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ORIGINAL ARTICLE

Nifedipine increases energy expenditure by increasing PGC-1α expression in skeletal muscle

Zhe Tian[1,2,5], Keishi Miyata[1,3,5], Mitsuhisa Tabata[1], Masato Yano[1], Hirokazu Tazume[1,2], Jun Aoi[1], Otowa Takahashi[1], Kimi Araki[4], Michio Kawasuji[2] and Yuichi Oike[1]

Nifedipine, an L-type calcium (Ca) channel blocker, is one of the most widely used Ca channel-blocking medications for hypertension. Previous studies have reported an association of nifedipine hypertensive treatment with decreased body weight in obese hypertensive humans and rat models. However, the precise mechanism underlying how nifedipine functions metabolically has not been elucidated. Here, we investigated the long-term effect of a non-hypotensive nifedipine dose using a mildly obese, endothelial NO synthase-deficient mouse model. Treating these mice with nifedipine decreased their body weight gain ratio, and white adipose tissue weight compared with the untreated controls. Metabolic analyses indicated that nifedipine treatment upregulated whole-body energy expenditure through increasing oxygen consumption and reducing the respiratory exchange ratio, suggesting that nifedipine promotes lipid oxidation rather than carbohydrate utilization. Furthermore, nifedipine treatment upregulated the expression of the peroxisome proliferator-activated receptor-γ coactivator -1α (PGC-1α) in skeletal muscle. Overall, these results suggest that a non-hypotensive dose of nifedipine has pleiotropic effects on energy expenditure that could ameliorate obesity.
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Keywords: lipid oxidation; nifedipine; obesity; skeletal muscle

INTRODUCTION

Obesity, the most prevalent nutritional disorder in industrialized countries, is a challenging medical problem associated with increased risks of hypertension, cardiovascular disease, hyperlipidemia, diabetes and mortality. Obesity is defined as excess body fat accumulation and results from a daily energy imbalance, such as excess calorie intake and/or decreased energy consumption. Thus, restoring energy expenditure is considered as an efficient strategy for preventing or treating obesity.

Skeletal muscle is a massive organ accounting for 40–50% of the total body mass and thus significantly expends whole-body energy expenditure even under sedentary conditions. In particular, lipid oxidation in skeletal muscle is critical and has received increased attention for its role in obesity. Specifically, in obese subjects, skeletal muscle has been described to reduce lipid oxidation.[1] A decreased ability to oxidize lipids has been linked with weight gain as well as a propensity towards obesity. Thus, developing effective treatments that can reverse and/or compensate for impaired lipid oxidation in the skeletal muscle of obese subjects is important.

Calcium (Ca) channel blockers are commonly used to treat patients with hypertension. Nifedipine, a widely used antihypertensive drug, is thought to act mainly by blocking dihydropyridine receptor/L-type Ca channels on vascular smooth muscle cells. Nifedipine treatment reduces atherosclerotic plaques in cholesterol-fed rabbits,[2,3] and suppresses development and progression of atherosclerosis in hypertensive patients.[4,5] Nifedipine has recently been reported to have pleiotropic effects on endothelial cells,[6,7] cardiac muscle cells,[8,9] mesangial cells[10–12] and neurons,[13,14] through mechanisms independent of blocking Ca channels. Previous studies have reported that nifedipine treatment decreases body weight in obese hypertensive humans[15] and rat models.[16] These data suggest that antihypertensive doses of nifedipine alter fat metabolism associated with anti-obesity. Interestingly, Iwai *et al.*[17] recently reported that a non-hypotensive dose of nifedipine decreased the weight of white adipose tissue (WAT) and enhanced insulin sensitivity in obese diabetic KK-A^y mice, suggesting that such treatment directly impacts fat metabolism. Nonetheless, the mechanism through which nifedipine treatment modulates energy expenditure has not been fully elucidated.

In this study, we examined the long-term effect of a non-hypotensive dose of nifedipine on mildly obese model mice in which endothelial NO synthase (eNOS) was deficient. Nifedipine treatment antagonized weight gain, and increased whole-body energy expenditure and lipid oxidation in skeletal muscle. Interestingly, we also observed an increase

[1]Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan; [2]Department of Cardiovascular Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan; [3]Department of Immunology, Allergy and Vascular Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan and [4]Division of Developmental Genetics, Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto, Japan
[5]These authors contributed equally to this work.
Correspondence: Dr K Miyata, Department of Immunology, Allergy and Vascular Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan.
E-mail: hully@gpo.kumamoto-u.ac.jp
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in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression in skeletal muscle of nifedipine-treated eNOS-deficient mice. These results indicate that nifedipine pleiotropically increases energy expenditure and could thereby counteract obesity.

METHODS

Animals

All experimental protocols were approved by the Kumamoto University Ethics Review Committee for Animal Experimentation. Male eNOS-deficient mice on a C57BL/6N background were used for all experiments. All animals were bred in a mouse house with automatically controlled lighting (12 h on, 12 h off), and a stable temperature of 23°C was maintained throughout. Mice were fed a normal diet (CE-2, CLEA, Tokyo, Japan).

Nifedipine treatment

Mice were divided into two groups (10 per group) and implanted with a mini-osmotic pump (Alzet Corp. Mini-osmotic pump, Model 2002, Palo Alto, CA, USA) in subcutaneous back tissue. Control mice were administrated a vehicle solution (polyethylene glycol 400 (WAKO, Osaka, Japan), ethanol and H_2O in the ratio 15:15:70). Nifedipine was administered at doses of 1 mg kg^{-1} per day in the vehicle solution (Bayer Yakuhin, Ltd, Osaka, Japan).

Metabolic measurements

Mice were subjected to metabolic analysis after 16 weeks of administration of either nifedipine or control vehicle. Insulin and glucose tolerance tests (ITT and GTT, respectively) were performed as previously described.[18] For ITT, mice were administered 1.0 U kg^{-1} of human insulin by intraperitoneal injection. For GTT, mice were deprived of food for 16 h and were injected with glucose intraperitoneally at 1.25 g kg^{-1}. Serum glucose was measured using the glucose oxidase method (Sanwa Kagaku, Nagoya, Japan), and serum insulin was measured using an insulin immunoassay (Morinaga, Yokohama, Japan). Insulin sensitivity was determined using homeostatic model assessment-insulin resistance (HOMA-IR).[19] The HOMA-IR index is calculated from both fasting glucose and insulin levels as follows: HOMA-IR index = fasting glucose (mg dl^{-1}) × fasting insulin (μU ml^{-1})/405. Mouse adiposity was assessed by computed tomography scanning (LaTheta, Hitachi, Aloka Medical, Ltd, Tokyo, Japan) as previously described.[18] Blood pressure was monitored every 4 weeks using a tail-cuff monitor (BP Monitor for Mice & Rats Model MK-2000, Muromachi Co., Ltd, Tokyo, Japan). Oxygen consumption (VO_2), carbon dioxide production (VCO_2), the respiratory exchange ratio (RER) and activity levels were determined (Light time; 13:00–17:00, Dark time; 1:00–5:00, air flow rate 0.50 l min^{-1}) as previously described.[18] For exercise experiments, mice were allowed to adapt to an air-tight treadmill chamber (Model MK-680AT/02 M, Muromachi Co., Ltd) for 30 min (air flow rate 0.90 l min^{-1}) at which point VO_2 and VCO_2 were stable; measurements were then continued for another 30 min while mice were in a sedentary state. Mice then exercised on a treadmill at a speed of 10 m min^{-1}, and VO_2, VCO_2 and RER were measured for 30 min as previously described.[20]

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Japan KK, Tokyo, Japan). DNase-treated RNA was reverse transcribed using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). PCR products were analyzed with a Thermal Cycler Dice Real-Time system (Takara Bio), and relative transcript abundance was normalized to that of β-actin mRNA. Oligonucleotides used for PCR are listed in Table 1.

Table 1 Primer sequences used in quantitative RT-PCR

Gene		*Sequences*
β-Actin	Forward	5′-CATCCGTAAAGACCTCTATGCCAAC-3′
	Reverse	5′-ATGGAGCCACCGATCCACA-3′
CD31	Forward	5′-CCGAAGCAGCACTCTTGCAG-3′
	Reverse	5′-CTGCAACTATTAAGGTGGCGATGA-3′
PGC-1α	Forward	5′-CCGTAAATCTGCGGGATGATG-3′
	Reverse	5′-CAGTTTCGTTCGACCTGCGTAA-3′
PGC-1β	Forward	5′-GTGCCAGGTGCTGACGAGAA-3′
	Reverse	5′-AGTGTATCTGGGCCAACGGAAG-3′
PPARα	Forward	5′-ACGCTCCCGACCCATCTTTAG-3′
	Reverse	5′-TCCATAAATCGGCACCAGGAA-3′
PPARδ	Forward	5′-CAGATGACCCTTGTGCTGCCTA-3′
	Reverse	5′-TCTGACCCTGGGACCTAAGTGTG-3′
Tie2	Forward	5′-TGCCCAGATATTGGTGTCCTTAAAC-3′
	Reverse	5′-TCCGCAGGGCAGTCAATTC-3′
UCP2	Forward	5′-GCAAGCATGTGTATGGCACAGTAAC-3′
	Reverse	5′-AAATGTGGGCCTTCGGTCAG-3′
UCP3	Forward	5′-GTGGTAAAGCCATGCACACCTG-3′
	Reverse	5′-CCTGCTGCTTTGAACTGATGGA-3′
VE-cadherin	Forward	5′-TGGCTTGTCGAATTTGAAGCA-3′
	Reverse	5′-TCTGGTGAGTGGGTTAGAGGCTATC-3′
CD36	Forward	5′-GATGGCCTTACTTGGGATTGGA-3′
	Reverse	5′-GGCTTTACCAAAGATGTAGCCAGTG-3′
FATP1	Forward	5′-GCAGCATTGCCAACATGGAC-3′
	Reverse	5′-GTGTCCTCATTGACCTTGACCAGA-3′
ACSL1	Forward	5′-TTTGCCTGCAGCGAGTGTG-3′
	Reverse	5′-GCCCTCGACTATCCCTATGGTAAGA-3′
FABP3	Forward	5′-TGGCTAGCATGACCAAGCCTACTAC-3′
	Reverse	5′-GTTCCACTTCTGCACATGGATGA-3′
CPT1-b	Forward	5′-GAGACAGGACACTGTGTGGGTGA-3′
	Reverse	5′-AGTGCCTTGGCTACTTGGTACGAG-3′
ACADS	Forward	5′-AAGTTTGGATCCGCACAGCAG-3′
	Reverse	5′-CAAGCTTTGGTGCCGTTGAG-3′
ACADM	Forward	5′-CGAGTATGTTATCAACGGCCAGAA-3′
	Reverse	5′-GCGGGTACTTTAGGATCTGGGTTAG-3′
ACADL	Forward	5′-GGACTCCGGTTCTGCTTCCA-3′
	Reverse	5′-TGCAATCGGGTACTCCCACA-3′
ACOX1	Forward	5′-AAGATGGATCCTAAGCCAGCTGAA-3′
	Reverse	5′-CAGCTTACCACAAAGCCAGCTACTC-3′
ACOX2	Forward	5′-CTGGGCTCAGATGAGCAGATTG-3′
	Reverse	5′-ACTCCTGGGTGGTTGCATCATAG-3′
Acaa1a	Forward	5′-TTCACGGCAGAAGCAGGATG-3′
	Reverse	5′-CACAATCTCAGCACGGAAGCA-3′
MFP2	Forward	5′-GCAGCATGGGACCATATGAAGA-3′
	Reverse	5′-ATGCCCAGCTTTGCAGCAC-3′

Abbreviations: Acaa1a, acetyl-CoA acyltransferase 1A; ACADL, acyl-CoA dehydrogenase, long chain; ACADM, acyl-CoA dehydrogenase, medium chain; ACADS, acyl-CoA dehydrogenase, short chain; ACOX, acyl-CoA oxidase; ACSL1, acyl-CoA synthetase long-chain family member 1; CD, cluster of differentiation; CPT1b, carnitine palmitoyl-transferase 1B; FABP, fatty acid binding protein; FATP1, fatty acid transport protein 1; MFP2, multifunctional protein 2; PGC1, proliferator-activated receptor-γ coactivator -1; PPAR, peroxisome proliferator-activated receptor; UCP, uncoupling protein; VE-cadherin, vascular endothelial cadherin.

Table 2 Body weight and systolic blood pressure before (pre) and after nifedipine administration

	Pre (8-week-old)	*2 months (16-week-old)*	*4 months (20-week-old)*
Body weight (g)			
Control	23.0 ± 0.3	26.7 ± 0.6	27.9 ± 0.8
Nifedipine	23.2 ± 0.1	26.9 ± 0.5	26.2 ± 0.4
Systolic blood pressure (mm Hg)			
Control	115.7 ± 2.2	117.4 ± 3.7	118.3 ± 6.3
Nifedipine	112.3 ± 1.8	116.8 ± 4.0	121.1 ± 5.5

Nifedipine was administered at 1 mg kg^{-1} per day for 4 months via an implanted osmotic pump.

Statistical analysis

All data are expressed as the means ± s.e.m.'s in the text and in figures. A value of $P<0.05$ was considered statistically significant.

RESULTS

Nifedipine treatment reduces adipose tissue weight and volume

After 16 weeks of nifedipine treatment ($1\,mg\,kg^{-1}$ per day), neither the systolic blood pressure (Table 2) nor the heart rate (data not shown) changed significantly in eNOS-deficient mice compared with the control mice. After 16 weeks of treatment, the overall body weight of the nifedipine-treated mice did not differ significantly from the control mice, although 16 weeks of nifedipine administration trended towards a reduction (P=0.090, Table 2). Indeed, the body weight gain ratio (increased weight per mouse body weight) of nifedipine-treated mice was significantly less than the control mice (P=0.018, Figure 1a), suggesting that nifedipine treatment antagonizes body weight increases. Significant reductions in the weight of epididymal WAT and brown adipose tissue proportional to body weight were observed in nifedipine-treated mice (P=0.0038 and P=0.0016, respectively, Figure 1b), whereas the skeletal muscle weight relative to body weight

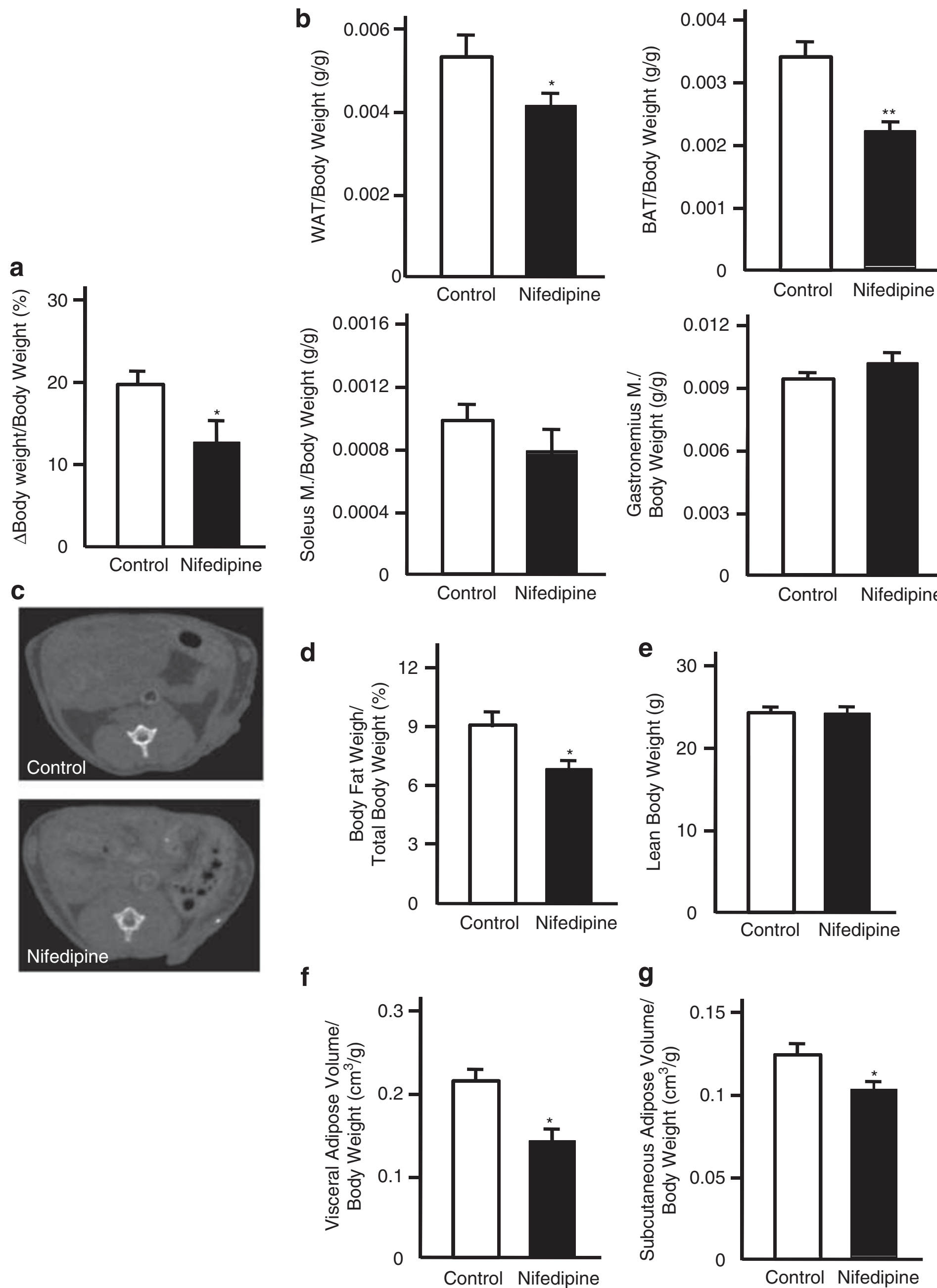

Figure 1 Effect of nifedipine on weight gain and percentage of body fat. Comparison of the ratio of body weight gain to overall body weight (ΔBody weight/Body weight) at the end of the 16 weeks (**a**) and weight of each tissue per overall body weight (**b**) between the control and nifedipine-treated mice (n=7–9 per group). Abdominal computed tomography analysis of control (top) and nifedipine-treated (bottom) mice (**c**). Comparison of the percentage of body fat (Body fat weight/Total body weight) (**d**), lean body weight (**e**), visceral adipose volume per body weight (**f**) and subcutaneous adipose volume per body weight (**g**) between control and nifedipine-treated mice (n=7–9 per group). $^{*}P<0.05$,$^{**}P<0.01$ *vs.* control.

was unchanged. Computed tomography scans indicated that the body fat volume of nifedipine-treated mice significantly decreased (P=0.021, Figures 1c and d), whereas the lean body weight remained unchanged (Figure 1e). Moreover, significant reductions in both visceral adipose and subcutaneous adipose volume per body weight were observed in nifedipine-treated mice (P=0.017 and P=0.047, respectively, Figures 1f and 1g, respectively). Taken together, these results indicate that nifedipine treatment antagonizes increases in body fat mass.

Nifedipine treatment enhances oxygen expenditure and reduces the RER

To investigate the mechanism by which nifedipine promotes WAT mass reduction, we analyzed basal metabolic rate, locomotor activity and food intake in eNOS-deficient mice (Figure 2). Nifedipine-treated mice exhibited a significant increase in whole-body VO_2 rates in dark-time measurements (P=0.027, Figure 2a) relative to control mice, whereas no significant changes were observed in VCO_2 rates (Figure 2b). Furthermore, the RER (VCO_2/VO_2) of nifedipine-treated mice was significantly decreased in dark time, suggesting a greater fat utilization as an energy source (P=0.025, Figure 2c). Nifedipine-treated mice exhibited a significant increase in locomotor activity compared with control mice, particularly in dark time (P=0.044, Figure 2d). No significant change was observed in food intake per body weight (Figure 2e) or rectal temperature (data not shown). Taken together, these results suggest that nifedipine treatment increases energy expenditure. We further investigated whether nifedipine treatment alters the oxygen consumption and carbon dioxide production during treadmill-induced exercise (Figure 3). To accomplish this, we monitored VO_2, VCO_2 and RER during 10 m min^{-1} treadmill running. Although no significant change was observed in VO_2 (Figure 3a), the VCO_2 of the nifedipine-treated mice was significantly decreased (25–30 min exercise; P=0.013, Figure 3b) compared to controls. Interestingly, after 10 min of exercise, the RER of

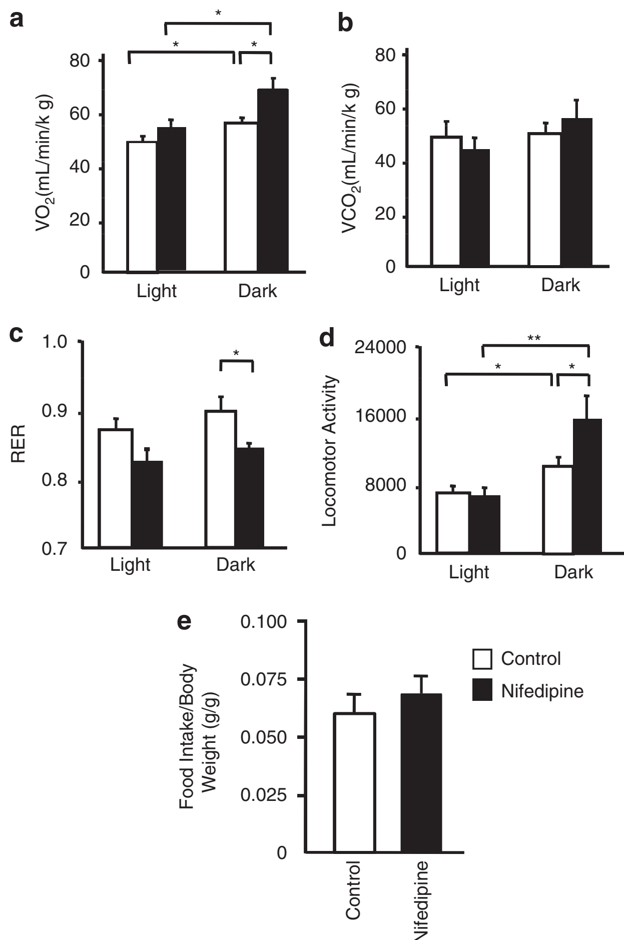

Figure 2 Effect of nifedipine on oxygen expenditure and the respiratory exchange ratio (RER). Comparison of oxygen consumption (**a**), carbon dioxide production (**b**), RER (**c**), locomotor activity (**d**) and food intake per body weight (**e**) between control and nifedipine-treated mice (n=7–8 per group). *P<0.05, **P<0.01 *vs.* control.

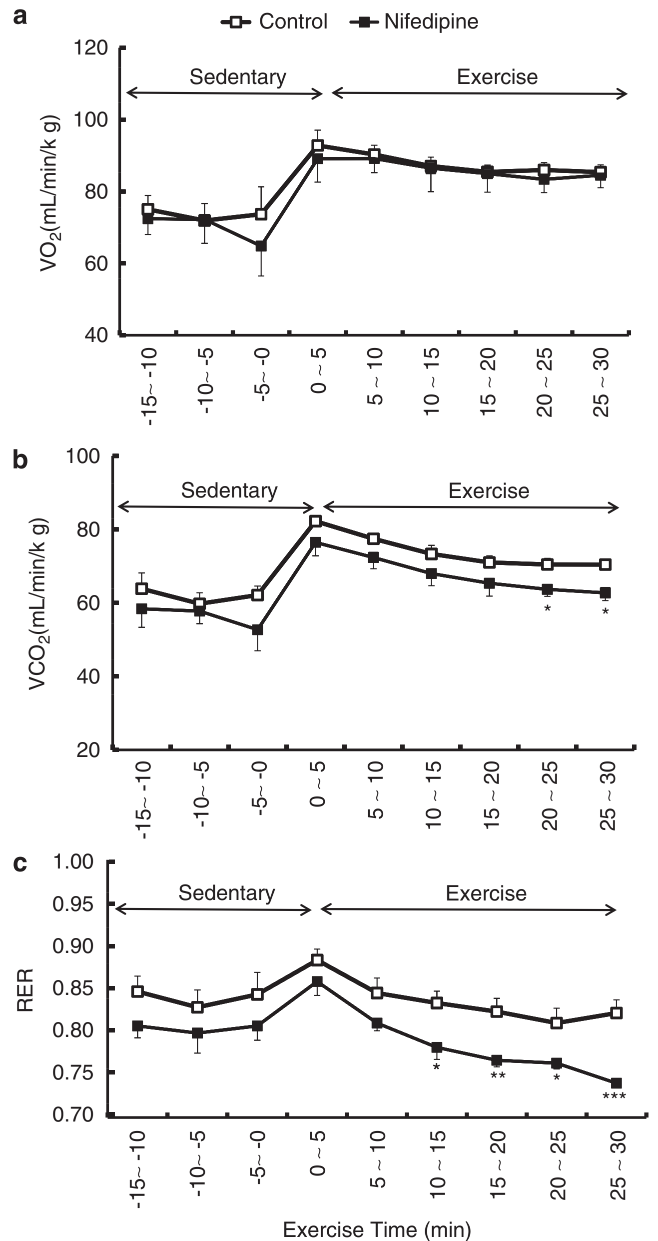

Figure 3 Effect of nifedipine on respiratory exchange ratio (RER) during exercise conditions. Comparison of oxygen consumption (**a**), carbon dioxide production (**b**) and RER during treadmill-induced exercise (10m min^{-1}) (**c**) between control and nifedipine-treated mice (n=6 per group). *P<0.05, **P<0.01, ***P<0.005 *vs.* control.

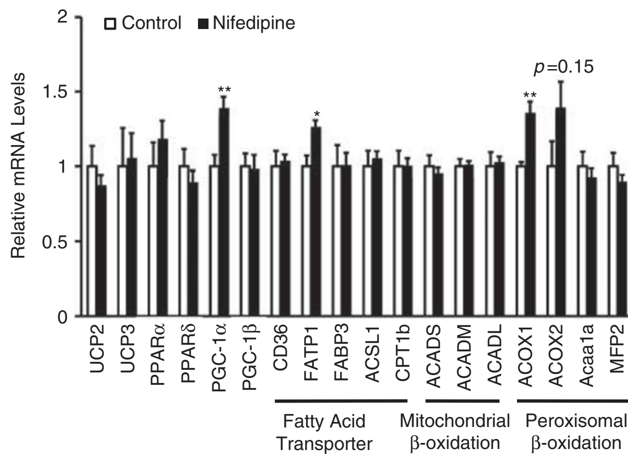

Figure 4 Effect of nifedipine on expression of markers indicating energy expenditure and fat metabolism in soleus muscle. Comparison of gene expression levels in control and nifedipine-treated mice (n=8 per group). Values were normalized to that of β-actin. *P<0.05, **P<0.01 *vs*. control. Acaa1a, acetyl-CoA acyltransferase 1A; ACADL, acyl-CoA dehydrogenase, long chain; ACADM, acyl-CoA dehydrogenase, medium chain; ACADS, acyl-CoA dehydrogenase, short chain; ACOX, acyl-CoA oxidase; ACSL1, acyl-CoA synthetase long-chain family member 1; CD, cluster of differentiation; CPT1b, carnitine palmitoyl-transferase 1B; FABP, fatty acid binding protein; FATP1, fatty acid transport protein 1; MFP2, multifunctional protein 2; PPAR, peroxisome proliferator-activated receptor; UCP, uncoupling protein.

nifedipine-treated mice significantly decreased compared with control mice (25–30 min exercise; P=0.0005, Figure 3c). These results agree with a spontaneous activity analysis showing reduced RER in nifedipine-treated mice (see Figure 2c). We also investigated the effect of nifedipine treatment on exercise tolerance (Supplementary Figure S1) and observed no significant change in endurance capacity compared with control mice. Overall, our findings suggest that nifedipine treatment increases lipid utilization rather than carbohydrates as an energy source during both spontaneous and treadmill-induced exercise, likely leading to decreased fat tissue weight.

Nifedipine treatment increases PGC-1α mRNA levels and β-oxidation in skeletal muscle

To determine the molecular basis for metabolic changes seen in nifedipine-treated mice, we undertook quantitative real-time PCR analysis to examine mRNA levels of factors regulating energy expenditure and metabolic activity in skeletal muscle using eNOS-deficient mice (Figure 4). PGC-1α expression was significantly increased in the soleus muscle of nifedipine-treated mice (P=0.0051, Figure 4). The expression of fatty acid transport protein 1 and acyl-coenzyme A oxidase 1, a peroxisomal β-oxidation-related gene, was significantly increased in nifedipine-treated mice compared with control mice (P=0.016 and P=0.008, respectively, Figure 4), suggesting that nifedipine-induced PGC-1α in skeletal muscle may activate fat utilization by enhancing fatty acid transport and β-oxidation. Expression levels of blood vascular markers, including CD31, Tie2 and VE-cadherin, in the soleus muscle were equivalent in nifedipine-treated and untreated mice, suggesting that non-hypotensive drug doses do not induce blood vessel formation in skeletal muscle (data not shown).

Nifedipine treatment increases insulin sensitivity

It has recently been reported that nifedipine administration significantly suppresses serum insulin levels in KK-A^y mice, suggesting that nifedipine treatment improves hyperinsulinemia.[17] Therefore, we investigated whether serum insulin or glucose levels would be altered by nifedipine treatment at the dosages outlined in the Methods section. Although nifedipine-treated mice exhibited no change in serum glucose levels, they did show lower serum insulin levels than control mice both after fasting and under *ad libitum* feeding conditions (P=0.024 and P=0.0032, respectively, Figure 5a). The HOMA-IR index was also significantly lower in nifedipine-treated mice (P=0.030, Figure 5b) than in control mice. These results indicate that nifedipine treatment promotes greater insulin sensitivity. To confirm this finding, we conducted ITT and GTT tolerance tests, and found a tendency toward increased insulin sensitivity in nifedipine-treated mice (P=0.089, Figures 5c and d). Serum glucose levels after glucose injection were significantly improved in nifedipine-treated mice compared with control mice (P=0.021 and P=0.024, respectively, Figures 5e and f). These results suggest that the attenuation of hyperinsulinemia by nifedipine treatment may contribute to its beneficial effects on energy expenditure.

DISCUSSION

In this study, we demonstrated that long-term treatment with non-hypotensive doses of nifedipine enhances whole-body energy expenditure by increasing oxygen consumption, resulting in reduced body fat gain in mildly obese mice. In a clinical trial, Tuck *et al.*[15] showed a significant decrease in body weight after nifedipine treatment (30–180 mg per day orally for 3 months) in patient groups classified as obese (BMI > 30) or overweight (25 ⩽ BMI ⩽ 30) with mild-to-moderate hypertension, whereas patients of normal weight (BMI < 25) did not show decreases in body weight. By contrast, nifedipine reduced blood pressure equally well in all hypertensive patient groups. Radin *et al.*[16] also reported that nifedipine treatment (average dose 114 mg kg^{-1} per day in rat chow for 3 months) decreased body weight and abdominal and subcutaneous fat masses of SHHF/Mcc-fa^{cp}/fa^{cp} rats, which are a spontaneous rat model of hypertension, obesity, insulin resistance, glucose intolerance and hyperlipidemia. These reports indicate that antihypertensive doses of nifedipine treatment can decrease body weight in obese hypertensive subjects. However, it is possible that nifedipine's activity as a vasodilator at these doses promotes blood flow into skeletal muscle and enhances the fatty acid supply as an energy source to these tissues. Therefore, we investigated whether nifedipine treatment directly suppresses body weight without an antihypertensive effect. The present study revealed that non-hypotensive doses of nifedipine (a dose of 1 mg kg^{-1} per day via infusion pump for 4 months) significantly reduced epididymal WAT and body weight gain, suggesting that the nifedipine has an anti-obesity effect. In agreement, Russell *et al.*[21,22] reported that nifedipine treatment (a dose of 15 mg kg^{-1} per day in food pellets for 7.5 months) moderately decreased the body weight of JCR:LA-cp/cp rats, a normotensive obese rat model with marked hyperlipidemia and insulin resistance, indicating that nifedipine directly affects weight loss. Iwai *et al.*[17] also reported that non-hypotensive doses of nifedipine (average dose 1.5 mg kg^{-1} per day in lab chow for 5 weeks) significantly decreased epididymal WAT weight and moderately decreased whole-body weight of obese diabetic KK-A^y mice by stimulating adipocyte differentiation. This observation strongly suggests that non-hypotensive doses of nifedipine treatment enhance fat metabolism.

The dihydropyridine receptor, a target of nifedipine, is expressed in skeletal muscle cells[23] although its function in those tissues remains unknown. A previous study reported that nifedipine treatment increases fatty acid oxidation in C2C12 myotubes.[24] Thus, nifedipine may directly inhibit the dihydropyridine receptor expressed in skeletal

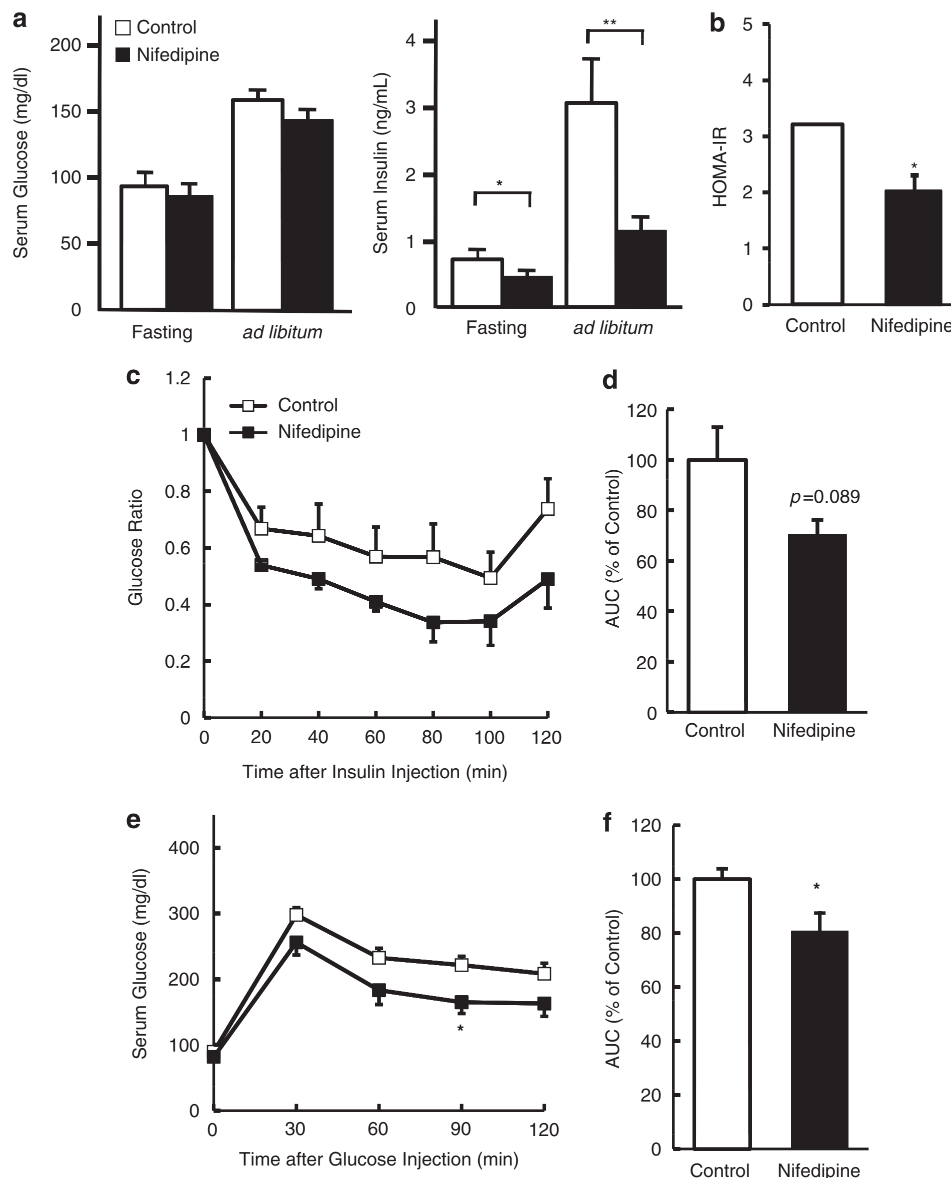

Figure 5 Effect of nifedipine on insulin sensitivity and glucose tolerance. Comparison serum glucose and insulin levels (**a**) and HOMA-RI index (**b**) after fasting (fasting) or under *ad libitum* feeding (*ad libitum*) between control and nifedipine-treated mice. Comparison of the results of insulin tolerance (**c**, **d**) and glucose tolerance (**e**, **f**) tests between control and nifedipine-treated mice (n=6–8 per group). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control. AUC, area under the curve.

muscle cells and may contribute to increased energy expenditure and fatty acid oxidation, although the detailed mechanism by which non-hypotensive doses of nifedipine impact obese subjects remains unclear.

Another dihydropyridine Ca antagonist, benidipine hydrochloride, an L-type Ca channel blocker, reportedly reduces blood pressure and inhibits weight gain in rats and dogs.[25,26] Benidipine treatment also seemingly decreases body weight and body fat in obese mice models after pretreatment with monosodium-L-glutamate.[27] However, only nifedipine and benidipine have been reported to have anti-obesity effects among Ca channel blockers. Therefore, the anti-obesity effect of both the drugs may be unique to these Ca channel blockers.

Peroxisomal β-oxidation reportedly functions in thermogenesis, because the first oxidation step catalyzed by fatty acyl-CoA oxidase is not coupled to ATP production, and thus energy is released as heat.[28] In this study, we found that PGC-1α and acyl-coenzyme A oxidase 1 expression levels were increased by nifedipine treatment. It has recently been reported that PGC-1α regulates factors required for peroxisomal β-oxidation, such as acyl-coenzyme A oxidase 1.[29] Therefore, nifedipine may exert an anti-obesity effect by upregulating PGC-1α, thus increasing thermogenesis mediated by acyl-coenzyme A oxidase 1 in skeletal muscle. In addition, we also observed fatty acid transport protein 1 upregulation in skeletal muscle of nifedipine-treated mice, suggesting that nifedipine enhances fatty acid transport into skeletal muscle cells.

Recently, studies have suggested that nifedipine pleiotropically activates the eNOS pathway.[7,30] Indeed, Iwai *et al.*[17] reported that nifedipine treatment reduced superoxide levels by increasing eNOS activity. However, in our study of eNOS-deficient mice, we found that the nifedipine has eNOS-independent effects on increasing the energy expenditure in skeletal muscle and insulin sensitivity. Thus, it remains unclear whether nifedipine-induced eNOS upregulation is a major pathway for countering obesity and improving insulin resistance.

Finally, we found that nifedipine treatment enhances energy expenditure by increasing PGC-1α expression in skeletal muscle. However, it is unclear whether nifedipine-induced PGC-1α upregulation has a direct effect on insulin sensitivity. Previous studies have reported that expression of PGC-1α is upregulated by prolonged exercise in rats[31] and that moderate PGC-1α overexpression in rat skeletal muscle *in vivo* improves insulin sensitivity.[32] Future investigations could address whether prolonged exercise combined with nifedipine treatment could synergistically elevate PGC-1α mRNA levels and improve insulin sensitivity in patients with obesity and/or type 2 diabetes.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)