

## Up-regulation of Muscle Uncoupling Protein 3 Gene Expression by Calcium Channel Blocker, Benidipine Hydrochloride in Rats

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**Abstract.** To examine whether benidipine hydrochloride, one of the calcium channel blockers, up-regulate uncoupling protein 3 (UCP3) expression in two skeletal muscles (gastrocnemius and soleus) in rats. Wistar rats were treated orally with benidipine hydrochloride at 4 mg/kg for 7 days. Blood pressure was measured after 4 days. At the end of experiments, the rats were weighed, and brown adipose tissue (BAT) and skeletal muscles (gastrocnemius and soleus muscles) were removed. The mRNA levels of uncoupling protein 1 (UCP1) and UCP3 were measured using the real-time quantitative reverse transcription-polymerase chain reaction method. Benidipine reduced body weight and also had a hypotensive effect. In rats treated with benidipine, UCP1 mRNA levels were significantly increased 1.4-fold in BAT, and UCP3 mRNA levels in BAT and gastrocnemius muscle were significantly increased 1.7 and 3.0-fold, respectively, compared with the control rats. There was no difference in UCP3 mRNA levels in soleus muscle between the two groups. We concluded that benidipine up-regulates not only UCP1 gene expression in BAT but also UCP3 gene expression in BAT and gastrocnemius muscle, which may contribute to thermogenesis in rats.

**Key words:** Obesity, Benidipine, Uncoupling protein, Brown adipose tissue, Skeletal muscle

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**CALCIUM** antagonists are generally thought to exert their therapeutic action by inhibiting L-type calcium channels in the heart and especially in the peripheral vascular system [1]. Benidipine hydrochloride is a dihydropyridine calcium antagonist used in treating hypertension [2, 3]. Its long-term administration to rats and dogs has an inhibitory effect on weight gain; body weight rapidly returns to the control value after withdrawal of the drug [4, 5]. In hypertensive patients with

mild obesity, benidipine induced a slight but significant body weight loss [6]. The mechanism by which this drug produces weight loss is unknown. We recently revealed that benidipine activated brown adipose tissue (BAT) to induce body weight loss in monosodium-L-glutamate (MSG)-obese mice [7], in which the activity of BAT is diminished.

Uncoupling proteins (UCPs) are thought to be a family of mitochondrial H<sup>+</sup>/fatty acid transporters that are expressed in a tissue specific manner. UCP1, a classic UCP, is present exclusively in BAT [8]. UCP2 is expressed in heart, skeletal muscle and a number of other tissues [9], and UCP3 expression is largely confined to skeletal muscle and heart, with small amounts being present in BAT [10]. In 1997, UCP2 was found to be highly expressed not only in rodents, but also in hu-

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mans, and UCP3 was also highly expressed in both rodents and humans, mainly in skeletal muscle [11]. However, the exact physiologic roles of UCP2 and UCP3 are not known and are under intense investigation. Moreover, it is unclear whether benidipine up-regulates UCP3 gene expression in skeletal muscle. Therefore, in this study, we tested the hypothesis that benidipine up-regulates UCP3 gene expression in skeletal muscle of rats.

In addition, this study using benidipine also examined the expression of UCP3 in the same two skeletal muscles that differ widely in their relative proportions of slow-oxidative (SO) fibers, fast-glycolytic (FG) fibers and fast-oxidative-glycolytic (FOG) fibers, namely, the soleus, consisting predominantly (84%) of SO fibers and a small proportion (16%) of FOG fibers, and the gastrocnemius, containing very few (4%) SO fibers and a high proportion (58% and 38%) of FG and FOG fibers [12].

## Materials and Methods

Male 18 weeks-old Wistar rats (Charles River Japan Inc., Tokyo, Japan) or male 17 weeks-old spontaneously hypertensive rats (SHRs, Hoshino Experimental Animals, Saitama, Japan) were housed in plastic cages at  $22 \pm 2^\circ\text{C}$  with a 12 h light-dark cycle and given free access to laboratory chow (CE-2; Clea Japan) and tap water. The rats were divided into two groups. One group was given benidipine (Kyowa Hakko Kogyo Co. Ltd., Tokyo) via gastric tube at a daily dose of 4 mg/kg dissolved in 0.5% methylcellulose for 7 days. The dose of benidipine was selected based on previous studies on anti-hypertensive and anti-obesity effects (decreases in body weight and visceral fat accumulation) [3, 7, 13]. The other group was given distilled water dissolved in 0.5% methylcellulose. After 4 days, blood pressure (BP) was measured 4 h after the drug treatment in all conscious rats using the indirect tail-cuff method on a preheated  $37^\circ\text{C}$  plate. At the end of 7 days, the body weight of the rats was measured. Moreover, skeletal muscles (gastrocnemius and soleus) of Wistar rats were removed rapidly, weighed and frozen in liquid nitrogen for real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis [14, 15]. Total RNA was extracted from 0.1–0.5 g of tissue using the TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA), according to the manufac-

ture's instructions. Total RNA (2  $\mu\text{g}$ ) was denatured at  $80^\circ\text{C}$  for 5 min, cooled immediately and mixed with reverse transcriptase, 50 pmol poly (dT) primer and 20 nmol dNTPs in a total volume of 20  $\mu\text{L}$  at  $37^\circ\text{C}$  for 1 h. A real-time quantitative PCR was performed in a fluorescence temperature cycler (LightCycler<sup>TM</sup>, Roche Diagnostics GmbH Mannheim, Germany), with 6  $\mu\text{L}$  of reaction mixture containing 3 mmol/L of  $\text{MgCl}_2$ , 50 mmol/L TrisHCL (pH 8.3), 500 ng/ $\mu\text{L}$  of bovine serum albumin, 200  $\mu\text{mol/L}$  of each dNTP, a 1 : 30,000 dilution of SYBR Green, 1.5  $\mu\text{mol/L}$  of each primer, 0.05 U/ $\mu\text{L}$  of Taq DNA polymerase, 11 ng/ $\mu\text{L}$  of TaqStart<sup>TM</sup> antibody (Clontech laboratories, Palo Alto, CA, USA) and template. Amplification was conducted with a three-cycle procedure (denaturing,  $95^\circ\text{C}$ , 1 sec, ramp rate  $20^\circ\text{C/sec}$ ; annealing  $60^\circ\text{C}$ , 10 sec, ramp rate  $20^\circ\text{C}$ ; and extension  $72^\circ\text{C}$ , 26 sec, ramp rate  $2^\circ\text{C/sec}$ ) for 40 cycles. The fluorescence signal was plotted against the cycle number for all samples and external standards. Primers for UCP1 cDNA were: forward 5'-GTGAAGGTCAGAATGCAAGCS-3' (position 409–428) and reverse 5'-AGGGCCCCCTTCA TGAGGTC-3' (position 586–605), chosen according to the rat UCP1 cDNA sequence (RNUCPG.PE1, EMBL). Primers for UCP2 cDNA were: forward 5'-CAAGACCATTGCACGAGAG-3' (position 788–807) and reverse 5'-CATGGTAAGGGCACAGTGCA-3' (position 1061–1080), chosen according to the rat UCP2 cDNA sequence (U69135, Genbank). Primers for UCP3 cDNA were: forward 5'-ATGCATGCCTAC AGAACCAT-3' (position 657–676) and reverse 5'-CTGGGCCACCATCCTCAGCA-3' (position 949–968), chosen according to the rat UCP3 cDNA sequence (U92069, Genbank) [15, 16]. Primers for  $\beta$ -actin cDNA were: forward 5'-ATGAAGATCCTG ACCGAGGGT-3' and reverse 5'-AACGCAGCTCA GTAACAGTCCG-3'. Some amplification products produced in the LightCycler were checked by electrophoresis on 1.5% ethidium bromide-stained agarose gels. The estimated size of the amplified fragments matched the calculated size for UCP1 (197 bp), UCP2 (293 bp), UCP3 (312 bp) and  $\beta$ -actin (584 bp) in all cases. UCP mRNA levels are expressed relative to controls. The Animal care and Use committee of Kyoto Prefectural University of Medicine approved the animal care and experimental procedures.

All calculations were performed using the SPSS/WIN program version 11.0 (SPSS, Chicago, IL). Values are expressed as mean  $\pm$  SE. A two-tailed unpaired

**Table 1.** Effects of benidipine on body weight and blood pressure in Wistar rats.

Parameters	Control	Benidipine	<i>P</i> value
Body weight (g)			
before	572 ± 12	555 ± 10	
after	582 ± 12	555 ± 9	
difference	10 ± 2	0 ± 3	<i>P</i> <0.05
Systolic blood pressure (mmHg)			
before	138 ± 5	134 ± 4	
after	136 ± 5	107 ± 5	
difference	-2 ± 6	-27 ± 4	<i>P</i> <0.01
Diastolic blood pressure (mmHg)			
before	108 ± 3	109 ± 3	
after	107 ± 6	87 ± 5	
difference	-1 ± 7	-22 ± 3	<i>P</i> <0.05

Data are means ± SE. *P* values compared to differences in the control groups.

**Table 2.** Effects of benidipine on body weight and blood pressure in SHR.

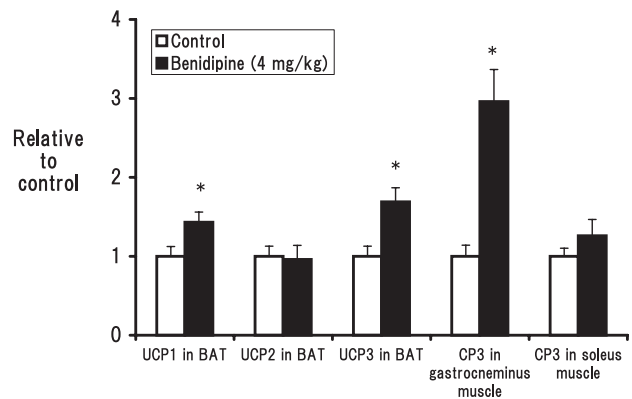
Parameters	Control	Benidipine	<i>P</i> value
Body weight (g)			
before	335 ± 10	335 ± 13	
after	339 ± 5	328 ± 8	
difference	4 ± 2	-7 ± 3	<i>P</i> <0.01
Systolic blood pressure (mmHg)			
before	186 ± 5	189 ± 4	
after	216 ± 6	111 ± 4	
difference	30 ± 4	-78 ± 5	<i>P</i> <0.01
Diastolic blood pressure (mmHg)			
before	160 ± 4	158 ± 5	
after	165 ± 6	84 ± 5	
difference	5 ± 3	-74 ± 6	<i>P</i> <0.01

Data are means ± SE. *P* values compared to differences in the control groups.

*t* test was used to analyze the difference between two groups. Results were considered significantly different at a *P* value of <0.05.

## Results

Control Wistar rats without benidipine administration showed approximately 10 gram increase in body weight during the experimental periods, whereas benidipine-administered rats did not show any increase in body weight during the same periods (Table 1). We also tested the effect of benidipine on body weight in

**Fig. 1.** Effects of benidipine on UCP1, UCP2 and UCP3 gene expression in BAT or skeletal muscles (gastrocnemius and soleus muscles) in Wistar rats.

Data are expressed as the mean ± SE, and the differences between control group and benidipine (4 mg/kg)-treated group were evaluated for significance using two-tailed unpaired *t* test (\**P*<0.05).

SHR. Control SHRs without benidipine administration showed a small increase in the body weight, but the benidipine-administered SHRs exhibited a decrease in body weight (Table 2). Food intakes were not different among the groups in two studies and mean food intake was 25 g/day/rat. The administration of benidipine decreased the arterial blood pressure in both Wistar rats and SHRs, confirming that benidipine at this time had significant effects on the cardiovascular system of the rats under the present experimental condition. The effects of benidipine on body weight and blood pressure were stronger in SHRs compared with Wistar rats (Table 1, 2).

In Wistar rats treated with benidipine, UCP1 mRNA levels were significantly increased by 1.4-fold in BAT compared with controls. In rats treated with benidipine (4 mg/kg), UCP3 mRNA levels were also significantly increased by 1.7 and 3.0-fold in BAT and gastrocnemius muscle, respectively, compared with the control rats (Fig. 1). There were no differences in UCP2 mRNA levels in BAT and UCP3 mRNA levels in soleus muscle between the groups.

## Discussion

It is probable that the anti-obesity effects of benidipine reported in several animal models of genetically obese are mediated via an indirect stimulation of BAT.

In this study, we clarified the mechanisms of anti-obesity effects of benidipine.

First, the present findings show that benidipine up-regulates UCP1 gene expression in BAT and reduces body weight in rats. These findings are consistent with previous studies [7, 15, 17], in which the activity of BAT was increased by benidipine. Using guanosine-5' diphosphate showed that benidipine activated BAT function in obese MSG mice [7]. Kajita *et al.* reported benidipine acutely stimulates blood flow to BAT in rats [18]. Karasawa *et al.* reported that benidipine did not significantly affect plasma NE concentrations, although nifedipine and amlodipine significantly increased plasma NE concentrations [19]. Zhao *et al.* using isolated brown fat cells from rats reported that benidipine itself has no thermogenic effect, and that the thermogenic response *in-vivo* is probably secondary to a release of NE from sympathetic nerves, here most likely directly from nerves in the BAT [17]. On the other hand, BAT is not only a target for pharmacotherapy of obesity and insulin resistance but also an endocrine tissue with leptin secretion and high insulin sensitivity. In fact, insulin and the adrenergic system is important in the regulation of energy homeostasis such as UCP1 expression in BAT [20], and benidipine is reported to improve insulin resistance [21, 22]. The mechanisms by which benidipine up-regulates UCP1 are thus unknown, but this is probably due to the improvement of insulin resistance not a calcium channel blocking effect.

Second, the present findings show that benidipine up-regulates UCP3 gene expression in BAT and gastrocnemius muscle. The underlying mechanism is unclear. Increases of 10-fold or more in UCP3 mRNA levels were observed upon fasting and diabetes, as well as the administration of  $\beta_3$ -agonist or T3 [23]. In many of these instances, the stimulation of UCP3 gene expression seemed to be mediated by an increase in circulating and/or intracellular fatty acid levels [24].

Moreover, from the preliminary study, benidipine increased UCP2 mRNA levels in white adipose tissue of Wistar rats. However, little is known about the association between benidipine and fatty acid levels. Further examination is needed to clarify these points.

Interestingly, UCP3 gene expression in soleus muscle did not change significantly, although that in gastrocnemius muscle did. The differential mRNA expression patterns of the two skeletal muscles are consistent with the heterogeneity in their glycolytic and oxidative enzymes activities and hence in their capacity to shift between lipids and glucose as fuel substrates. Soleus muscle is an oxidative type muscle with higher dependency on lipids than the gastrocnemius, hence it has a lower capacity to shift between lipids and glucose as fuel substrates [25]. Samec *et al.* reported that the changes of UCPs mRNA expressions in gastrocnemius muscle were greater than those in soleus muscle [25]. We reported that  $\beta_3$ -adrenergic agonist induces a functionally active uncoupling protein in fat and slow-twitch muscle fibers in gastrocnemius muscle of obese MSG mice [26]. Because the role of muscle UCP3 as a thermogenic and/or thermoregulatory protein remains to be established, further examination is needed.

In this study, we clarified the hypothesis that benidipine up-regulates UCP3 gene expression in skeletal muscle of rats. Further studies are needed to clarify the mechanism of the anti-obesity effect and UCPs up-regulation of benidipine. Moreover, we must prove the physiological activities of UCPs in each tissue and the differences of UCPs mRNA expression between Wistar rats and SHR.

In conclusion, benidipine up-regulates not only UCP1 gene expression in BAT but also UCP3 in BAT and gastrocnemius muscle, which may contribute to a reduction in weight, possibly by activating thermogenesis in skeletal muscle through UCP3. These findings suggest that benidipine may be useful in treating hypertensive patients with obesity.

## References

- Scholz H (1997) Pharmacological aspects of calcium channel blockers. *Cardiovasc Drug Ther* 10: 869–872.
- Naokajima O, Akioka H, Miyazaki M (2000) Effect of the calcium antagonist benidipine hydrochloride on 24-h ambulatory blood pressure in patients with mild to moderate hypertension in a double-blind study against placebo. *Arzneimittel Forschung* 50: 620–625.
- Kitakaze M, Karasawa A, Kobayashi H, Tanaka H, Kuzuya T, Hori M (1999) Benidipine: A new  $\text{Ca}^{2+}$  channel blocker with a cardioprotective effect. *Cardiovasc Drug Rev* 17: 1–15.
- Tanaka H, Waki Y, Ito R, Kashitani J, Yamanami S, Saijo T, Ikenaga J, Hara T (1990) Toxicity study of benidipine hydrochloride (1<sup>st</sup> report): acute toxicity in

- mice and rats, subacute and chronic oral toxicity studies of benidipine hydrochloride in rats. *Clin Rep* 24: 1045–1109. (in Japanese)
5. Tanaka H, Waki Y, Ikenaga T, Kashitani J, Yamanami S, Saijo T, Ikenaga J, Hara T (1990) Toxicity study of benidipine hydrochloride (2<sup>nd</sup> report): acute, subacute and chronic oral toxicity studies of benidipine hydrochloride in dogs. *Clin Rep* 24: 1811–1860. (in Japanese)
  6. Yoshida T, Kondo M, Yoshikawa T, *et al.* (2002) Utility of benidipine hydrochloride for obese subjects with hypertension — multicenter study —. *J News Rem Clin* 51: 399–408. (in Japanese)
  7. Yoshida T, Umekawa T, Wakabayashi Y, Sakane N, Kondo M (1994) Mechanism of anti-obesity action of benidipine hydrochloride in mice. *Int J Obes Relat Metab Disord* 18: 776–779.
  8. Kozak LP, Koza RA (1999) Mitochondria uncoupling proteins and obesity: molecular and genetic aspects of UCP1. *International Journal of Obesity & Related Metabolic Disorders: Journal of the International Association for the Study of Obesity* 23 Suppl 6: S33–S37.
  9. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, Bouillaud F, Seldin MF, Surwit RS, Ricquier D, Warden CH (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* 15: 269–272.
  10. Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP (1997) Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 12: 39–42.
  11. Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP (1997) Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 408: 39–42.
  12. Samec S, Seydoux J, Dulloo A (1999) Skeletal muscle UCP3 and UCP2 gene expression in response to inhibition of free fatty acid flux through mitochondrial beta-oxidation. *Eur J Physiol* 438: 452–457.
  13. Sakamoto S, Murakado M, Utsumi E, Miyoshi S, Minami K, Okada K, Ohnaka S, Nakaya Y (1996) Long-term effects of oral benidipine hydrochloride on plasma lipids and visceral fat accumulation in rats. *Jpn Pharmacol Ther* 24: 1257–1261. (in Japanese)
  14. Kogure A, Sakane N, Takakura Y, Umekawa T, Yoshioka K, Nishino H, Yamamoto T, Kawada T, Yoshikawa T, Yoshida T (2002) Effects of caffeine on the uncoupling protein family in obese yellow KK mice. *Clin Exp Pharmacol Physiol* 29: 391–394.
  15. Denjean F, Lachuer J, Gélöën A, Cohen-Adad F, Moulin C, Barre H, Duchamp C (1999) Differential regulation of uncoupling protein-1, -2 and -3 gene expression by sympathetic innervation in brown adipose tissue of thermoneutral or cold-exposed rats. *FEBS Lett* 444: 181–185.
  16. Strömmer L, Abou El-Ella G, Kamel A, Marcus C, Hager P, Adrian TE, Permert J (2001) Upregulation of uncoupling protein homologues in skeletal muscle but not adipose tissue in posttraumatic insulin resistance. *Biochem Biophys Res Comm* 281: 334–340.
  17. Zhao J, Golozubova V, Bengtsson T, Cannon B, Nedergaard J (1999) Benidipine induces thermogenesis in brown adipose tissue by releasing endogenous noradrenalin: a possible mechanism for the anti-obesity effect of calcium antagonists. *Int J Obes Relat Metab Disord* 23: 238–245.
  18. Kajita J, Kobayashi S, Yoshida T (1994) Effect of benidipine hydrochloride on regional blood flow of the adipose tissue in anesthetized rats. *Arzneim-Forsch/Drug Res* 44: 297–300.
  19. Karasawa A, Nomura H, Nito M, Sonoda R, Tanaka H, Kosaka N, Yamaguchi K, Kobayashi S (1999) Effects of benidipine hydrochloride (Coniel®) on blood pressure, heart rate and plasma norepinephrine concentration in spontaneously hypertensive rats. *Folia Pharmacol Jpn* 113: 317–326.
  20. Klein J, Fasshauer M, Klein HH, Benito M, Kahn CR (2002) Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action. *Bioessays* 24: 382–388.
  21. Higashiura K, Ura N, Takada T, Agata J, Yoshida H, Miyazaki Y, Shimamoto K (1999) Alteration of muscle fiber composition linking to insulin resistance and hypertension in fructose-fed rats. *Am J Hypertens* 12: 596–602.
  22. Suzuki M, Kanazawa A, Hasegawa M, Harano Y (1999) Improvement of insulin resistance in essential hypertension by long-acting Ca antagonist benidipine. *Clin Exp Hypertens (New York)* 21: 1327–1344.
  23. Giacobino JP (2002) Uncoupling proteins, leptin, and obesity. *Ann New York Acad Sci* 967: 398–402.
  24. Weigle DS, Selfridge LE, Schwartz MW, Seeley RJ, Cummings DE, Havel PJ, Kuijper JL, BeltrandelRio H (1998) Elevated free acids induce uncoupling protein 3 expression in muscle. *Diabetes* 47: 298–302.
  25. Samec S, Seydoux J, Dulloo AG (1998) Role of UCP homologues in skeletal muscles and brown adipose tissue: mediators of thermogenesis or regulators of lipids as fuel substrate? *FASEB J* 12: 715–724.
  26. Yoshida T, Umekawa T, Kumamoto K, Sakane N, Kogure A, Kondo M, Wakabayashi Y, Kawada T, Nagase I, Saito M (1998)  $\beta_3$ -adrenergic agonist induces a functionally active uncoupling protein in fat and slow-twitch muscle fibers. *Am J Physiol* 274: E469–E475.