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Brain mitochondrial uncoupling protein 2 (UCP2): a protective stress signal in neuronal injury

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Abstract

Mitochondrial uncoupling proteins (UCPs) can dissociate oxidative phosphorylation from respiration, and they appear to be critical for energy balance. One of these proteins, UCP2, is also expressed in neurons of subcortical brain regions of healthy subjects. Here, we report on the protective role of UCP2 in brain injury by revealing its early induction after lesions and its inverse relationship with activation of an apoptotic signal, caspase 3, in wild-type and *UCP2* overexpressing transgenic mice.

Keywords: Mitochondrial uncoupling proteins; Brain injury; Neuronal degeneration; Microglia; Hippocampus

1. Introduction

UCPs are located in the inner membrane of the mitochondria, and one of their main functions is to leak hydrogen ions from the intermembrane space to the matrix, thereby; dissipating the proton gradient energy and reducing the production of free radicals [1–7]. To date, most of the attention given to UCPs has been to their role in peripheral energy metabolism, where their energy-dissipating function has been explored primarily in the context of metabolic disorders [1–5]. One of these proteins, UCP2, is expressed in a variety of tissues, including subcortical brain structures and the immune system [3,8–10]. Interestingly, in the healthy brain, UCP2 is expressed selectively in neuronal populations of the central nervous system that are responsible for the minute-by-minute regulation of homeostasis and represent fast firing neurons under the influence of massive neuronal and humoral regulation [9,10]. We sought to explore the function of UCP2 in the central nervous system and its involvement in the course of brain injury, particularly, since UCP2 has been shown to affect the production of free radicals [6,7], molecules that have been linked in a causal manner to neurodegenerative processes [11]. We studied rats and mice using an acute neurodegeneration model, the entorhinal cortex lesion paradigm. The entorhinal cortex is connected to the hippocampus via the perforant path and is critical for spatial memory and learning. Stereotaxic transection of this pathway induces anterograde and retrograde degeneration accompanied by glial cell activation followed by reactive synaptogenesis [12,13].

2. Materials and methods

2.1. Animals

Adult male Wistar rats (290–310 g) and C57BL6/6J and *UCP2/UCP3* overexpressing male C57BL6/6J mice (30–35 g), housed under standard laboratory conditions, were

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Abbreviations: UCP, uncoupling protein; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3; PB, phosphate buffer

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used for this study. All experimental procedures were conducted in accordance with approved institutional animal care protocols from both Yale and Humboldt Universities.

2.2. hUCP2 and hUCP3 expressing transgenic animals

Transgenic animals were produced using an 80-kb bacterial artificial chromosome containing the human *UCP2* and *UCP3* genes to construct an overexpressing transgenic mouse line. The transgene comprised the *UCP2* and *UCP3* genes, which are adjacent on chromosome 11, with their native promoters and *cis*-acting elements. Detailed descriptions of the production of these animals and their phenotypic characterization can be found elsewhere [14]. For these experiments, heterozygous mice of N6 or higher backcross generation and their wild-type littermates were used.

2.3. Entorhinal cortex lesion

Stereotaxic surgery was performed under deep ketamine/xylazine anesthesia. In all animals, the right medial entorhinal cortex was lesioned using a 2-mm broad knife for rats and a 1.5-mm broad knife for mice. The medial edge of the knife was adjusted to the following coordinates as measured from the lambda: anterior-posterior 1.2 mm, lateral 3.0 mm, dorsoventral down to the base of the skull for rats and anterior-posterior 0.8 mm, lateral 1.0 mm, dorsoventral down to the base of the skull for mice. Following entorhinal cortex lesion, animals were allowed to survive for either 24 or 48 hr. In all cases, care was taken to minimize any pain or discomfort to the animals.

2.4. Immunocytochemistry

Four experimental animals from each group (experimental and control rats, experimental and control mice) were killed. The animals were deeply anesthetized with dimethyl ether and transcardially perfused with 100 mL (20 mL for mice) of 0.9% NaCl followed by 500 mL (80 mL for mice) of a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) (0. 1 M PB). Brains were removed and post-fixed for several hours. Horizontal 60-μm sections of the brain were cut using a vibratome. The sections were washed several times in PB and stored at 4°. Free-floating sections were incubated for 15 min in PB containing 0.5% Triton X-100 and 10% normal serum of the species of the secondary antibody to block non-specific binding of the antibodies. The primary antibodies (rabbit anti-UCP2 [9,10], rabbit anti-activated caspase 3, rabbit anti-glial fibrillary acidic protein (GFAP), and mouse anti-OX-42—all from the Sigma Chemical Co.) were diluted in a dilution buffer (0.1 M PB) containing 0.5% Triton X-100 and 2% normal serum of the species of the secondary antibodies in 0.1 M PB. GFAP labels astrocytes, OX42 visualizes microglia (macrophages), and activated caspase 3 is a downstream inducer of apoptosis. Sections were incubated for 12 hr at room temperature in PB containing the primary antibodies. After washing the sections thoroughly with PB, the primary antibodies were visualized using either rhodamine- or fluorescein-coupled secondary antibodies. Primary antibodies were also detected using biotinylated secondaries and diaminobenzidine (DAB) as chromogen. Nonspecific binding of all secondary antibodies was tested by omitting the primary antibodies. Fluorescence microscopy was performed on an Olympus BX-50 microscope.

Narrow-band filters were used (U-MNIBA, U-MWIG) to guarantee the selectivity of fluorescent signals. Pictures were taken using either analog cameras (double-fluorescence) or an Olympus digital camera. The effect of UCP2 overexpression on apoptotic mechanisms was quantified by counting activated caspase 3-positive neurons in *UCP2* overexpressing ($N = 4$) and control mice ($N = 4$) at 48 hr post-lesion. An investigator blinded to the experimental groups counted five arbitrary fields (1 μm^2) around the lesion site in five sections from each animal. The mean numbers of immunopositive cells were compared between groups using a *t*-test. Differences were concluded significant if P values were below 0.05.

2.5. RT-PCR analysis of UCP2 mRNA expression

Total RNA was extracted from the lesioned and contralateral sides of mice, which included the entorhinal cortex and dentate gyrus as well as the hippocampus proper by the guanidine isothiocyanate-phenol-chloroform method using TRIzol reagent (Life Technologies) and transcribed using the First-Strand cDNA synthesis kit (Pharmacia). A fragment of 404 bp of UCP2 cDNA was amplified by RT-PCR using specific oligonucleotide primers derived from the coding region of the rat UCP2 sequence (5′-GTC GAA TTC TAC AAG ACC ATT GCA CGA-3′ and 5′-TGG GAT CCT CAT AGG TGA CAA ACA TTA-3′). As a control, a 603-bp cDNA of rat β-actin was amplified using the following primers: 5′-TAC AAC CTC CTT GCA GCT CC-3′ and 5′-GGA TCT TCA TGA GGT AGT CAG TC-3′. The PCR reaction was performed using the following protocol: 3 μg of cDNA templates reacted with 500 nM primers, 1.25 mM $MgCl_2$, 80 μM dNTP, and 2 U *Taq* DNA polymerase. Thermal profiles were 94° for 1 mm, 60° for 1 mm, and 72° for 1 mm for 30 cycles with a final 10-mm extension period.

2.6. Statistical analysis

Data were expressed as means ± SEM and the Kolgomorow-Smirnoff test was used to analyze the normal distribution. Statistical analysis was performed by ANOVA. Only groups from the same genetic background were compared, and a $P \leq 0.05$ was considered significant.

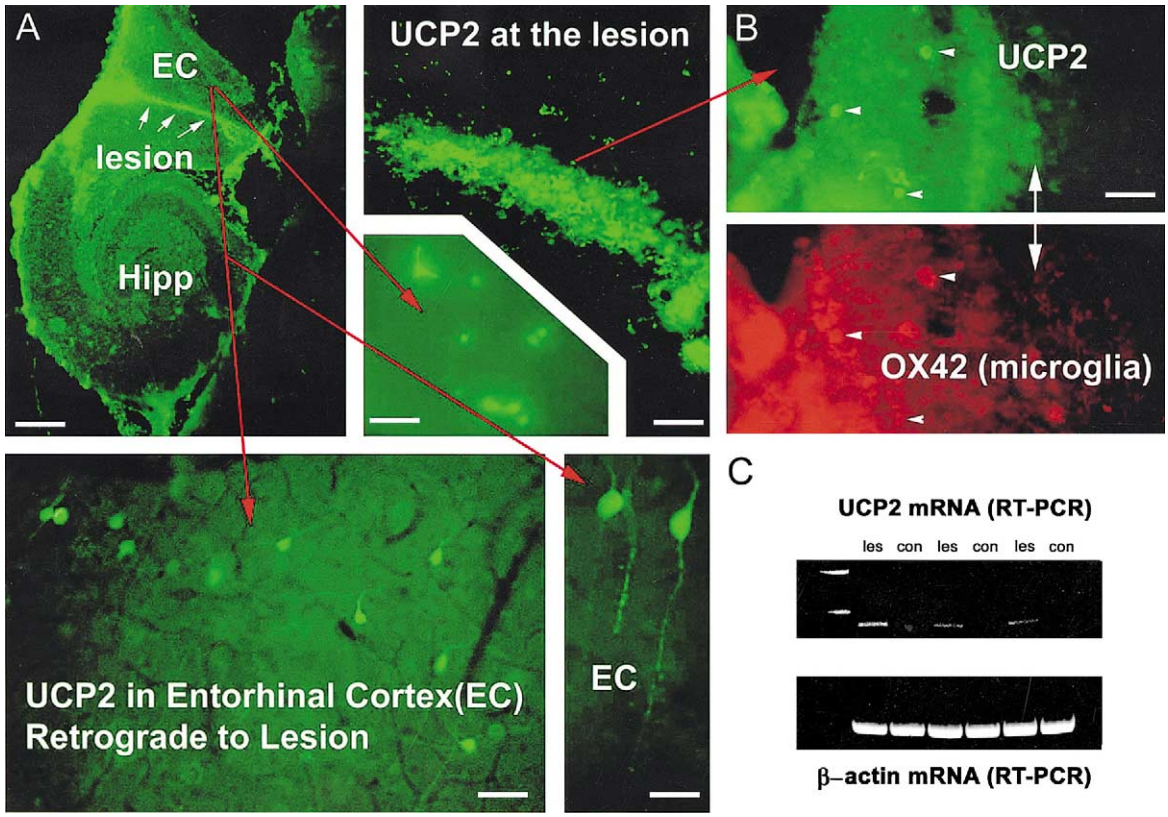

Fig. 1. Lesion-induced UCP2 expression. (A): A typical lesion of the entorhinal cortex is seen in the upper left panel. The bar scale represents 500 μm. The middle upper insert of panel A shows UCP2-imnmunopositive cells retrograde to the lesion resembling microglia cells. The bar scale represents 15 μm. The bottom panels of (A) show neurons of the entorhinal cortex retrograde to the lesion immunolabeled for UCP2. The bar scales on the lower left and right panels represent 30 and 15 μm, respectively. UCP2 immunolabeling was strong at the site of the lesion [upper right panel of (A); the bar scale represents 30 μm]. (B) At the site of the lesion, numerous UCP2-immunolabeled monocyte-like structures (green fluorescent profiles in the upper panel) were also immunolabeled for OX42, a macrophage marker (red fluorescent profiles on lower panel). Arrows indicate some of the double-labeled cells. The bar scale represents 30 μm. (C) RT-PCR analyses of the lesioned (les) and intact control (con) entorhinal cortex reveal a robust induction of UCP2 mRNA at the lesion site.

3. Results

In normal rat and mouse brains, immunocytochemistry for UCP2 resulted in labeled neurons and neuronal processes in a variety of basal brain structures corresponding to earlier descriptions [9,10]. UCP2-immunopositive profiles were sporadic in other areas, such as the cerebral cortex and the hippocampal formation. In the rat ($N = 5$) and mouse ($N = 4$) hippocampal formation, 24 hr after entorhinal lesion, strong UCP2 immunolabeling was present alongside the lesion (Fig. 1A and B). The immunolabeled cells in this region were small and round (Fig. 1B), resembling monocytes and microglia cells, and were identified as such using the microglia/macrophage marker OX-42 (Fig. 1B). In the zone of anterograde degeneration, i.e. the dentate gyrus and CA regions of the hippocampus, where microglia are known to be highly activated after entorhinal lesions [12,13], UCP2 induction was not observed. In the entorhinal cortex of both rats and mice, retrograde and ipsilateral to the lesion, neurons exhibited UCP2 immunoreactivity 24 hr after lesioning (Fig. 1A), and this signal was still present 48 hr post-lesion. While labeled cells could be found in different layers of the entorhinal cortex, most were located in layer 3. In control rats and mice, no UCP2 immunoreactive cells were detected in the entorhinal cortex.

To correlate the increased UCP2 protein expression in the hippocampal formation with increased transcription of UCP2 mRNA, we carried out mRNA analyses using the RT-PCR method on post-lesion day 2 ($N = 4$). Corresponding to the immunocytochemical findings, 48 hr after entorhinal lesioning, there was a robust induction of UCP2 mRNA at the site of the lesion, while contralateral to the lesion, UCP2 mRNA expression remained minimal (Fig. 1C).

To test the role that UCP2 plays in injured brain cells, we studied mice that overexpress human UCP2 in the brain and their wild-type littermates. We analyzed the pattern of activation of caspase 3 (Fig. 2), a downstream regulator of apoptosis after entorhinal lesions in these transgenic mice and their wild-type littermates. Activated caspase 3 immunopositive cells were not found in unlesioned animals (Fig. 2A). Strikingly, in *UCP2* overexpressing transgenic animals that underwent entorhinal lesioning, the mean number of neurons that were immunopositive for activated caspase 3 in the immediate vicinity of the lesion was significantly lower ($24 \pm 5/1\ mm^2$; $P < 0.05$) compared with the number of activated caspase 3-labeled cells

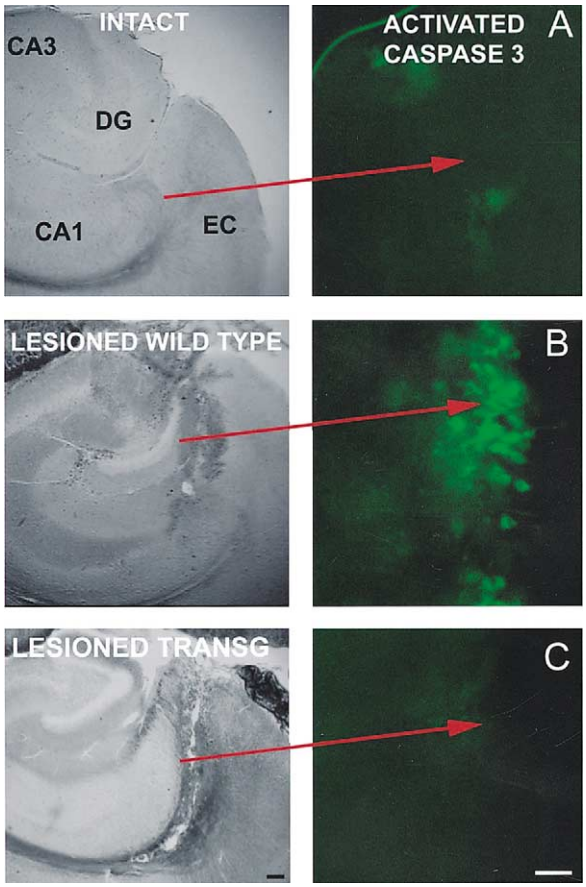

Fig. 2. Suppression of caspase 3 by UCP2. Black and white panels on the left show low power magnifications of the hippocampus of an intact (A), lesioned wild-type (B) and lesioned *UCP2* overexpressing animal (C). Lesioning of the entorhinal cortex resulted in activated caspase 3 immunoreactivity (green fluorescent images) in the vicinity of the lesions in wild-type mice 48 hr following surgery (left panel of B). However, in UCP2 overexpressing transgenic animals (C panels), the number of activated caspase 3-immunoreactive cells at the site of the lesion was significantly lower compared with that of the wild-type littermates. The bar scales on the C panels represent 100 μm on black and white inserts and 30 μm on fluorescent images.

found in wild-type control mice (168 ± 32/1 mm^2; Fig. 2B and C).

4. Discussion

These results demonstrated that UCP2 expression is induced during neurodegeneration at brain sites where it is not normally expressed. However, it could not be deciphered whether UCP2 promotes degeneration or protection of cells within the damaged area. A recent study revealed early induction of UCP2 by irradiation in non-neuronal cell lines vulnerable to x-ray-induced apoptosis [15]. Furthermore, collapse of the inner membrane potential of the mitochondria is thought to be one of the initiators of apoptotic cascades [16]. In fact, the lower number of activated caspase 3-immunoreactive neurons in the *UCP2* transgenic animals could have been the consequence of either accelerated or attenuated cell death. In support of attenuated cell death patterns, however, we found no evidence for decreased overall cell number in the transgenic animals nor did we observe colocalization of UCP2 and activated caspase 3 immunoreactivities (data not shown). In addition, electron microscopic analysis revealed no apoptotic chromatin segregation in neuronal nuclei of UCP2-containing cells, while this typical feature of apoptosis was readily visible in the cell nuclei of activated caspase 3-immunopositive cells (data not shown). Furthermore, the fact that the type of brain injury studied in the present experiments is accompanied by a robust increase in free radical production [12,13] that is suppressed by UCP2 [6,7] further argues that in the central nervous system; UCP2 may be neuroprotective, but a role for UCP3 cannot be ruled out.

The major finding of this study was that UCP2 is induced in subpopulations of neurons and microglia following brain lesions and that UCP2 expression levels are inversely correlated with caspase 3 activation. These observations lend support to the novel hypothesis that UCP2 is a cellular stress signal that, if up-regulated, may protect cells against degeneration that accompanies physiological processes such as aging or pathological mechanisms, including Alzheimer's, Huntington's, and Parkinson's Disease, hypoxia, ischemia, and temporal lobe epilepsy.

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