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Altered lipid metabolism in vasopressin V_{1B} receptor-deficient mice

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ABSTRACT

We previously reported that insulin sensitivity was increased in vasopressin V_{1B} receptor-deficient ($V_{1B}R^{-/-}$) mice. Here, we investigate the lipid metabolism in $V_{1B}R^{-/-}$ mice. Despite having lower body weight, $V_{1B}R^{-/-}$ mice had significantly greater fat weight of the epididymal white adipose tissue than $V_{1B}R^{+/+}$ mice. Glycerol production and β-oxidation were suppressed in $V_{1B}R^{-/-}$ mice under a fasting condition, and isoproterenol-stimulated lipolysis in differentiated adipocytes was significantly decreased in $V_{1B}R^{-/-}$ mice. These results indicated that lipolysis was inhibited in $V_{1B}R^{-/-}$ mice. On the other hand, lipogenesis was promoted by the increased metabolism from glucose to lipid. Furthermore, our *in vivo* and *in vitro* analyses showed that the secretion of adiponectin was increased in $V_{1B}R^{-/-}$ mice, while the serum leptin level was lower in $V_{1B}R^{-/-}$ mice. These findings indicated that the insulin sensitivity and lipid metabolism were altered in $V_{1B}R^{-/-}$ mice and that the increased insulin sensitivity could contribute to the suppressed lipolysis and enhanced lipogenesis, which consequently resulted in the increased fat weight in $V_{1B}R^{-/-}$ mice.

1. Introduction

The neurohypophyseal peptide [Arg^8]-vasopressin (AVP) is involved in diverse functions, such as the regulation of body fluid homeostasis, vasoconstriction, and adrenocorticotrophic hormone (ACTH) release (Michell et al., 1979). These physiological effects are mediated by three subtypes of AVP receptors designated V_{1A}, V_{1B}, and V_2, all of which belong to G protein-coupled receptors (Birnbaumer, 2000). The vasopressin V_{1A} receptor is expressed ubiquitously, while the vasopressin V_{1B} receptor is specifically expressed in pituitary corticotrophs and pancreatic islets (Oshikawa et al., 2004). Both of them bring about phosphatidylinositol hydrolysis, leading to the mobilization of intercellular Ca^{2+}. The vasopressin V_2 receptor is primarily found in the kidney and is linked to adenylate cyclase and the production of cAMP, in association with antidiuresis (Thibonnier, 1988).

AVP plays a crucial role in the regulation of the blood levels of glucose and free fatty acid. AVP infusions induce an increase in circulating glucose levels (Rofe and Williamson, 1983; Spruce et al., 1985). This effect is supposed to be due to two distinct actions. AVP stimulates the glucagon and insulin releases from pancreatic islet cells *in vitro* (Oshikawa et al., 2004; Yibchok-anun and Hsu, 1998) via the vasopressin V_{1B} receptor (Fujiwara et al., 2007b; Oshikawa et al., 2004; Richardson et al., 1995). In addition, AVP can act directly in the liver to stimulate glucose production. In hepatocytes, AVP interacts with specific vasopressin V_1 receptor sites (Keppens and de Wulf, 1979) and promotes glycogenolysis and gluconeogenesis (Hems, 1977). These actions of AVP in the liver are distinct from those of glucagon and are mediated by a calcium-dependent pathway (Garrison and Wagner, 1982) via the vasopressin V_{1A} receptor. With regard to the lipid metabolism, AVP is known to decrease circulating ketone bodies (Rofe and Williamson, 1983) and to suppress isoproterenol-induced lipolysis depending on the existence of calcium ion (Tebar et al., 1996) via the vasopressin V_{1A} receptor. This finding indicates that AVP stimulates the lipid metabolism via the vasopressin V_{1A} receptor. Consisting with the finding, vasopressin V_{1A} receptor-deficient ($V_{1A}R^{-/-}$) mice exhibit a phenotype with the hypermetabolism of fat (Hiroyama et al., 2007a).

We generated $V_{1A}R^{-/-}$ and V_{1B} receptor-deficient ($V_{1B}R^{-/-}$) mice, which are not lethal and have no apparent anatomical anomalies. $V_{1A}R^{-/-}$ mice exhibit the hypermetabolism of fat and muscular protein, and insulin resistance (Aoyagi et al., 2007; Hiroyama et al., 2007a,b). These characteristics are in part due to an interference of insulin signaling by a deficiency of the vasopressin V_{1A} receptor, which could inhibit the activation of Gs signaling to hormone-sensitive lipase (Hiroyama et al., 2007a). On the other hand, $V_{1B}R^{-/-}$ mice exhibit the impairment of ACTH, corticosterone, and insulin secretion (Oshikawa et al., 2004; Tanoue et al., 2004). Recently, we have reported that $V_{1B}R^{-/-}$ mice have enhanced insulin sensitivity (Fujiwara et al., 2007a), which is a contrastive phenotype compared to $V_{1A}R^{-/-}$ mice. Here, we investigated the lipid metabolism in $V_{1B}R^{-/-}$ mice and found suppressed lipolysis and enhanced lipogenesis due to increased insulin sensitivity, leading to increased fat weight.

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2. Materials and methods

2.1. Animals

The generation of vasopressin $\mathbf{V_{1B}}$ receptor-deficient ($\mathbf{V_{1B}}R^{-/-}$) mice was described previously (Tanoue et al., 2004). Briefly, by homologous recombination, we disrupted exon 1, which contains the translation initiation codon. The generated mutant mice were of a mixed genetic background of 129 Sv and C57BL/6. Non-$\mathbf{V_{1B}}$ receptor-deficient littermates ($\mathbf{V_{1B}}R^{+/+}$) were used as age-matched control subjects for $\mathbf{V_{1B}}R^{-/-}$ mice and maintained on the 129 Sv and C57BL/6. Animals were housed in micro-isolator cages in a pathogen-free barrier facility. $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice were housed on a 12 h light/dark cycle with *ad libitum* access to food and water except when the experimental protocol specified otherwise. Male mice were used for this study under a feeding condition with a normal chow diet. Male $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice were weighed once weekly from weaning at 3 weeks of age. The body weights were measured using a microbalance. The epididymal white adipose tissue deposits were dissected immediately after mice were killed by cervical dislocation under anesthesia with diethyl ether. All experimentation was performed under the guidelines for the Care and Use of Laboratory Animals of the National Research Institute for Child Health and Development.

2.2. Biochemical analysis

The serum free fatty acid, triglyceride, and cholesterol obtained from the inferior vena cava of mice at 8–10 weeks of age under the feeding or 24 h-fasting condition were measured using the NEFA *C*-test Wako, triglyceride *E*-test Wako, and cholesterol *E*-test Wako (Wako, Tokyo, Japan), respectively. Serum adiponectin and leptin were measured using an Adiponectin EIA kit (SPI-BIO, Montigny Le Bretonneux, France), and a Leptin EIA kit (SPI-BIO, Montigny Le Bretonneux, France).

2.3. Electrospray tandem mass spectrometry

Electrospray tandem mass spectrometry for the analysis of carnitine and acylcarnitines in dried blood specimens collected from the tail vein of mice at 8–10 weeks of age under the feeding or 24 h-fasting condition was carried out as described previously (Hiroyama et al., 2007a).

2.4. Isoproterenol-induced lipolysis assay in differentiated white fat precursor cells

White fat precursor cells were isolated from $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice at 3–4 weeks of age by collagenase digestion as described previously (Hiroyama et al., 2007a). The precursor cells were seeded on a 96-well plate at $1.2–2.0\times10^4$/well, and the medium was changed to a fresh medium 20 h later. Adipocyte differentiation was induced by treating confluent cells in an induction medium (10% FBS-DMEM containing 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethazone, 0.125 mM indomethacine, 20 nM insulin (Sigma, MO, USA), and 1 nM T3 for 3 days, and the lipolysis assay was then carried out in a phenol red free-DMEM medium. Isoproterenol was added at the indicated concentrations for 5 h, and the glycerol content in the culture medium was measured with a lipolysis assay kit (Zen-Bio Inc., NC, USA).

For oil-red O staining, cells fixed using 10% formalin for 1 h were soaked in 3 mg/ml oil-red O in 60% isopropanol for 1 h and then washed with 60% isopropanol for 2 min. For the quantification of intracellular triglyceride, the living cells were washed with PBS once, and then 200 μl of PBS was added into each well. Then, 5 μl of AdipoRed (BioWhittaker Inc., MD, USA) was added into each well, and the cells were incubated for 10 min at room temperature. After 10 min, the fluorescence was measured with excitation at 485 nm and emission at 535 nm. To determine the protein concentration, separate wells were prepared, and the protein concentration was quantified using the BCA protein assay (Pierce, IL, USA).

2.5. Insulin-induced lipogenesis and 2-deoxyglucose uptake assay in differentiated white fat precursor cells

For the glucose incorporation experiment, the precursor cells were seeded on a 6-well plate, and the medium was changed to a fresh medium 20 h later. Adipocyte differentiation was induced by treating confluent cells with an induction medium for 3 days for the glucose incorporation experiment. After a preincubation period of 30 min at 37 °C in Krebs–Ringer buffer containing 0.55 mM D-glucose, 15 mM sodium bicarbonate, 10 mM Hepes, and 1% BSA (pH 7.4), the cells were incubated with the indicated concentration of insulin for 5 min at 37 °C. The adipocytes were then incubated with 0.12 μCi D-[U–^{14}C] glucose/ml (Perkin Elmer, MA, USA) for 60 min. The supernatant was removed, and then the reaction was terminated by the addition of 1 ml chloroform:methanol (1:2). The extracts were transferred into 15 ml tubes. Three hundred μl chloroform was added, and then 600 μl of 1 N HCl was added to separate the layers by centrifugation (1000 *g*, 5 min). The lower phase was measured. Glucose incorporation into lipids is expressed as nano-moles of glucose incorporated per milligram of lipid.

For the 2-deoxyglucose uptake experiment, the precursor cells were seeded on a 6-well plate and differentiated for 3 days. The differentiated cells were then used for the 2-deoxyglucose uptake experiment. After a preincubation period of 30 min at 37 °C in Krebs–Ringer buffer containing 15 mM sodium bicarbonate, 10 mM Hepes, and 1% BSA (pH 7.4), the cells were incubated with the indicated concentration of insulin for 25 min at 37 °C. The adipocytes were then incubated with 50 μM 2-deoxy-D-[^{3}H] glucose (0.5 μCi/ml) (Perkin Elmer, MA, USA) for 30 min, and the reaction was terminated by the addition of 10 μm cytochalasin B (Sigma, MO, USA). Cells were washed three times with ice-cold PBS and lysed with 0.5 ml Solvable (Perkin Elmer, MA, USA). The radioactivity taken up by the cells was measured using a liquid scintillation counter.

2.6. Tissue triglyceride, free fatty acid, and cholesterol contents

The liver and skeletal muscle of mice at 8–10 weeks of age under the feeding or 24 h-fasting condition were dissected, and the exact mass of the sample was then determined using a microbalance. The tissues were homogenated in 100 μl of H_2O, and 600 μl of chloroform: methanol (1:2) was then added to the homogenates. The homogenates were mixed and placed overnight at room temperature to extract the total lipids. Two hundred ml chloroform was added, and then 200 μl of 1 N HCl was added to separate the layers by centrifugation. The lower phase was dried and resolved in the appropriate volume of isopropanol. The solutions were used for triglyceride, free fatty acid, and cholesterol measurements using the triglyceride *E*-test Wako (Wako, Tokyo, Japan), NEFA *C*-test Wako, cholesterol *E*-test Wako, respectively.

2.7. Adipokine secretion from adipocytes

For this experiment, the precursor cells were seeded on a 6-well plate, and the medium was changed to a fresh medium 20 h later. Adipocyte differentiation was induced by treating confluent cells with an induction medium for 5 days. After the differentiation, the culture medium was replaced with 0.5% BSA-DMEM. The cells were then incubated for 4 h. The level of adiponectin was measured using the Adiponectin EIA kit (SPI-BIO, Montigny Le Bretonneux, France).

2.8. Adipokine expression during the differentiation of adipocytes derived from $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice

For this experiment, the precursor cells were seeded on 10-cm dishes, and the medium was changed to a fresh medium 20 h later. Adipocyte differentiation was induced by treating confluent cells in an induction

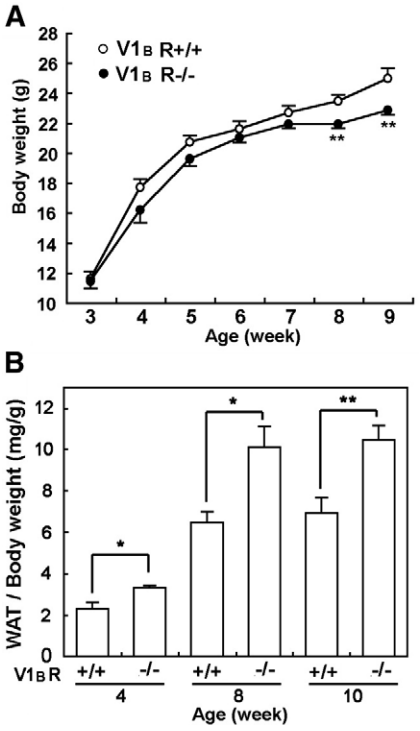

Fig. 1. Body weights (BW) and ratio of epididymal fat deposits to BW. (A) The body weights were measured in $\mathbf{V_{1B}}R^{+/+}$ ($n=17$) and $\mathbf{V_{1B}}R^{-/-}$ ($n=22$) mice on a normal diet from 3 to 9 weeks of age. (B) The epididymal fat weights were measured, and the ratios of epididymal fat deposits to BW were plotted on age (week) as the *x* axis. The *y* axis is expressed per unit of fat mass to body weight in $\mathbf{V_{1B}}R^{+/+}$ mice and $\mathbf{V_{1B}}R^{-/-}$ mice. Each point represents one observation. Values are the means ± S.E.M. Significance: **, $0.001<P<0.01$ vs $\mathbf{V_{1B}}R^{+/+}$ mice by the unpaired Student's *t*-test.

medium for 1, 3, and 5 days. After the differentiation, the cells were harvested, and then the RNAs were purified using ISOGENE (Nippon Gene, Tokyo, Japan).

2.9. RNA expression analysis by RT-PCR

The RNAs were purified from the liver and white adipose tissue of mice at 8–10 weeks of age under the feeding or 24 h-fasting condition using ISOGENE (Nippon Gene, Tokyo, Japan). The RNAs were reverse-transcribed using Superscript III (Invitrogen, CA, USA). The amplification reactions were conducted on a PCR thermal cycler (TaKaRa, Kyoto, Japan) at 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min for 25 cycles using specific primer sets: GAPDH, 5′-GGTCATCATCTCCGCCCCTTC-3′ and 5′-CCACCACCCTGTTGCTGTAG-3′; adiponectin, 5′-AAGGACAAGGCCGT-TCTCT-3′ and 5′-TATGGGTAGTTGCAGTCAGTTGG-3′; PPARγ2, 5′-GCTGT-TATGGGTGAAACTCTG-3′ and 5′-ATAATAAGGTGGAGATGCAGG-3′; HMG CoA reductase, 5-GATTCTGGCAGTCAGTGGGAA-3′ and 5′-GTTGTAGCCG-CCTATGCTCC-3′; AACS, 5′-GTCAGTGCTGGAGGAGAAGG-3′ and 5′-TGTC-AGTGCCTCCTGAGATG-3′; LPL, 5′-AGGGCTCTGCCTGAGTTGTA-3′ and 5′-AGAAATTTCGAAGGCCTGGT-3′; UCP1, 5′-GAGCCATCTGCATGGGA-TC-3′ and 5′-GTACGCTTGGGTACTGTCC-3′. The products were detected under UV illumination.

2.10. Statistical analysis

All values are expressed as the means ± standard error (S.E.M.). Statistical analyses were performed using the unpaired Student's *t*-test or two-way analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference, used as a *post hoc* test. $P<0.05$ by unpaired Student's *t*-test was considered statistically significant.

3. Result

3.1. Increased ratio of epididymal white adipose tissue depots/body weights

Growth curves in weight gain were measured from 3 to 9 weeks of age in $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice under the condition with a normal chow diet. The weights of $\mathbf{V_{1B}}R^{-/-}$ mice tended to be lower than those of $\mathbf{V_{1B}}R^{+/+}$ mice from 3–7 weeks of age, and the weights of $\mathbf{V_{1B}}R^{-/-}$ mice

Table 1
Analysis of (acyl) carnitines under the feeding and fasting conditions

		FC	C2	C3	C4	C5	C5:1	OH–C5
	$\mathbf{V_{1B}}R^{+/+}$(Feed)	41.63±0.775	30.04±0.811	1.0875±0.0799	0.514±0.037	0.309±0.019	0.028±0.0022	0.115±0.0031
	$\mathbf{V_{1B}}R^{-/-}$(Feed)	39.84±1.137	28.54±0.43	0.974±0.0465	0.6585±0.033	0.335±0.0106	0.025±0.0018	0.086±0.0044
	$\mathbf{V_{1B}}R^{+/+}$(Fast)	40.28±1.705	39.36±1.135	1.022±0.0651	0.35±0.0169	0.325±0.0273	0.031±0.0013	0.128±0.0024
	$\mathbf{V_{1B}}R^{-/-}$(Fast)	36.95±0.845	33.69±0.806	0.843±0.072	0.389±0.0255	0.355±0.0251	0.026±0.0024	0.085±0.0005
Significance	Feed($\mathbf{V_{1B}}R^{+/+}$ vs $^{-/-}$)	0.2281	0.1399	0.2545	0.0194[a]	0.2659	0.3126	0.0007[c]
	Fast($\mathbf{V_{1B}}R^{+/+}$ vs $^{-/-}$)	0.1181	0.0036[b]	0.1023	0.2325	0.4415	0.0795	<0.0001[c]
	$\mathbf{V_{1B}}R^{+/+}$(Feed vs Fast)	0.4917	0.0002[c]	0.5429	0.0037[b]	0.6428	0.2645	0.0104[a]
	$\mathbf{V_{1B}}R^{-/-}$(Feed vs Fast)	0.0761	0.0005[c]	0.1649	0.0002[c]	0.4835	0.8718	0.8281
		C5DC	C6	C8	C10	C10:1	C12	C14:1
	$\mathbf{V_{1B}}R^{+/+}$(Feed)	0.042±0.0059	0.113±0.0038	0.07±0.0018	0.06±0.0029	0.078±0.0072	0.089±0.0048	0.094±0.0073
	$\mathbf{V_{1B}}R^{-/-}$(Feed)	0.042±0.0024	0.114±0.004	0.062±0.0032	0.062±0.0037	0.072±0.0038	0.071±0.0019	0.075±0.004
	$\mathbf{V_{1B}}R^{+/+}$(Fast)	0.027±0.0019	0.129±0.0142	0.098±0.0055	0.099±0.0055	0.117±0.0024	0.162±0.0069	0.237±0.0125
	$\mathbf{V_{1B}}R^{-/-}$(Fast)	0.034±0.003	0.101±0.0057	0.084±0.0044	0.085±0.0034	0.111±0.0141	0.137±0.0095	0.192±0.0197
Significance	Feed($\mathbf{V_{1B}}R^{+/+}$ vs $^{-/-}$)	0.9399	0.7924	0.063	0.7553	0.4813	0.0069[b]	0.0567
	Fast($\mathbf{V_{1B}}R^{+/+}$ vs $^{-/-}$)	0.0843	0.1044	0.092	0.0545	0.711	0.0706	0.0928
	$\mathbf{V_{1B}}R^{+/+}$(Feed vs Fast)	0.0428[a]	0.308	0.0014[b]	0.0002[c]	0.0009[c]	<0.0001[c]	<0.0001[c]
	$\mathbf{V_{1B}}R^{-/-}$(Feed vs Fast)	0.0595	0.0875	0.0032[b]	0.0015[b]	0.027[a]	0.0001[c]	0.0004[c]
		C14	C16	OH–C16	C18	OH–C18:1	C18:1	Total
	$\mathbf{V_{1B}}R^{+/+}$(Feed)	0.234±0.0155	1.248±0.0412	0.035±0.0031	0.256±0.01	0.023±0.0018	0.452±0.0347	34.4±0.91
	$\mathbf{V_{1B}}R^{-/-}$(Feed)	0.198±0.004	1.155±0.0609	0.025±0.0018	0.28±0.0117	0.023±0.0009	0.374±0.0147	32.7±0.52
	$\mathbf{V_{1B}}R^{+/+}$(Fast)	0.404±0.0152	1.706±0.0734	0.055±0.0017	0.401±0.0337	0.045±0.0048	0.957±0.0461	44.6±1.16
	$\mathbf{V_{1B}}R^{-/-}$(Fast)	0.331±0.02	1.415±0.0401	0.04±0.0027	0.347±0.0241	0.034±0.003	0.699±0.0329	38.2±0.75
Significance	Feed($\mathbf{V_{1B}}R^{+/+}$ vs $^{-/-}$)	0.0541	0.2418	0.0242[a]	0.1578	1	0.071	0.1549
	Fast($\mathbf{V_{1B}}R^{+/+}$ vs $^{-/-}$)	0.0204[a]	0.0084[b]	0.0019[b]	0.2326	0.0888	0.0018[b]	0.0017[b]
	$\mathbf{V_{1B}}R^{+/+}$(Feed vs Fast)	<0.0001[c]	0.0006[c]	0.0005[c]	0.0034[b]	0.0027[b]	<0.0001[c]	0.0001[c]
	$\mathbf{V_{1B}}R^{-/-}$(Feed vs Fast)	0.0002[c]	0.0073[b]	0.0015[b]	0.0369[a]	0.0083[b]	<0.0001[c]	0.0003[c]

Values are the means ± S.E.M. These results were obtained from five mice. Unit: micromoles/liter. FC, free carnitine; C, Carbon of carnitine; OH, hydroxy. Significance: [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ vs $\mathbf{V_{1B}}R^{+/+}$ mice by the unpaired Student's *t*-test.

were significantly lower than that of $\mathbf{V_{1B}}R^{+/+}$ mice after 8 weeks (8 weeks; 23.4±0.42 g in $\mathbf{V_{1B}}R^{+/+}$ mice vs 21.9±0.3 g in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.0043, 9 weeks; 24.9±0.68 g in $\mathbf{V_{1B}}R^{+/+}$ mice vs 22.8±0.34 g in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.0049) (Fig. 1A). However, the group comparison by two-way ANOVA followed by the *post hoc* test (Fisher's PLSD) revealed that there was no significant difference in weights ($F_{6,\ 268}$=0.810, P=0.5632). The volumes of food intake were not different between $\mathbf{V_{1B}}R^{-/-}$ and $\mathbf{V_{1B}}R^{+/+}$ mice (data not shown). The epididymal white adipose tissue deposits were weighed, and then the ratios for the fat mass against the body weight in $\mathbf{V_{1B}}R^{+/+}$ mice and $\mathbf{V_{1B}}R^{-/-}$ mice were calculated. The ratios for the fat mass against the body weight in $\mathbf{V_{1B}}R^{-/-}$ mice were significantly higher than those of $\mathbf{V_{1A}}R^{+/+}$ mice at 4, 8 and 10 weeks of age (Fig. 1B), indicating that $\mathbf{V_{1B}}R^{-/-}$ mice were underweight even though $\mathbf{V_{1B}}R^{-/-}$ mice had accumulated more fat deposits.

3.2. Inhibited β-oxidation in $\mathbf{V_{1B}}R^{-/-}$ mice

We investigated the blood levels of free carnitine and acylcarnitines in mice on a normal diet, which indicate the state of β-oxidation, by tandem mass spectrometry (Table 1). The analysis with tandem mass spectrometry revealed that the level of free carnitine (C_0) in $\mathbf{V_{1B}}R^{-/-}$ mice was similar to that of $\mathbf{V_{1B}}R^{+/+}$ mice under both the feeding and fasting conditions and that the free carnitine levels were constant under both the feeding and fasting conditions. Most acylcarnitine levels of $\mathbf{V_{1B}}R^{-/-}$ mice were lower than those of $\mathbf{V_{1B}}R^{+/+}$, especially in the case of C2, OH–C5, C12, C14, C16, and C18:1 under the fasting condition. Consistent with this finding, the total acylcarnitine level was lower in $\mathbf{V_{1B}}R^{-/-}$ mice than in $\mathbf{V_{1B}}R^{+/+}$ mice under the fasting condition, while the total acylcarnitine level was not different between $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice under the feeding condition (the feeding condition; 34.4±0.91 in $\mathbf{V_{1B}}R^{+/+}$ mice vs 32.7±0.52 in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.1549, the fasting condition; 44.6±1.16 in $\mathbf{V_{1B}}R^{+/+}$ mice vs 38.2±0.74 in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.0017). This finding shows that β-oxidation in $\mathbf{V_{1B}}R^{-/-}$ mice was suppressed. In addition, the serum levels of glycerol were examined under the fasting and feeding conditions on a normal diet to investigate the lipid metabolism in $\mathbf{V_{1B}}R^{-/-}$ mice. The glycerol level in $\mathbf{V_{1B}}R^{-/-}$ mice was similar to that in $\mathbf{V_{1B}}R^{+/+}$ mice under the feeding condition (155.5±17.04 μM in $\mathbf{V_{1B}}R^{+/+}$ mice vs 154.5±7.37 μM in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.9588), while the glycerol level in $\mathbf{V_{1B}}R^{-/-}$ mice was significantly lower than that in $\mathbf{V_{1B}}R^{+/+}$ mice under the fasting condition (245.5±28.5 μM in $\mathbf{V_{1B}}R^{+/+}$ mice vs 152.6±24.29 μM in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.0412). This result showed that the glycerol level was increased under the fasting condition in $\mathbf{V_{1B}}R^{+/+}$ mice (vs under the feeding condition, P=0.0351) but not in $\mathbf{V_{1B}}R^{-/-}$ mice (vs under the feeding condition, P=0.9483), indicating that lipolysis was not enhanced in $\mathbf{V_{1B}}R^{-/-}$ mice under the fasting condition and that lipolysis was suppressed in $\mathbf{V_{1B}}R^{-/-}$ mice.

3.3. In vitro analysis for lipolysis

To investigate lipolysis in response to stimulation with a sympathetic β adrenergic receptor agonist, isoproterenol, we carried out an *in vitro* lipolysis assay using differentiated adipocytes. We assessed the differentiation of preadipocytes by oil-red O staining and observed the adipocytes that differentiated from the preadipocytes (Fig. 2A). The triglyceride contents in the adipocytes were measured using an AdipoRed kit. The triglyceride content in adipocytes from $\mathbf{V_{1B}}R^{-/-}$ mice was higher than that in adipocytes from $\mathbf{V_{1B}}R^{+/+}$ mice (8900±421.9 RFU/protein (μg) in $\mathbf{V_{1B}}R^{+/+}$ mice vs 14009±1390.7 RFU/protein (μg) in $\mathbf{V_{1B}}R^{-/-}$ mice, P=0.0126). The rates of lipolysis were significantly lower in $\mathbf{V_{1B}}R^{-/-}$ mice than in $\mathbf{V_{1B}}R^{+/+}$ mice (Fig. 2B). A group comparison by two-way ANOVA followed by the *post hoc* test (Fisher's PLSD) revealed that there was a significant difference between $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice ($F_{4,\ 30}$=9.374, P<0.0001). These results indicated that *in vitro* lipolysis was also suppressed in $\mathbf{V_{1B}}R^{-/-}$ mice.

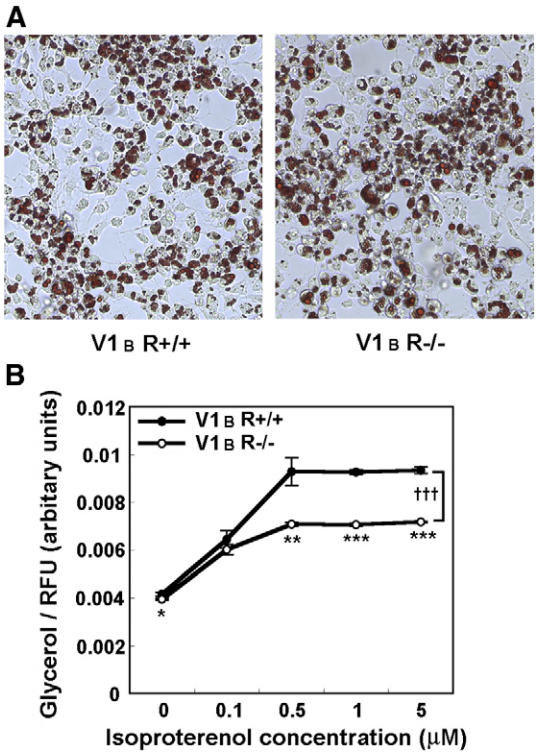

Fig. 2. *In vitro* lipolysis assay using differentiated adipocytes. (A) The precursor cells of white adipocytes from $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice at 8–10 weeks of age on a normal diet were differentiated and then stained using the oil Red O staining method. (B) The rate of lipolysis against the total triglyceride was calculated (n=4 in $\mathbf{V_{1B}}R^{+/+}$, and n=4 in $\mathbf{V_{1B}}R^{-/-}$) and expressed as glycerol/RFU. Values are the means±S.E.M. Significance: *, 0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001 vs $\mathbf{V_{1B}}R^{+/+}$ mice by the unpaired Student's t-test. Furthermore, †††, P<0.001 vs $\mathbf{V_{1B}}R^{+/+}$ mice by two-way ANOVA.

3.4. In vitro lipogenesis and glucose uptake

To investigate lipogenesis in response to insulin stimulation, we carried out an *in vitro* lipogenesis assay using the differentiated adipocytes. [^{14}C]-glucose was incorporated into lipids in a dose-dependent manner. The group comparison by two-way ANOVA followed by the *post hoc* test (Fisher's PLSD) revealed that [^{14}C]-glucose incorporation in $\mathbf{V_{1B}}R^{-/-}$ mice was significantly higher than that in $\mathbf{V_{1B}}R^{+/+}$ mice ($F_{5,24}$=3.849, P=0.0124) (Fig. 3A). Thus, the response to insulin stimulation in $\mathbf{V_{1B}}R^{-/-}$ mice was enhanced, compared to that in $\mathbf{V_{1B}}R^{+/+}$ mice. Furthermore, we assessed whether this higher lipogenesis response to insulin in $\mathbf{V_{1B}}R^{-/-}$ mice resulted from higher glucose uptake using an *in vitro* glucose uptake assay. The glucose uptake in response to insulin stimulation in $\mathbf{V_{1B}}R^{-/-}$ mice was also higher than that in $\mathbf{V_{1B}}R^{+/+}$ mice (Fig. 3B). A group comparison by two-way ANOVA followed by the *post hoc* test (Fisher's PLSD) revealed that there was a significant difference between $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice ($F_{2,12}$=5.167, P=0.0241) (Fig. 3B). Thus, these results indicate that lipogenesis in response to insulin stimulation was promoted in $\mathbf{V_{1B}}R^{-/-}$ mice.

3.5. Cholesterol synthesis enzyme gene expression in the liver

The gene expression levels of lipogenesis enzymes (cholesterol synthesis enzymes), such as HMG CoA reductase (HMG CoA R) and acetyl CoA synthetase (AACS), in the liver were evaluated with a semi-quantitative PCR using samples from mice before or after the 24 h fasting. The expression level of HMG CoA R of $\mathbf{V_{1B}}R^{-/-}$ mice was slightly higher than that of $\mathbf{V_{1B}}R^{+/+}$ mice under the feeding condition (Fig. 3C). The expression level of AACS of $\mathbf{V_{1B}}R^{-/-}$ mice was higher than that of $\mathbf{V_{1B}}R^{+/+}$ mice under both the fasting and feeding conditions (Fig. 3C).

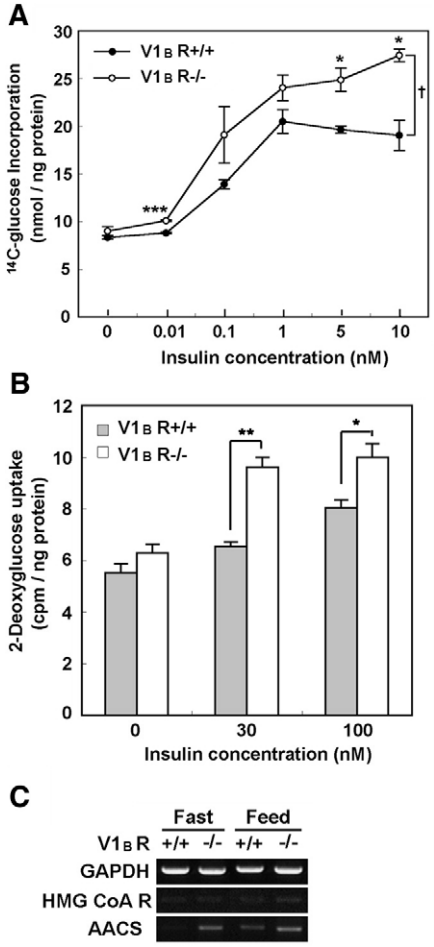

Fig. 3. Enhanced glucose uptake and lipid syntheses in $\mathbf{V_{1B}}R^{-/-}$ differentiated adipocytes. (A) Glucose incorporation assay into the lipid of adipocytes was carried out. The incorporated ^{14}C-glucose into the lipid of adipocytes from $\mathbf{V_{1B}}R^{-/-}$ and $\mathbf{V_{1B}}R^{+/+}$ mice at 8–10 weeks of age on a normal diet ($n=3$ in $\mathbf{V_{1B}}R^{+/+}$, and $n=3$ in $\mathbf{V_{1B}}R^{-/-}$) was measured ($n=3$ in $\mathbf{V_{1B}}R^{+/+}$, and $n=3$ in $\mathbf{V_{1B}}R^{-/-}$). (B) Glucose uptake assay in response to insulin stimulation was performed with mouse adipocytes. The uptaken 2-deoxyglucose in adipocytes from $\mathbf{V_{1B}}R^{-/-}$ and $\mathbf{V_{1B}}R^{+/+}$ mice ($n=3$ in $\mathbf{V_{1B}}R^{+/+}$, and $n=3$ in $\mathbf{V_{1B}}R^{-/-}$) was counted using a scintillation counter. Values are the means±S.E.M. Significance: *, $0.01<P<0.05$; **, $0.001<P<0.01$; ***, $P<0.001$ vs $\mathbf{V_{1B}}R^{+/+}$ mice by the unpaired Student's *t*-test. Furthermore, †, $0.01<P<0.05$ vs $\mathbf{V_{1B}}R^{+/+}$ mice by two-way ANOVA. (C) The gene expression levels of cholesterol synthesis enzymes, HMG CoA R and AACS, in the liver were examined using a semi-quantitative PCR.

These results suggest that the production of cholesterol in the liver was promoted in $\mathbf{V_{1B}}R^{-/-}$ mice, which is consistent with the result of the analysis for cholesterol content in the liver, muscle, and serum and *in vitro* lipogenesis (Fig. 3A and B).

3.6. Serum and tissue triglyceride, free fatty acid, and cholesterol contents

We measured the triglyceride, free fatty acid, and cholesterol levels in the serum, liver, and muscle to investigate the lipid metabolism (Table 2). The triglyceride in the liver of $\mathbf{V_{1B}}R^{+/+}$ mice was increased under the fasting condition, but that in the serum and muscle was not. The triglyceride in the liver and muscle of $\mathbf{V_{1B}}R^{-/-}$ mice was increased under the fasting condition but decreased in the serum. The triglyceride in the serum, liver, and muscle of $\mathbf{V_{1B}}R^{-/-}$ mice was higher than that of $\mathbf{V_{1B}}R^{+/+}$ mice under both the feeding and fasting conditions, but that in serum under the fasting condition was not. The free fatty acid in the serum, liver, and muscle of $\mathbf{V_{1B}}R^{+/+}$ mice was increased under the fasting condition. The free fatty acid in the liver and muscle of $\mathbf{V_{1B}}R^{-/-}$ mice was increased, but that in serum was not changed at all. As in case of the

Table 2
Triglyceride, free fatty acids and cholesterol contents in serum, liver and muscle

	Lipids	Status	$V1_BR^{+/+}$	$V1_BR^{-/-}$	*P* value	*n*
Serum	Triglyceride	Feed	150.5±7.96	177.5±13.45	0.0634	9
		Fast	162.9±15.38	126.3±8.149	0.0441[a]	9
	Free fatty acids	Feed	1.54±0.088	1.92±0.083	0.0006[c]	9
		Fast	2.50±0.084	1.95±0.125	0.002[b]	9
	Cholesterol	Feed	83.6±3.81	111.9±6.82	0.0019[b]	9
		Fast	93.0±4.66	122.2±4.91	0.0006[c]	9
Liver	Triglyceride	Feed	3.78±0.212	6.11±0.514	0.003[b]	5
		Fast	23.1±4.80	31.2±2.22	0.1615	5
	Free fatty acids	Feed	2.21±0.106	2.30±0.109	0.5692	5
		Fast	8.46±0.964	9.78±1.000	0.3801	5
	Cholesterol	Feed	1.56±0.043	1.66±0.121	0.4877	5
		Fast	4.63±0.965	7.67±0.645	0.0310[a]	5
Muscle	Triglyceride	Feed	5.93±0.971	7.40±1.354	0.4023	5
		Fast	5.46±0.526	10.47±1.553	0.0157[a]	5
	Free fatty acids	Feed	1.61±0.094	1.66±0.203	0.8091	5
		Fast	1.82±0.135	2.20±0.115	0.0682	5
	Cholesterol	Feed	0.40±0.032	0.60±0.097	0.0906	5
		Fast	0.35±0.036	0.62±0.114	0.055	5

Values are the means±S.E.M. Significance: [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ vs $\mathbf{V1_BR}^{+/+}$ mice by the unpaired Student's *t*-test.

triglyceride, the free fatty acid in the serum, liver, and muscle of $\mathbf{V_{1B}}R^{-/-}$ mice was higher than that of $\mathbf{V_{1B}}R^{+/+}$ mice under both the feeding and fasting conditions, but that in the serum under the fasting condition was not. Cholesterol in the serum and liver of $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice was increased under the fasting condition, but that in the muscle was not changed. Cholesterol in the serum, liver, and muscle of $\mathbf{V_{1B}}R^{-/-}$ mice was higher than that of $\mathbf{V_{1B}}R^{+/+}$ mice under both the feeding and fasting conditions. These results showed that the contents of triglyceride, free fatty acids, and cholesterol in tissues were increased in $\mathbf{V_{1B}}R^{-/-}$ mice, suggesting that lipid synthesis was promoted and lipolysis was decreased in $\mathbf{V_{1B}}R^{-/-}$ mice.

3.7. Adipokine levels in serum

Since $\mathbf{V_{1B}}R^{-/-}$ mice have enhanced insulin sensitivity (Fujiwara et al., 2007a) and adipokines are known to influence insulin sensitivity (Farooqi et al., 2002; Kubota et al., 2002) as well as lipolysis, we measured the serum levels of adipokines, such as adiponectin and leptin. The level of adiponectin in $\mathbf{V_{1B}}R^{-/-}$ mice was not different from that in $\mathbf{V_{1B}}R^{+/+}$ mice under the fasting condition, but the level of adiponectin in $\mathbf{V_{1B}}R^{-/-}$ mice was higher than that in $\mathbf{V_{1B}}R^{+/+}$ mice under the feeding condition (Fig. 4A) (feeding; 5.01±0.304 μg/ml in $\mathbf{V_{1B}}R^{+/+}$ mice vs 5.95±0.136 μg/ml in $\mathbf{V_{1B}}R^{-/-}$ mice, $P=0.0169$, fasting; 5.9±0.061 μg/ml in

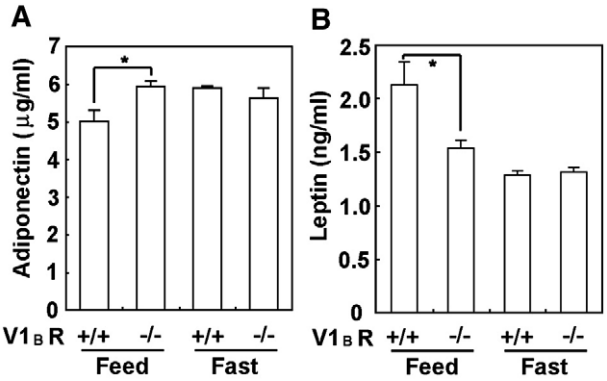

Fig. 4. Measurements of the serum levels of adiponectin and leptin. The serum levels of adiponectin (A) and leptin (B) in serum were analyzed in $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ male mice at 8–10 weeks of age on a normal diet under the feeding and fasting (for 24 h) conditions ($n=5$ in $\mathbf{V_{1B}}R^{+/+}$, and $n=5$ in $\mathbf{V_{1B}}R^{-/-}$). Values are the means±S.E.M. Significance: *, $0.01<P<0.05$; **, $0.001<P<0.01$ vs $\mathbf{V_{1B}}R^{+/+}$ mice by the unpaired Student's *t*-test.

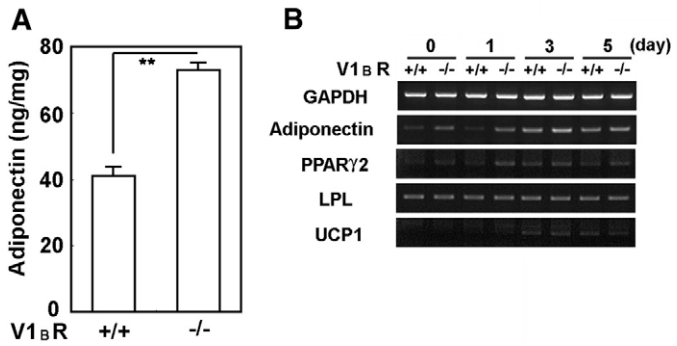

Fig. 5. Secretion of adipokines from adipocytes and the genes expressions in them. Preadipocytes were isolated from 8–10-week-old mice on a normal diet as described in the method, and (A) the secretion of adiponectin from differentiated adipocytes was examined (adiponectin; $n=3$ in $\mathbf{V_{1B}}R^{+/+}$, and $n=3$ in $\mathbf{V_{1B}}R^{-/-}$). Values are the means±S.E.M. Significance: **, $0.001<P<0.01$ vs $\mathbf{V_{1B}}R^{+/+}$ mice by the unpaired Student's t-test. (B) The gene expressions of adipokines and differentiation markers were examined using a semi-quantitative PCR with RNA from adipocytes at 0, 1, 3, or 5 days after the onset of differentiation.

$\mathbf{V_{1B}}R^{+/+}$ mice vs 5.63±0.273 μg/ml in $\mathbf{V_{1B}}R^{-/-}$ mice, $P=0.4086$). The levels of leptin in $\mathbf{V_{1B}}R^{-/-}$ mice were not different from those in $\mathbf{V_{1B}}R^{+/+}$ mice under the fasting condition, but leptin was lower in $\mathbf{V_{1B}}R^{-/-}$ mice than that in $\mathbf{V_{1B}}R^{+/+}$ mice under the feeding condition (feeding; 2.13±0.213 ng/ml in $\mathbf{V_{1B}}R^{+/+}$ mice vs 1.54±0.072 ng/ml in $\mathbf{V_{1B}}R^{-/-}$ mice, $P=0.0314$, fasting; 1.29±0.043 ng/ml in $\mathbf{V_{1B}}R^{+/+}$ mice vs 1.32±0.042 ng/ml in $\mathbf{V_{1B}}R^{-/-}$ mice, $P=0.6379$) (Fig. 4B). The levels of adiponectin and leptin in $\mathbf{V_{1B}}R^{-/-}$ mice were consistent with the finding that $\mathbf{V_{1B}}R^{-/-}$ mice have high sensitivity to insulin (Fujiwara et al., 2007a).

3.8. Adipokine secretion and expression during the differentiation of adipocytes derived from $\mathbf{V_{1B}}R^{+/+}$ and $\mathbf{V_{1B}}R^{-/-}$ mice

We measured the secretions of adiponectin with cultured adipocytes. The levels of adiponectin in $\mathbf{V_{1B}}R^{-/-}$ adipocytes were higher than those in $\mathbf{V_{1B}}R^{+/+}$ adipocytes (Fig. 5A) (adiponectin; 41.0±2.7 ng/mg protein in $\mathbf{V_{1B}}R^{+/+}$ mice vs 73.0±2.2 ng/mg protein in $\mathbf{V_{1B}}R^{-/-}$ mice, $P=0.0028$). We also tried to measure the leptin level in adipocytes, but it was not detected in the culture medium.

Next, we examined the expression of adipokines and differentiation markers in adipocytes during the differentiation from 0 to 5 days using a semi-quantitative PCR. The expression of adiponectin, or PPARγ2, increased during the differentiation and was higher in the adipocytes of $\mathbf{V_{1B}}R^{-/-}$ mice than in those of $\mathbf{V_{1B}}R^{+/+}$ mice (Fig. 5B). The expression of leptin could not be detected in the adipocytes by RT-PCR. The mRNA levels of adiponectin were consistent with the secretion levels of those from adipocytes (Fig. 5A).

4. Discussion

Our *in vivo* and *in vitro* studies revealed that the lipid metabolism was altered in $\mathbf{V_{1B}}R^{-/-}$ mice. The serum glycerol level and β-oxidation did not increase in $\mathbf{V_{1B}}R^{-/-}$ mice under the fasting condition when compared with those under the feeding condition, however, both levels were significantly increased in $\mathbf{V_{1B}}R^{+/+}$ mice. Moreover, the *in vitro* assay in differentiated adipocytes showed that isoproterenol-stimulated glycerol production decreased significantly in $\mathbf{V_{1B}}R^{-/-}$ mice. These results indicate that lipolysis was inhibited in $\mathbf{V_{1B}}R^{-/-}$ mice *in vitro* as well as *in vivo*. In addition to inhibited lipolysis, enhancement of lipogenesis was observed because the glucose uptake into adipocytes and incorporation into the lipid of adipocytes were increased. Thus, lipolysis was suppressed, but lipogenesis was enhanced in $\mathbf{V_{1B}}R^{-/-}$ mice. Since insulin has long-term effects on the expression of lipogenic genes (Assimacopoulos-Jeannet et al., 1995) via the transcription factor sterol regulatory element-binding protein-1 (SREBP1) (Kersten, 2001), the promoted lipogenesis could be caused by the enhanced insulin sensitivity. On the other hand, the suppressed lipolysis could be in part due to the decreased leptin level in $\mathbf{V_{1B}}R^{-/-}$ mice. It has been reported that leptin strongly stimulates lipolysis (Siegrist-Kaiser et al., 1997) and that the plasma leptin level is affected by insulin sensitivity (Considine et al., 1996). Therefore, increased insulin sensitivity could cause a decreased leptin level, leading to suppressed lipolysis in $\mathbf{V_{1B}}R^{-/-}$ mice. The following are two possible explanations for the altered lipid metabolism in $\mathbf{V_{1B}}R^{-/-}$ mice are included; I) the increased insulin sensitivity and II) the decreased production of leptin (Siegrist-Kaiser et al., 1997).

Plasma levels in adipocytokines are known to vary depending on the insulin sensitivity. For instance, under an insulin hypersensitive condition, adiponectin is increased, and leptin is decreased (Considine et al., 1996; Hotamisligil et al., 1995; Kadowaki and Yamauchi, 2005; Steppan et al., 2001). Because the insulin sensitivity and lipid metabolism were altered in $\mathbf{V_{1B}}R^{-/-}$ mice, we measured the serum level of adiponectin and found that it had increased. Since it is known that adiponectin promotes insulin sensitivity (Kubota et al., 2002), which in turn, stimulates adiponectin secretion (Kadowaki and Yamauchi, 2005), the increased adiponectin level could result from and/or in the increased insulin sensitivity. We also measured the serum level of leptin and found that it decreased in $\mathbf{V_{1B}}R^{-/-}$ mice. Leptin has been reported not only to promote the lipolysis in adipocytes (Siegrist-Kaiser et al., 1997) but also to inhibit the lipogenesis (Bai et al., 1996; Wang et al., 1999). Therefore, the suppressed lipolysis and the enhanced lipogenesis observed in $\mathbf{V_{1B}}R^{-/-}$ mice could be caused by the decreased leptin level. Taking these findings together, we conclude that the lipid metabolism was altered, at least in part, due to the decreased leptin level.

Furthermore, glucagon is known to influence the insulin sensitivity: for examples, glucagon receptor knockout mice exhibit a lower level of blood glucose and hyperglucagonemia (Gelling et al., 2003), accompanied with the increased insulin sensitivity (Sorensen et al., 2006). As described in our previous report, the glucagon secretion was impaired in $\mathbf{V_{1B}}R^{-/-}$ mice (Fujiwara et al., 2007a; Oshikawa et al., 2004). Thus, the decreased glucagon level observed in $\mathbf{V_{1B}}R^{-/-}$ mice could contribute to the insulin sensitivity. In addition, it is also known that growth hormone (GH) affects the weight gain; for example, GH-deficient mice exhibit the decreased body weight and the increased fat weight (Liu et al., 2004). GH also stimulates lipolysis and ketogenesis in humans (Moller et al., 2003). Since we have observed in other experiments that a plasma GH level in $\mathbf{V_{1B}}R^{-/-}$ mice were altered (Nakamura et al., manuscript in preparation), the altered GH level in $\mathbf{V_{1B}}R^{-/-}$ mice could affect the insulin sensitivity as well as weight gain.

Autonomic function and physical activity could influence lipolysis and body weights. We examined the body temperature and found that there was no difference between $\mathbf{V_{1B}}R^{-/-}$ and $\mathbf{V_{1B}}R^{+/+}$ mice (data not shown). In addition, UCP1 expression in the interscapular brown adipose tissue was not different between $\mathbf{V_{1B}}R^{-/-}$ and $\mathbf{V_{1B}}R^{+/+}$ mice (data not shown). These findings suggest that autonomic function and basal metabolic rates were not affected in $\mathbf{V_{1B}}R^{-/-}$ mice. Furthermore, there was no difference in activity as analyzed by analysis with an open-field test (Egashira et al., 2005) as well as in 24 h motor activity as analyzed by analysis with an automated activity counter with infrared sensor (NS-AS01, Neuroscience Inc, Tokyo, Japan) (unpublished data), suggesting that the physical activity was not altered in $\mathbf{V_{1B}}R^{-/-}$ mice. Taken together, the altered weight gain and lipolysis observed in $\mathbf{V_{1B}}R^{-/-}$ mice could be in part due to the decreased glucagon and insulin levels, but not due to differences in autonomic function, physical activity, or basal metabolic rates.

In conclusion, we showed that lipolysis was suppressed and lipogenesis was promoted in $\mathbf{V_{1B}}R^{-/-}$ mice, at least partly due to their altered insulin sensitivity and glucagon and leptin secretion. Thus, blockade of the vasopressin $\mathbf{V_{1B}}$ receptor could influence the lipid metabolism as well as glucose homeostasis.

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