IC_{50} = 1.5$ μM. It is more effective than its effect in a purely chemical system generating superoxide or in cells capable of oxidative burst. DHSB also attenuated ROS formation caused by 7 μM rotenone in primary cultures of neonatal rat cardiomyocytes. We infer that the apparent uncoupler-like activity of DHSB is the basis of its ROS modulation effect in neonatal rat cardiomyocytes and may also be the culprit in DHSB diminishing the thyroid hormone-mediated UCP2 up-regulation.

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10P.7 Nitrolinoleic acid is a cycling substrate of mitochondrial uncoupling protein UCP2

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Nitrated derivatives of fatty acids (FA-NO_2) are pluripotent cell-signaling mediators that exhibit anti-inflammatory properties. Experimental evidence suggests that nitrolinoleic acid (LNO_2) stimulates mitochondrial uncoupling *via* adenine nucleotide translocase (ANT) and UCP2-dependent mechanism and that LNO_2 nitroalkylates ANT and possibly UCP2 (Nadtochiy SM *et al.*, 2009, *Cardiovasc Res.* **82**, 333–340). Here we tested the hypothesis that LNO_2 acid induces the UCP2-mediated proton transport and that LNO_2 is transported *via* UCP2 by the FA-protonophore cycling mechanism. Using protein-free liposomes, we determined that the addition of synthesized LNO_2 caused fast acidification of liposomal interior, indicating spontaneous transport of protonated LNO_2 across the phospholipid bilayer. Using *E. coli*-expressed UCP2 reconstituted into liposomes, we found that LNO_2 induced purine nucleotide-sensitive H^+ uniport. In parallel experiments, LNO_2 induced K^+ influx balanced by anionic charge transfer in UCP2-proteoliposomes, indicating that LNO_2 was transported as an anion. Additional experiments using isolated lung mitochondria from *Ucp2*-WT and *Ucp2*-KO mice verified UCP2-dependent, LNO_2-induced uncoupling. Taken together, these data are consistent with LNO_2 being transported by UCP2 as a cycling substrate by the FA-protonophore mechanism. The data further indicate an active role of UCP2 in mediating the FA-NO_2 signal transduction.

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10P.8 Investigation into the role of uncoupling protein 3 in the thymus and spleen

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UCP3 is predominantly associated with skeletal muscle and brown adipose tissue (BAT). Our laboratory has also demonstrated the presence of UCP3 protein in thymus and spleen mitochondria of rats [1]. The aim of this study was to determine the level of apoptosis in cells isolated from these tissues and whether the absence of UCP3 affected the degree of apoptosis. To that end, we utilized propidium iodide and annexin V staining of thymocytes and splenocytes from wild type and *Ucp3* knockout ($UCP3^{-/-}$) mice followed by FACS analysis to determine the levels of apoptosis. Thymocytes and splenocytes were treated for 18 h with dexamethasone (0.1 μM) or vehicle (EtOH, to measure spontaneous apoptosis). We were able to detect a significant increase in spontaneous apoptosis in thymocytes isolated from $UCP3^{-/-}$ mice compared to those from wild type mice (33% increase, $p = 0.04$) We detected no significant difference in spontaneous apoptosis in splenocytes or in apoptosis due to dexamethsone in splenocytes and thymocytes in a comparison of $UCP3^{-/-}$ and wild-type mice. These preliminary data suggest that UCP3 plays a role in protection against apoptosis in thymocytes but not in splenocytes.

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