Induction of uncoupling protein (UCP) 2 in primary cultured hepatocytes

Kazuhiro Kimura*, Bae Dong Jung, Katsushi Kanehira, Yukiko Irie, Xavier Cañas, Masayuki Saito

Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan

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Abstract Uncoupling protein 2 (UCP2) mRNA expression and function was examined in rat primary cultured hepatocytes. UCP2 mRNA was not expressed in freshly isolated hepatocytes, but appeared during a 24–144 h primary culture period. Isolated mitochondria from 144 h cultured hepatocytes showed a lower oxygen consumption rate in the presence of succinate and ADP. However, the ratio of the oxygen consumption rate when media contained succinate alone to that with succinate and ADP was increased by 166% versus control mitochondria. Moreover, the mitochondrial potential in the presence of succinate was decreased by 60%, indicating the potential role of UCP2 in hepatocyte mitochondria as an active uncoupler.

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Key words: Adenosine triphosphate; Hepatocyte; Mitochondrial respiration; Uncoupler; Uncoupling protein 2

1. Introduction

Uncoupling protein 1 (UCP1) is exclusively expressed in brown adipose tissue (BAT) and located in the mitochondrial inner membrane. Its main function is to allow proton re-entry into the mitochondrial matrix, resulting in dissipation of the proton gradient formed during respiration. This uncouples respiration from ATP synthesis and the liberated energy is dissipated as heat. Therefore, UCP1 functions as a key molecule in the thermogenic function of BAT [1–4].

The uncoupling mechanism of UCP1 is not persistently active. Thermogenesis in BAT is activated by sympathetic nerves. Norepinephrine released from sympathetic nerve endings stimulates the β-adrenergic receptors, followed by accelerating lipolysis. Then, liberated fatty acids are used as substrates for mitochondrial oxidation and UCP1 activation. Since active UCP1 is supposed to prevent formation of ATP, more fuel (fatty acid) can be oxidized without phosphorylating ADP. In contrast, the uncoupling activity of UCP1 is inhibited by purine nucleotides such as GDP. Thus, the activity of UCP1 is controlled positively by free fatty acids (FFA) and negatively by nucleotides [1–5].

UCP1 plays an important role in the regulation of energy expenditure in mammals, and genetic ablation of BAT by modifying the UCP1 gene causes obesity in mice [6]. However, since the amount of BAT varies from species to species, and with age and body size [4], the relative importance of UCP1 in weight regulation and thermogenesis in large mammals, including humans, remains uncertain.

Recently, novel members of the UCP family have been cloned [7–10]. The newly identified UCP2 and UCP3 show 59% and 57% homology with UCP1, respectively, and function as partial uncouplers when expressed in yeast [7,8,11]. The expression of UCP2 and UCP3 is not limited to BAT. UCP2 is widely expressed in many human and rodent tissues with higher levels in white adipose tissue (WAT), spleen and lung [7,8], whereas UCP3 is specific to skeletal muscles and BAT [9,10]. Moreover, UCP2 and UCP3 genes are located adjacent to each other on mouse chromosome 7, which is tightly linked to the *tubby* mutation (a genetic marker of hyperinsulinemia) [7]. It has also been reported that there is significant linkage between the genetic markers located near the UCP2 gene and the resting metabolic rate [12], although no linkage was found of the UCP2 gene with a propensity to type 2 (non-insulin-dependent) diabetes mellitus in morbidly obese patients [13] or with juvenile- and mature-onset forms of obesity and insulin resistance [14]. Therefore, it has been suggested that UCP2 and UCP3 may play roles in the control of the basal metabolic rate and body weight in mammals, including humans.

Hepatocytes of the adult rat liver are unique cells that do not express UCP2, while non-parenchymal cells do [15]. In contrast, UCP2 is expressed in transformed hepatocytes displaying a fetal phenotype as regards energy metabolism [16], in fetal liver [17], and also in regenerating liver [18]. Moreover, when given bacterial lipopolysaccharide, UCP2 appears in adult hepatocytes via a tumor necrosis factor-α-dependent mechanism [19]. All these findings suggest that UCP2 expression may be suppressed in mature hepatocytes, except those of *ob/ob* mice [20]. However, the expression and function of UCP2 in the physiology and pathophysiology of the liver remain to be elucidated.

In the present study, we found that UCP2 was expressed in hepatocytes during primary culture. Using these cultured cells, we have demonstrated that UCP2 functions as an uncoupler in hepatocytes.

2. Materials and methods

2.1. Animals

Male Wistar rats (180–230 g) were maintained on a 12:12-h light-dark cycle (light on from 7.00 h to 19.00 h) with free access to standard laboratory chow and water.

2.2. Isolation of hepatocytes and primary culture

Hepatocytes were isolated from liver by the collagenase perfusion

*Corresponding author. Fax: (81) (11) 757-0703.
E-mail: k-kimura@vetmed.hokudai.ac.jp

Abbreviations: BAT, brown adipose tissue; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COX, cytochrome *c* oxidase; DNP, dinitrophenol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FFA, free fatty acid; RCR, respiratory control ratio; UCP, uncoupling protein; WAT, white adipose tissue

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method [21]. Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and the liver was perfused through the portal vein, initially with HEPES-buffered Hanks' solution (pH 7.4), containing 0.4 mM EGTA, 5 mM glucose, equilibrated with 95% O_2-5% CO_2 at 37°C, and then with collagenase (0.05%, type IV, Wako Pure Chemical Co., Osaka, Japan). After digestion, cells were separated by low-speed centrifugation ($30\times g$). The pellet, mainly consisting of hepatocytes, was subsequently centrifuged with Percoll at $400\times g$ to remove residual non-parenchymal liver cells. After being washed three times with phosphate-buffered saline (PBS), hepatocytes were plated at a density of 1.2×10^6 cells per 10 ml of Williams E medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, 1 μM dexamethasone (Wako) and 1 μM insulin (Sigma). After 3 h at 37°C, the medium was aspirated and then replaced with fresh medium, and changed every 48 h.

2.3. Isolation of mitochondria

Mitochondria were isolated from rat liver starved overnight, freshly isolated hepatocytes, or primary cultured hepatocytes according to the method of Negre-Salvayre et al. [22]. The liver was carefully dissected, minced, and homogenized using a Potter-Elvehjem homogenizer with a Teflon pestle in 0.22 M mannitol, 70 mM sucrose, 0.1 mM EDTA, 2 mM HEPES (pH 7.4) and 1% fatty acid-free bovine serum albumin (isolation buffer). The primary cultured hepatocytes were washed twice with HEPES-buffered Hanks' solution (pH 7.4), scraped into ice-cold isolation buffer using a cell scraper, and homogenized as described above. The homogenates were then centrifuged at $800\times g$ for 10 min to remove nuclei and debris. Mitochondria were separated from the supernatant by centrifugation at $10\,000\times g$ for 15 min and washed in the same buffer. The pellet was resuspended in a respiration buffer (10 mM K_2HPO_4-KH_2PO_4, 0.225 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) at a concentration of 4–5 mg/ml of protein. Protein was determined by the method of Lowry et al. [23] using BSA as a standard.

2.4. Oxygen consumption and rhodamine 123 uptake

Isolated mitochondria (~1 mg/ml) were incubated in the respiration buffer containing 5 μM rotenone, and further in 2.5 mM succinate and 0.2 mM ADP at 25°C. Changes in oxygen consumption were measured using a Clark-type oxygen electrode [24].

The uptake of rhodamine 123 by respiring mitochondria was determined as described by Johnson et al. [25]. The mitochondrial fraction (~0.5 mg/ml of protein) was suspended in the respiration buffer containing 5 μM rotenone and 5 mM succinate with 1 μM rhodamine 123 (Wako) at 37°C. The uptake of rhodamine 123 was measured in the presence or absence of 0.4 mM GDP (Sigma). After 15 min of incubation with rhodamine 123, mitochondria were carefully washed three times in the respiration buffer. Remaining fluorescence of rhodamine 123 in the mitochondria was measured with a fluorescence spectrophotometer (excitation wavelength 520 nm; emission wavelength 580 nm). To confirm that mitochondrial uptake of rhodamine 123 was related to membrane potential, a control study using a well known uncoupling agent, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 1 μM, Sigma), was carried out simultaneously.

2.5. Northern blot analysis

Total RNA was prepared according to the method of Chomczynski and Sacchi [26]. Thirty micrograms of each RNA sample was loaded on a 1% formaldehyde gel, and electrophoresed for 4–6 h. After blotting of the gel onto a nylon membrane (Amersham, Buckinghamshire, UK), the RNAs were UV cross-linked to the membrane. The UCP2 cDNA probe corresponding to the region of nucleotides 742–963 was obtained by the RT-PCR method using RNA from mouse BAT as a template, with 5′-GGCTGGTGGTGGTCGGAGAT-3′ as a sense primer and 5′-CCGAAGGCAGAAGTGAAGTG-3′ as an antisense primer. Hybridizations were performed in hybridization buffer with the probe labeled with [α-^{32}P]dCTP. The membranes were then washed in 2×SSC (20×SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% SDS twice for 15 min at 42°C, and twice with 0.1×SSC, 0.1% SDS at 52°C. The blots were exposed to a phospho-imaging plate and were analyzed using a BAS 1000 Image analyzer. Blots were then exposed to X-ray films (Kodak X-Omat S film) and developed later. Other probes used were: rat albumin cDNA (1200 bp), a 452 bp PCR product of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA and a 581 bp PCR product of mouse cytochrome *c* oxidase (COX, subunit IV) cDNA.

2.6. RT-PCR analysis

Expression of UCP1 and UCP3 mRNAs was determined by RT-PCR. Total RNA (2 μg) was denatured at 70°C for 10 min, cooled immediately and reverse-transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco), 50 pmol of poly(dT) primer and 20 nmol of dNTPs in a total volume of 10 μl at 37°C for 1 h. After heating at 95°C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase Gold (Perkin-Elmer, Tokyo, Japan), 3 mM $MgCl_2$ and 50 pmol of forward and reverse primers for mouse UCPs in a total volume of 50 μl. UCP1 primers used were: 5′-GTGAAGGTCAGAATGC-3′; 5′-AAGGGCCCCCTTCATGAGGT-3′. Mouse UCP3 primers used were: 5′-GAGCGGACCACTCCAGCGTC-3′; 5′-TGAGACTCCAGCAACTTCTC-3′. PCR was conducted for 25 cycles (UCP1) and for 30 cycles (UCP3), each consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and DNA extension at 72°C for 1 min. The PCR product (10 μl) was analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

2.7. Statistical analysis

Data were expressed as means ± S.E.M., and analyzed by Student's *t*-test. A P value of less than 0.05 was considered to be statistically significant.

3. Results

In accordance with the results reported previously, hepatocytes freshly isolated from rat liver did not express UCP2 mRNA (Fig. 1A). However, when cultured in vitro, hepatocytes expressed UCP2 mRNA 24 or 48 h after the start of culture. The expression of UCP2 mRNA dramatically increased further at 72–120 h (Fig. 1A,B). In contrast, albumin mRNA in hepatocytes gradually decreased during the culture, being about one-third of the initial level at 120 h (Fig. 1A,B). COX VI and GAPDH mRNAs were increased 2–4-fold at 8–24 h, faster than UCP2 (Fig. 1A,B). To determine whether primary cultured hepatocytes also expressed UCP1 and UCP3 mRNAs, RT-PCR was performed. As seen in Fig. 2, neither UCP1 nor UCP3 mRNA was detected in the isolated or cultured hepatocytes.

Although UCP2 was reported to function as an uncoupling protein in transfected yeast [7], it remained obscure whether UCP2 uncouples mitochondrial respiration in hepatocytes. To test this, mitochondria from hepatocytes cultured for 144 h were isolated and their respiration was measured, using mitochondria isolated from fasted rat liver as a control (Fig. 3). In the latter, oxygen consumption was increased after the addition of succinate and further after the addition of ADP. The respiration rate in the presence of ADP was not different from that in the presence of a chemical uncoupling agent (10 μM dinitrophenol, DNP). Mitochondria isolated from cultured hepatocytes showed a much slower rate of succinate oxidation compared to the control mitochondria (Fig. 3). The respiration rate of cultured hepatocyte mitochondria increased after the addition of ADP, but to a lesser extent than the control. Moreover, the chemical uncoupler DNP did not further increase the rate, suggesting that the oxidative phosphorylation capacity of mitochondria isolated from cultured hepatocytes was impaired. To assess the uncoupling activity, the ratio of succinate oxidation to the maximal rate of respiration was calculated (Table 1). The ratio in mitochondria from cultured hepatocytes expressing UCP2 was higher than in control mitochondria, in line with respiratory changes in UCP2-trans-

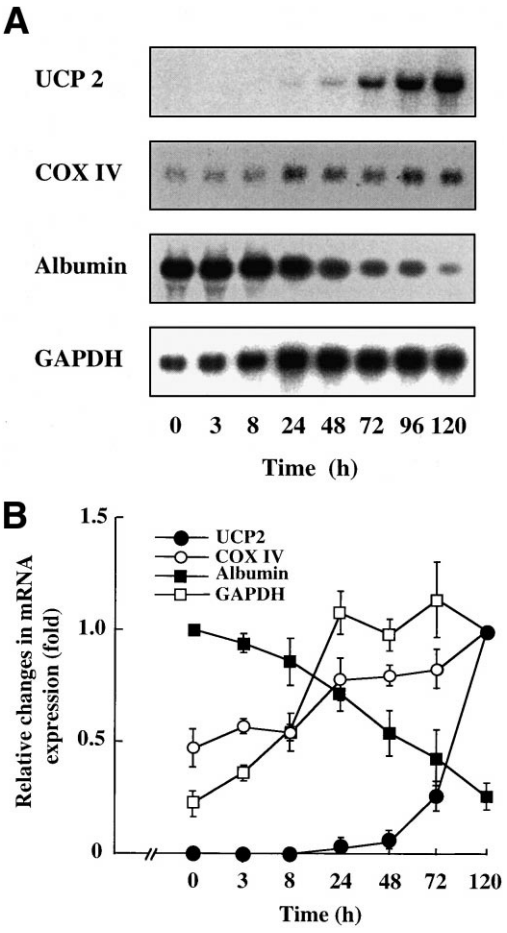

Fig. 1. Northern blot analysis of UCP2, GAPDH, albumin and COX VI expression during hepatocyte culture. Hepatocytes were cultured for 0 h (isolated) to 6 days and then total RNA was extracted at the indicated time point. Northern blots were performed using respective probes. Typical results from four series of preparations are shown in A. Quantitative changes in UCP2, GAPDH, and COX VI mRNA expression were estimated by referring to the amount of the radioactivity at 120 h as 1, and the changes in albumin mRNA expression were calculated by referring to the amount of the radioactivity at 0 h as 1 ($n=4$, B).

fected yeast mitochondria [7]. In addition, the respiratory control ratio (RCR) was decreased in mitochondria from cultured hepatocytes.

The membrane potential during succinate oxidation was assessed in mitochondria from freshly isolated hepatocytes and those cultured for 144 h by measuring rhodamine 123

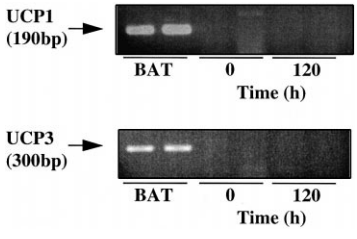

Fig. 2. Lack of expression of UCP1 and UCP3 mRNAs in primary cultured hepatocytes. Total RNA was extracted from isolated hepatocytes (0 h) and those cultured for 120 h, and RT-PCR was performed utilizing the primer sets for UCP1 or UCP3. RNA from BAT was used as a positive control.

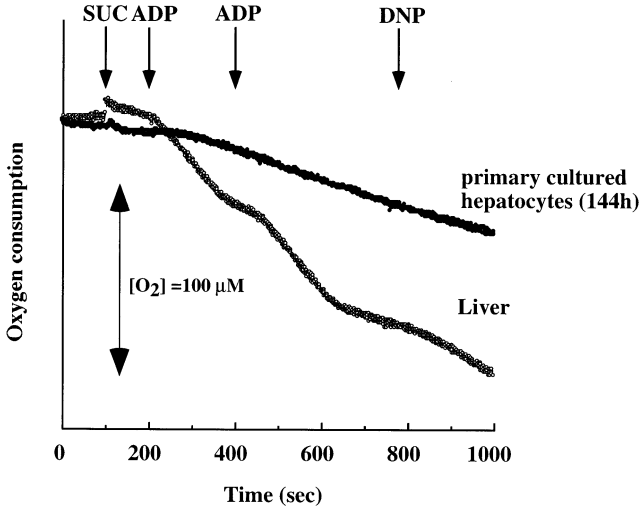

Fig. 3. Mitochondrial oxygen consumption during respiration. Mitochondria were isolated from fasted rat liver or hepatocytes cultured for 144 h, and changes in oxygen concentrations were monitored during mitochondrial respiration in the presence of rotenone. Representative records are shown. Succinate (2.5 mM), ADP (0.2 mM) and DNP (10 μM) were added where indicated.

uptake. Mitochondria from cultured hepatocytes accumulated 40% less rhodamine 123 than those from isolated hepatocytes (Fig. 4). The rhodamine 123 uptake was not changed even in the presence of GDP, which is known to inhibit UCP1.

4. Discussion

Liver parenchymal cells are unique in terms of UCP2 expression, though almost all other cells and tissues express it. Actually, UCP2 expression by freshly isolated hepatocytes

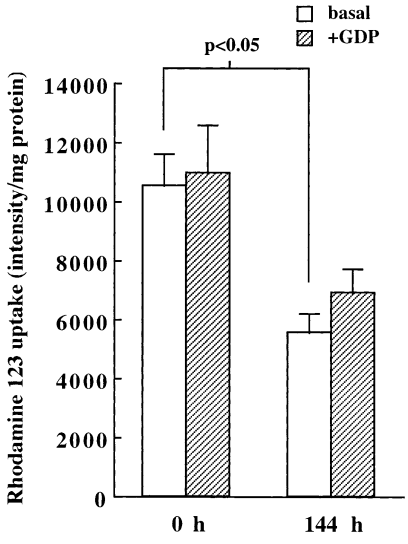

Fig. 4. Estimation of membrane potential by rhodamine 123 uptake. Mitochondria were obtained from isolated hepatocytes and those cultured for 144 h, and rhodamine 123 uptake of respiring mitochondria in the presence or absence of 0.4 mM GDP was determined as described in Section 2. Simultaneously, rhodamine 123 uptake was determined in the presence of CCCP (1 μM) as an uncoupling agent, and the fluorescence intensity obtained with CCCP was subtracted from that without CCCP. The data ($n=7$) are expressed after adjusting for protein content.

Table 1
Comparison of basal rate of respiration and RCR between fasted liver mitochondria and hepatocyte mitochondria cultured for 144 h

Mitochondrial source	*n*	Basal rate of respiration (% of basal/ADP addition)	RCR
Fasted liver	3	25.5 ± 2.3	6.4 ± 0.8
Cultured hepatocytes (144 h)	4	42.4 ± 4.6*	2.0 ± 0.1**

Rates of respiration in the basal condition (succinate and rotenone) and in the presence of ADP were determined, and the rate of basal respiration is shown as % of the rate in the presence of ADP. As seen in Fig. 3, the rate of respiration in the presence of a chemical uncoupler (10 μM DNP) was almost the same as that in the presence of ADP. RCR is the respiratory control ratio determined by the ratio of the respiratory rate of state 3 to that of state 4. Statistical significance between fasted liver mitochondria and cultured hepatocyte mitochondria is indicated (*$P < 0.05$; **$P < 0.01$).

was not detected. However, when cultured for a long period, hepatocytes started to express UCP2 mRNA. Recently, it has been reported that transformed hepatocytes express UCP2 [16]. Since transformed cells are known to display many metabolic characteristics of the embryonic and fetal stages of development [27,28] where UCP2 is highly expressed [17], similar changes might induce UCP2 expression in hepatocytes during culture.

Although induction of UCP2 protein was not determined because a specific antibody was not available, the above results prompted us to examine the functions of mitochondria from UCP2-expressing and non-expressing hepatocytes. Unfortunately, there was a decrease in the total capacity of oxidative phosphorylation in mitochondria from hepatocytes cultured for 144 h. However, it might be expected that, if the uncoupling protein was active, substrate (basal) oxidation without production of ATP might increase as in UCP2-expressing yeast mitochondria. Thus, the ratio of the basal oxidation rate to that with ATP production was calculated. Basal oxidation was significantly increased in mitochondria from UCP2-expressing cells compared to the control mitochondria lacking UCP2. Moreover, the membrane potential during succinate oxidation was reduced in mitochondria from UCP2-expressing cells. Collectively, these results suggest that UCP2 indeed causes the membrane potential to collapse in hepatocyte mitochondria. As proof of this, very recently, Chavin et al. demonstrated that UCP2 was highly expressed in the *ob/ob* obese mouse liver [20]. Mitochondria isolated from the liver, which contained non-parenchymal cells, also exhibited an increased rate of proton leakage, which partially dissipated the mitochondrial membrane potential [20].

The uncoupling mechanism of UCP1 is not persistently active, and is regulated by FFA and nucleotides [1–5]. UCP1 regulation by FFA seems to be relevant, because the function of UCP1 is controlled by sympathetic nerves, resulting in high availability of FFA via the norepinephrine effect. In contrast, it is unlikely that FFA is provided from intracellular sources in UCP2-expressing cells and tissues except WAT. Even in WAT, the main part of FFA released during lipolysis enters into circulating blood; hence, UCP2 activity would be regulated by extracellular FFA, if present. However, our results suggest that UCP2 is active without adding FFA, being coincident with UCP2 in yeast and the *ob/ob* obese mouse liver [7,20]. Thus, the amount of UCP2 expression itself, but not the fuel substrate, may control the capacities of mitochondrial respiration and ATP synthesis. Moreover, the uncoupling activity was not inhibited by GDP, though there is a putative nucleotide binding domain in UCP2 [29]. Since other nucleotides were not tested, more accurate examination is necessary to determine the effect of nucleotides on UCP2 function.

In summary, our results suggest that UCP2 is an active uncoupler, resulting in decreased capacity of oxidative phosphorylation in hepatocytes. However, high expression of UCP2 did not affect the intracellular ATP levels in cultured hepatocytes (Kimura et al., unpublished observation). This may be explained by some compensatory mechanism such as a glycolytic pathway that overcomes the decrease. The roles of UCP2 are still obscure, and primary cultured hepatocytes may be a useful model system for analyzing the regulatory mechanisms of UCP2 expression and its functions.

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