Effects of dietary nutrients on UCP family expressions in rat tissues

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Summary  We investigated that the effects of dietary nutrients on uncoupling protein (UCP)-1, 2, 3 gene expressions of white (epididymal) and brown adipose tissues of 8 wk-old rats fed diets containing various fats (hydrogenated fat or corn oil) and proteins (casein or soybean protein) for 1 wk. The mRNA expressions of UCPs were elevated in the white adipose tissues of the rats fed soybean protein in comparison with those fed casein, and that of UCP-2 was elevated also in the brown adipose tissue. The UCP-2 expression was more markedly elevated by dietary soybean protein than the others, particularly in the white adipose tissue. The effects of dietary fat type on the expressions were not clear. The UCPs protein levels were elevated in the adipose tissues of the rats fed soybean protein in comparison with those fed casein, in parallel with the mRNA expressions. Moreover, in fasted-refed experiment of the rats, the UCPs mRNA inductions reached maximal levels at 2 h, whereas the protein inductions reached at 4 h in the tissues and were stimulated by dietary fat or soybean protein in the white adipose tissues. Thus, the UCP-2 expression was markedly affected by dietary nutrients, particularly in the white adipose tissue.

Introduction
Uncoupling protein (UCP)-1 is a trans membrane proton transporter in the mitochondria of brown adipose tissue, a specialized tissue which functions in temperature homeostasis and energy balance (1). Recently, two proteins related to UCP-1 have been identified and designated UCP-2 (2) and UCP-3 (3). The UCP-2 protein is widely expressed in human and rodent tissues and was postulated to play an important role in energy balance, body weight regulation and thermoregulation (4). UCP-2 is highly expressed in white adipose tissue (2, 4, 5). UCP-3 was reported as being highly specific of skeletal muscle (3). The gene expression of these UCPs appears to be differentially regulated (6-8). However, the differences in the roles
of three UCPs and the correlation of their gene expressions remain to be elucidated.

Fatty acids have been reported to act as the transcriptional regulators of the expression of lipid-related genes in adipose cells (9). Physiological concentrations of polyunsaturated and monounsaturated fatty acids up-regulated UCP-2 mRNA levels in 3T3-L1 preadipocytes (10). Dietary fatty acids may be a possible physiological regulator of UCP-2 expression. Although there have been numerous publications describing regulation of UCP-2 or UCP-3 mRNA expression, the results are not always consistent (11, 12). Therefore, the physiological significance and regulation of UCP-2 and UCP-3 expressions remain to be further elucidated. In the present study, we investigated the effects of dietary nutrients on the UCP-1, 2, 3 mRNA and protein levels in tissues of steady state rats fed synthetic diets, and in fasted-refed rats.

MATERIALS AND METHODS

**Materials.** [α-32P]dCTP (111 TBq/mmol) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, U.S.A.). Nylon filter (Hybond N) was purchased from Amersham (Buckinghamshire, U.K.). Most other reagents were obtained from Wako (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A).

**Animals.** **Experiment 1.** Male Wistar rats (Japan SLC Co., Hamamatsu, Japan), 7 wk-old, fed on a commercially available non-purified diet (No. MF, Oriental Shiryou Co., Osaka, Japan) were fasted overnight and then fed a 10 % hydrogenated fat or corn oil diet for 1 or 2 wk. The diet compositions were 563 g sucrose, 200 g casein, 100 g hydrogenated fat or corn oil, 95 g cellulose, 40 g salt mixture (13), 1 g choline chloride and 1 g vitamin mixture (13). Rats were individually housed in wire-bottomed cages in a temperature-controlled room (24°C) under an automatic lighting schedule (08:00 h to 2000 h). Each rat had free access to water and was given an equal energy-containing diet per body weight per day.

The rats were killed between 9 00 h and 10 00 h to measure the mRNA concentrations of UCP-1, 2, 3, insulin receptors and leptin. The rats were decapitated after blood was taken using a heparinized syringe from the inferior vena cava while under diethyl ether anesthesia. Plasma was obtained by centrifuging the heparinized blood at 4°C for 20 min at 1200 x g. Brown and white (epididymal) adipose tissues were immediately removed, frozen in liquid nitrogen and stored at -80°C until used to measure the relative mRNA concentrations by dot or Northern blot hybridization analysis. Care and treatment of experimental animals were in accordance with the Guide for the Care and Use of Laboratory Animals (14).

**Experiment 2.** In time course studies, the 8 wk-old male Wistar rats were fasted for 2 days,
fed 1 g of the fat-free diet or 0.88 g of the corn oil diet (an equal energy to 1 g of the fat-free diet) per 100 g body weight every 30 min for 8 h, and were then killed at different times 0, 1, 2, 4, 8 h after refeeding. Brown and white adipose tissues were immediately removed, frozen in liquid nitrogen and stored at -80°C until used to measure the mRNA concentrations by dot or Northern blot hybridization analysis.  

**Dot blot and Northern blot hybridization assays.** The cDNA fragments of UCP-1, 2, 3 spanning nucleotides +100 to +865, +100 to +871 and +100 to +868, respectively, were cloned from rat adipose tissue by reverse transcription and polymerase chain reaction amplification according to Boss et al. (15). Human insulin receptor cDNA (16) was a generous gift from Professor Y. Ebina (Institute for Enzyme Research, University of Tokushima, Japan). A rat leptin cDNA fragment spanning nucleotides -59 to +540 was cloned from adipose tissue by reverse transcription and polymerase chain reaction amplification according to Murakami et al. (17). The genomic clone of rat rRNA was obtained from the Japanese Cancer Research Resources Bank (Mishima, Japan). About a 1 kb BamHI/EcoRI fragment of this clone was isolated and used as a probe for 18S rRNA. Total RNA was isolated from the tissues by acid guanidium thiocyanate-phenol-chloroform extraction (18). To measure the relative mRNA concentrations, the total RNA (10-30 μg) was denatured with formamide, spotted on a nylon filter and then radiated with ultraviolet light for 5 min. The filter was prehybridized and then hybridized with 32P-labeled cDNA as described previously (19). Relative densities of the hybridization signals were determined by scanning the autoradiograms at 525 nm and normalized to the values of 18S rRNA. The mRNA concentrations were measured by the dot blot hybridization method, and many were confirmed by Northern blot analysis.  

Northern blot analysis of RNA was performed as described by Gonzales and Kasper (20). Total RNA was denatured and electrophoresed on a 1.0% agarose gel containing 2.2 mol/L formaldehyde. The gel was blotted onto a nylon filter according to Thomas (21). Prehybridization, hybridization, and autoradiography were carried out as described previously (19).  

**Western blot.** Extract protein from tissues were separated by 8% SDS-polyacrylamide gel electrophoresis according to Laemmli (22) and blotted onto nitrocellulose using a Bio-Rad semidry transfer apparatus according to the manufacturer’s instructions. The membranes were blocked with 5% Halmein Casein in Tris-buffer and saline containing 0.1%Tween 20 (blocking solution), and then incubated with anti-UCP1, -2 or -3 followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz) in blocking solution. Bound
antibody was detected by enhanced chemiluminescence following the manufacturer’s directions (Amersham).

**Analyses.** Plasma glucose concentrations were determined by the glucose-oxidase method (23). Plasma insulin concentrations were measured by a two-antibody system radioimmunoassay according to the method of Morgan and Lazarow (24). Total hepatic lipids were extracted by the methods of Folch et al (25). Plasma and hepatic triacylglycerol concentrations were determined by the method of Fletcher (26).

**Statistical analysis.** Two-way ANOVA was followed by inspection of all differences between pairs of means using the least significant difference test (27). Differences were considered significant at *p*<0.05.

**RESULTS**

**Animal profiles.** The final body weights, white (epididymal) and brown adipose tissue weights of rats (Experiment 1) are shown in Table 1. The body weights, white and brown adipose tissue weights were not significantly different due to dietary fat and protein. Plasma glucose and insulin concentrations were not significantly affected by dietary protein or fat in rats (Table 1). The plasma triacylglycerols concentrations were significantly lowered by soybean protein. The liver triacylglycerols concentrations were also significantly lowered by soybean protein.

**Effects of dietary nutrients on UCP-1, 2, 3 mRNA and protein levels (Experiment 1).**

The rats were fed experimental diets for 1 or 2 wk. The mRNA and protein levels of UCP-1, 2, 3 in brown and white adipose tissues were not significantly different in rats fed the diets for 1 and 2 wk. Therefore, only the relative mRNA and protein levels in rats fed experimental diets for 1 wk are shown.

In the white adipose tissues, the mRNA expressions of UCP-1, 2, 3 were elevated in rats fed soybean protein in comparison with those fed casein (Fig. 1). The UCP-2 mRNA expressions were significantly elevated by corn oil compared with hydrogenated fat in rats fed soybean protein in the white adipose tissue. No effects of dietary fat types on the UCP-1, 3 expressions were found. In the brown adipose tissues, the UCP-2 mRNA expressions were elevated in rats fed soybean protein in comparison with those fed casein in rats (Fig. 2). No effects of dietary fat types on the UCP-1, 2, 3 expressions were found in the brown adipose tissue. Thus, the soybean protein effects on the UCPs mRNA expressions were greater in white adipose tissue than in the other tissues. The UCP-2 mRNA expressions in white
Table 1. Effects of dietary nutrients on body weights, white (epididymal) and brown adipose tissue weights and plasma data

<table>
<thead>
<tr>
<th>Diet</th>
<th>Casein Hyd. fat</th>
<th>Casein Corn oil</th>
<th>Soy Hyd. fat</th>
<th>Soy Corn oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>228 ± 24.3</td>
<td>236 ± 16.8</td>
<td>225 ± 30.8</td>
<td>225 ± 18.6</td>
</tr>
<tr>
<td>White adipose tissue weight (g/100 g bw)</td>
<td>0.86 ± 0.17</td>
<td>0.88 ± 0.15</td>
<td>0.70 ± 0.07</td>
<td>0.87 ± 0.16</td>
</tr>
<tr>
<td>Brown adipose tissue weight (x10⁻²g/100 g bw)</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>0.17 ± 0.03</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>11.7 ± 1.00</td>
<td>11.1 ± 1.08</td>
<td>10.7 ± 0.53</td>
<td>11.3 ± 1.77</td>
</tr>
<tr>
<td>Plasma insulin (nmol/L)</td>
<td>0.40 ± 0.07</td>
<td>0.41 ± 0.11</td>
<td>0.33 ± 0.05</td>
<td>0.37 ± 0.13</td>
</tr>
<tr>
<td>Plasma triacylglycerols (mg/ml)</td>
<td>1.92 ± 0.42</td>
<td>1.43 ± 0.40</td>
<td>1.59 ± 0.17</td>
<td>1.20 ± 0.27</td>
</tr>
<tr>
<td>Liver triacylglycerols (mg/g)</td>
<td>16.9 ± 2.30</td>
<td>12.8 ± 2.65</td>
<td>14.3 ± 1.40</td>
<td>11.2 ± 0.87</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD, n=6. Rats were pair-fed the casein or soybean protein diets containing 10% hydrogenated fat (Hyd. fat) or 10% corn oil for 1 wk and then killed.

Fig. 1. Effects of dietary nutrients on UCP-1, 2, 3 mRNA (upper) and protein (lower) levels in white adipose tissues of rats. Rats were fed the casein or soybean protein diets containing 10% hydrogenated fat (Hyd. fat) or 10% corn oil for 1 wk and then killed. The mRNA and protein levels were normalized to the rats fed the casein/hydrogenated fat diet. The values are means ± SD, n=8. Two-way ANOVA for mRNA and protein levels: protein main effect, p<0.05 in each panel.
adipose tissue were more markedly elevated by the dietary soybean protein than the UCP-1, 3 mRNA expressions. The UCPs protein levels were elevated in the adipose tissues of the rats fed soybean protein in comparison with those fed casein, in parallel with the mRNA expressions.

**Insulin receptor and leptin mRNA expressions.** The insulin receptor mRNA expressions in the liver were not significantly affected by dietary fat types but were induced by dietary soybean protein (Fig.3). The insulin receptor mRNA expressions in the white adipose tissues were not affected by protein and fat. However, the expressions in both tissues of the hydrogenated fat groups were significantly (by t-test) elevated in rats fed soybean protein in comparison with those fed casein. The leptin mRNA expressions were not significantly affected by the diet (Fig. 3).

**UCPs mRNA and protein inductions after refeeding a fat-free or corn oil diet to fasted rats. (Experiment 2).**

The time courses for UCP-1, 2, 3 mRNA and protein inductions were measured after refeeding a fat-free or 10% corn oil diet to 8 wk-old fasted rats (Fig. 4). The mRNA inductions of the UCPs in the white adipose tissues were already elevated 1 h after the refeeding, and reached a maximal level at 2 h.
The mRNA and protein inductions of UCP-2 were markedly elevated, particularly at the peak, in the white adipose tissues of rats fed the corn oil diet. The mRNA inductions of UCP-2 were significantly elevated by dietary soybean protein in comparison with casein in the white adipose tissues (data not shown).

Fig. 3. Effects of dietary nutrients on insulin receptor mRNA concentrations in livers (left), white adipose tissue (middle) and leptin mRNA concentrations in white adipose tissue (right) of rats. The mRNA concentrations were normalized to those in rats fed the casein/hydrogenated fat (Hyd. fat) diet. The values are means ± SD, n=8. Two-way ANOVA for liver insulin receptor mRNA: protein main effect, p<0.05.

Fig. 4. UCPs mRNA (upper) and protein (lower) inductions in white adipose tissue after feeding a fat-free (open circle) or corn oil (closed circle) diet containing casein to fasted rats. The mRNA and protein inductions were normalized to each value at 0 time (fasted rats). The values are means ± SD, n=4. Two-way ANOVA for UCP mRNA and protein levels: fat main effect, time main effect, p<0.05.
DISCUSSION

In Experiment 1, the mRNA expressions of UCP-2 were elevated in the white and brown adipose tissues of the rats fed soybean protein in comparison with those fed casein, and those of UCP-1 were elevated only in the white adipose tissue. We previously found that the conversion rates of thyroxine to triiodothyronine by liver microsomes and plasma triiodothyronine concentrations were significantly higher in rats fed soybean protein than in those fed casein (28). The plasma and liver triacylglycerol concentrations were lower in soybean protein fed rats. Triiodothyronine-treatment stimulated UCP-1 mRNA expression in fetal brown adipocytes in culture (29), UCP-2, 3 mRNA expressions in human skeletal muscle (30), and UCP-2 mRNA expression in rat white and brown adipose tissues and skeletal muscle (31). Therefore, it is suggested that dietary soybean protein stimulated the UCP-1, 2, 3 expressions by increased conversion rates of thyroxine to triiodothyronine.

We previously found that plasma and liver triacylglycerol concentrations and lipogenic enzyme activities were significantly suppressed by dietary soybean protein (28). Therefore, dietary soybean protein appeared to reduce the triacylglycerol levels by suppression of fatty acid synthesis and stimulation of energy expenditure.

Chevillotte et al. (32) reported that n-6 polyunsaturated fatty acids induced a 3-fold rise in UCP-2 expression in primary cultures of human muscle cells. In the present experiment, the UCP-2 mRNA expressions were significantly elevated by dietary corn oil compared with hydrogenated fat in white adipose tissue, but were not elevated by any dietary fat in brown adipose tissues. The UCP-1, 3 mRNA expressions were not significantly affected by any dietary oil in the tissues. Thus, the effects of dietary fatty acid type on the stimulation of UCPs expressions did not always coincide and still remains to be elucidated.

Leptin impairs several metabolic actions of insulin (33), whereas insulin increased the leptin mRNA levels in adipocytes (34, 35) and increased both leptin secretion and production by rat white adipose tissue (36). These results support the possibility that insulin is an important regulator of leptin gene expression but leptin down-regulates insulin functions. The insulin actions are controlled by insulin receptor expression and binding capacity to insulin. Insulin directly stimulates UCP-2 and UCP-3 mRNA expression in skeletal muscle in vitro (37). Leptin increased UCP-1, 3 mRNA levels in brown adipose tissues and UCP-2 mRNA levels in epididymal white adipose tissues (38).

In fasted-refed rats (in Experiment 2), the UCP-1, 2, 3 mRNA and protein inductions in white adipose tissue reached a peak at 2 h and 4 h respectively, and then decreased. The induction
of UCPs mRNA and protein was rapid, probably due to rapid turnover and rapid response of the mRNAs and proteins to dietary protein and fat. The UCP-2 mRNA inductions were more markedly elevated in rats fed the corn oil diet, particularly at the peak, in comparison with rats fed the fat-free diet. The inductions of UCP-2 mRNA in white adipose tissues were more markedly elevated in rats fed soybean protein than in those fed casein. Thus, dietary soybean protein and corn oil stimulated the UCP-1, 2, 3 expressions more clearly in the fasted-refed rats than in the steady state.

Thus, the UCP-2 expression is markedly affected by dietary nutrients, particularly in the white adipose tissue. Moreover, the turnover of UCP-1, 2, 3 appeared to be rapid and to be rapidly involved in thermogenesis after the nutritional manipulation.

REFERENCES
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