Insulin hypersensitivity in mice lacking the V1b vasopressin receptor

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We have reported that [Arg^8]-vasopressin-stimulated insulin release is blunted in islet cells isolated from V1b receptor-deficient ($V1bR^{-/-}$) mice. In this study, we used $V1bR^{-/-}$ mice to examine the physiological role of the V1b receptor in regulating blood glucose levels *in vivo*, and we found that the fasting plasma glucose, insulin and glucagon levels were lower in $V1bR^{-/-}$ mice than in wild-type ($V1bR^{+/+}$) mice. Next, we evaluated glucose tolerance by performing an intraperitoneal glucose tolerance test (GTT). The plasma glucose and insulin levels during the GTT were lower in $V1bR^{-/-}$ mice than in $V1bR^{+/+}$ mice. An insulin tolerance test (ITT) revealed that, after insulin administration, plasma glucose levels were lower in $V1bR^{-/-}$ mice than in $V1bR^{+/+}$ mice. In addition, a hyperinsulinaemic–euglycaemic clamp study showed that the glucose infusion rate was increased in $V1bR^{-/-}$ mice, indicating that insulin sensitivity was enhanced at the *in vivo* level in $V1bR^{-/-}$ mice. Furthermore, we found that the V1b receptor was expressed in white adipose tissue and that insulin-stimulated phosphorylation of Akt as an important signaling molecule was increased in adipocytes isolated from $V1bR^{-/-}$ mice. Thus, the blockade of the V1b receptor could result, at least in part, in enhanced insulin sensitivity by altering insulin signalling in adipocytes.

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The neurohypophysial hormone [Arg^8]-vasopressin (AVP) is known to promote vascular smooth muscle cell contraction, water reabsorption in the kidney and adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland (Altura & Altura, 1977; Gillies *et al.* 1982; Schrier *et al.* 1993; Nielsen *et al.* 1995). AVP also regulates the blood glucose level by stimulating gluconeogenesis and glycogenolysis in the liver (Hems & Whitton, 1973; Michell *et al.* 1979) and by stimulating insulin and glucagon secretion in the pancreas (Dunning *et al.* 1984*a*,*b*; Mineo *et al.* 1997; Yibchok-Anun *et al.* 1999; Abu-Basha *et al.* 2002). These diverse functions of AVP are mediated by specific G protein-coupled receptors. AVP has a high affinity for the oxytocin receptor and three vasopressin-receptor subtypes: the V1a, V1b and V2 receptors. One of these, the V1b receptor, is mainly expressed in the pituitary gland, adrenal gland and pancreas (Ventura *et al.* 1999; Oshikawa *et al.* 2004).

In *in vitro* and *ex vivo* experiments using several AVP-receptor agonists and antagonists, it was demonstrated that AVP-induced insulin and glucagon secretion is mediated via the V1b receptor (Lee *et al.* 1995; Richardson *et al.* 1995; Yibchok-anun & Hsu, 1998; Yibchok-Anun *et al.* 1999; Folny *et al.* 2003). These AVP-induced insulin and glucagon releases are affected by changes in glucose concentrations (Gao *et al.* 1990; Abu-Basha *et al.* 2002). That is, AVP increases glucagon release at low glucose concentrations (5.6 mM or below), while AVP enhances insulin release at high glucose concentrations (more than 7 mM) (Gao *et al.* 1990, 1992; Abu-Basha *et al.* 2002). In addition, intravenous AVP administration increases plasma insulin and glucagon levels in rat and sheep (Dunning *et al.* 1984*a*; Spruce *et al.* 1985; Mineo *et al.* 1997) and plasma glucagon levels in human (Spruce *et al.* 1985). However, little is known about the effects of AVP mediated via the V1b receptor on glucose homeostasis *in vivo*.

In our previous study, we generated V1b receptor-deficient ($V1bR^{-/-}$) mice and demonstrated that the V1b receptor plays a crucial role in maintaining

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basal ACTH secretion as well as in regulating hypothalamic–pituitary–adrenal (HPA) axis activity (Tanoue *et al.* 2004). In addition to the distinct phenotype in the HPA axis, our *in vitro* study with pancreatic islets showed that AVP caused insulin release from the islet cells of wild-type ($V1bR^{+/+}$) mice, while its effect was completely lost in the islet cells of $V1bR^{-/-}$ mice (Oshikawa *et al.* 2004). In pharmacological studies, SSR149415, a selective antagonist for the V1b receptor, inhibited AVP-induced insulin release from islet cells of $V1bR^{+/+}$ mice, whereas OPC-21268, a V1a receptor antagonist, had no effect (Oshikawa *et al.* 2004). Thus, studies of $V1bR^{-/-}$ mice have provided convincing evidence that the V1b receptor participates in AVP-induced insulin release from pancreatic islets, and this finding in the *in vitro* study using $V1bR^{-/-}$ mice led us to investigate the influence of V1b receptor deficiency on the regulation of insulin release and consequent glucose homeostasis *in vivo*.

Methods

Materials

Human insulin used for the glucose tolerance test was purchased from Novo Nordisk (Novolin R; Bagsvared, Denmark). Ascensia Dexter ZII, used for measuring blood glucose in the hyperinsulinaemic–euglycaemic clamp test, was from Bayer Medical (Tokyo, Japan). SuperScript III reverse transcriptase and DMEM culture medium were from Invitrogen (Carlsbad, CA, USA). ISOGEN was from Nippon Gene (Tokyo, Japan) and Ex Taq polymerase was from Takara (Tokyo, Japan). Anti-Akt monoclonal antibody was obtained from Santa Cruz Biotechnology (CA, USA), and anti-phospho-Akt (Ser473) monoclonal antibody was from Cell Signalling Technology (Danvers, MA, USA). Human insulin used for culture was from Sigma-Aldrich (St Louis, MO, USA). The insulin ELISA kit was from Morinaga (Tokyo, Japan). The glucose CII-test kit, glucagon ELISA kit, and all other reagents were from Wako Pure Chemicals (Tokyo, Japan).

Animals

The generation of V1b receptor-deficient ($V1bR^{-/-}$) mice was previously described (Tanoue *et al.* 2004). Briefly, by homologous recombination, we disrupted exon 1, which contains the translation initiation codon. The non-V1b receptor-deficient littermates ($V1bR^{+/+}$) were used as age-matched control subjects for $V1bR^{-/-}$ mice. The genetic background of the animals used for all experiments was a mixture of 129Sv and C57BL/6J. In some sets of experiments, we analysed $V1bR^{-/-}$ on the congenic C57BL/6J background ($V1bR(C57BL/6J)^{-/-}$), which was generated by backcrossing with C57BL/6J mice more than six times, to evaluate the fasting plasma glucose and insulin levels. Animals were housed in micro-isolator cages in a pathogen-free barrier facility and were placed on a 12 h light–dark cycle with *ad libitum* access to food and water except when an experimental protocol was specified. All data presented here were obtained from male mice. All experiments were performed under the approved guidelines for the Care and Use of Laboratory Animals of the National Research Institute for Child Health and Development.

Measurement of plasma glucose, insulin and glucagon

After male mice aged 8–10 weeks had fasted for 18 h (19:00–13:00 h), blood samples were collected from the tail vein in the conscious state in a rodent restrainer (Harvard Apparatus, Inc., MA, USA). For the measurement of plasma glucose levels, blood samples (10 μl) were immediately mixed with 10 volumes of 0.33 M $HClO_4$ to inhibit the utilization of glucose before measurement. The mixtures were centrifuged at 800*g* for 5 min, the supernatants (10 μl) were taken up, and the glucose concentrations were determined by the glucose oxidase method (Glucose CII-test kit) with an identically treated standard. For the measurement of plasma insulin and glucagon levels, blood samples were centrifuged for 10 min at 800*g* and 4°C, and the supernatants were collected and stored at −80°C until use. Plasma insulin and glucagon levels were measured by ELISA assay.

Intraperitoneal glucose tolerance test (GTT)

Male mice aged 8–10 weeks were fasted for 18 h before receiving intraperitoneal (I.P.) administration of 1.5 g glucose (kg body weight)$^{-1}$ in saline (0.9% NaCl). Blood samples of conscious mice were taken from the tail vein in heparinized microcapillary tubes at 0, 10, 30, 60 and 120 min after glucose loading, and the plasma glucose and insulin levels were determined.

Intraperitoneal insulin tolerance test (ITT)

The insulin-tolerance test was performed with male mice aged 8–10 weeks after a 4 h fast. The animals were intraperitoneally injected with 1.0 U (kg body weight)$^{-1}$ of insulin. Blood samples (10 μl) of conscious mice were drawn from the tail vein for the measurement of plasma glucose levels at 0, 10, 30, 60 and 120 min after the insulin injection.

Hyperinsulinaemic–euglycaemic clamp test

The hyperinsulinaemic–euglycaemic clamp test was carried out in non-anaesthetized male mice aged 8–10 weeks according to the method reported previously (Kim *et al.* 2000; Voshol *et al.* 2001). In brief, at least

2 days before the experiments, 10-week-old mice were anaesthetized with sodium pentobarbital (40 mg kg^{-1}, I.P.), and an indwelling catheter was inserted in the left jugular vein as described in previously (Tanoue *et al.* 2002; Aoyagi *et al.* 2007). The catheters were externalized through an incision in the skin flap behind the head, and the animals were returned to individual cages after surgery. The mice showed normal behaviour after 2 days of surgery. Experiments were started after a 16 h fast. A 120 min hyperinsulinaemic condition was conducted with a continuous infusion of insulin at a rate of 15 mU kg^{-1} min^{-1} after 100 mU kg^{-1} bolus administration. Blood samples (20 μl) were collected at 5 min intervals for the immediate measurement of blood glucose concentrations using Ascensia Dexter ZII, and 20% glucose in saline (0.9% NaCl) was infused at variable rates to maintain the blood glucose in a range from 80 to 100 mg dl^{-1}. After the clamp test, mice were then anaesthetized with ether and killed by cervical dislocation. The glucose infusion rate (GIR) was determined during the final 20 min.

Plasma glucose and insulin response to AVP

In male mice aged 8–10 weeks, an aliquot of 100 ng kg^{-1} of AVP was injected into the tail vein, as described by Mineo *et al.* (1997) with some modifications. Food was withdrawn during assay. Blood (100 μl) samples of conscious mice were collected from the tail vein before and 15 min after the AVP injection. Plasma glucose and insulin levels were measured by the ELISA method.

RT-PCR

Mice aged 8–10 weeks were anaesthetized with ether and killed by cervical dislocation. Then, total RNA was isolated from heart, liver, femoral muscle, epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) of mice using ISOGEN. First-strand cDNA was synthesized from 5 μg of DNase-digested total RNA by SuperScript III reverse transcriptase in 20 μl of a reaction volume. The primers were designed as follows: 5′-CTCTGCTGGACACCTTTCTTCATCG-3′ upstream and 5′-CTGATGGACTTTGAAGATTTAGGTG-3′ downstream for the V1a receptor; 5′-ACAGGTGCTCAGCATGTTTG-3′ upstream and 5′-CATCTTCACGGTTCGGATCT-3′ downstream for the V1b receptor; 5′-GTCTCCTCGGAGTTGCGTAG-3′ upstream and 5′-CTGGGAGTCTTCCTCACCTG-3′ downstream for the V2 receptor; 5′-TTCTTCGTGCAGATGTGGAG-3′ upstream and 5′-AGGACGAAGGTGGAGGAGTT-3′ downstream for the oxytocin receptor; and 5′-GCTTCTGCCAAGACCTTCAC-3′ upstream and 5′-CAGTAGGGTTCCCACCTCAA-3′ downstream for the insulin receptor. The GAPDH primers were 5′-CCATCACCATCTTCCAGGAG-3′ upstream and 5′-TCCAGCTCTGGGATGACCTT-3′ downstream as a standard. The PCR reactions were carried out with Ex Taq polymerase. After the reaction mixture was treated at 95°C for 5 min, 30 cycles of amplification were performed as follows: 30 s at 95°C, 30 s at 65°C, and 1 min at 72°C. The final extension step was performed at 72°C for 10 min. The PCR products were electrophoresed and visualized on a 1.5% agarose gel stained with ethidium bromide.

Primary culture of isolated adipocytes

Isolation of adipocytes was carried out as described previously (Hiroyama *et al.* 2007). WAT was isolated from surrounding epididymus, femoral and subcutaneous upper back areas of 3-week-old male mice, which were anaesthetized with ether and killed by cervical dislocation, and was minced finely. Samples were incubated in 2 mg ml^{-1} collagenase in Krebs–Ringer buffer (120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgCl_2$, 4.75 mM KCl, and 1.2 mM KH_2PO_4) containing 15 mM sodium bicarbonate, 10 mM Hepes (pH 7.4), 2 mM sodium pyruvate and 1% BSA for 60 min at 37°C with shaking (100 r.p.m. min^{-1}). After incubation, samples were filtered through a nylon mesh (70 μm) and centrifuged at 80*g* for 5 min at room temperature. A precipitate including immature adipocytes was washed twice with the same buffer. Cells were suspended in DMEM containing 10% fetal bovine serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were then seeded in a 12-well dish at a concentration of 1.2–1.8 $\times$ 10^4 cells per well. At the confluent condition, adherent preadipocytes were cultured in differentiation-inducing DMEM medium containing 10% fetal bovine serum, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 20 nM insulin, 1 nM triiodothyronine, 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethazone and 0.125 mM indomethacin for 3 days. The differentiation to mature adipocytes was checked with Oil-Red O staining. Assays were carried out using cells differentiated to mature adipocytes with lipid droplets.

Western blotting

The cultured adipocytes were incubated in DMEM (low glucose) containing 0.3% BSA for 3 h at 37°C and stimulated with insulin at a final concentration of 50 nM for 5 min at 37°C. After incubation, the dishes were placed on ice and washed with ice-cold PBS. Cells were collected in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin and 1 mM PMSF) and disrupted using a Branson sonicator (Sonifier 250; Branson, Danbury, CT, USA) at setting 5 for 5 s. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA), and 20 μg of protein was

applied. SDS-PAGE and protein transfer were carried out according to the procedures previously described (Shibata *et al.* 2003) and processed using Enhanced Chemiluminescence (ECL) plus reagents (GE Healthcare, Buckinghamshire, UK) for detection with specific antibodies against Akt (diluted 1 : 1000 in TBS-T buffer; 10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20) or phospho-Akt (diluted 1 : 1000 in TBS-T buffer containing 5% milk).

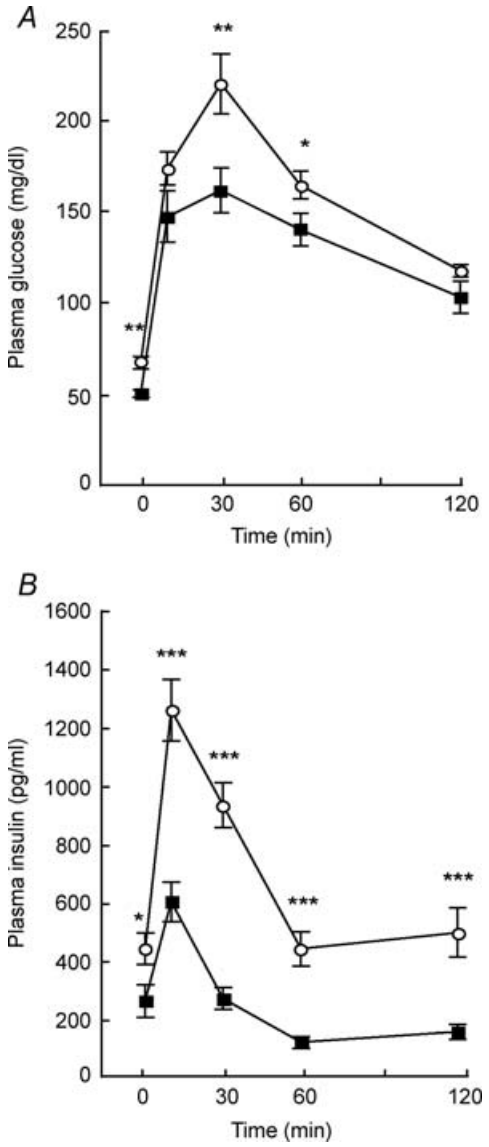

Figure 1. Intraperitoneal glucose tolerance test (GTT) for *V1bR*$^{-/-}$ mice
Plasma glucose (*A*) and insulin levels (*B*) during GTT in *V1bR*$^{+/+}$ (○) and *V1bR*$^{-/-}$ (■) mice ($n = 16$ of each). Values are the means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* *V1bR*$^{+/+}$ mice by *post hoc* comparison with Fisher's PLSD.

Table 1. Plasma glucose, insulin, and glucagon, levels in V1bR$^{+/+}$ and *V1bR*$^{-/-}$ mice

	V1bR$^{+/+}$	*V1bR*$^{-/-}$
4 h fasting		
Glucose (mg dl^{-1})	118.3±4.8 (n = 16)	90.5± 4.1 (n = 15)***
18 h fasting		
Glucose (mg dl^{-1})	66.3±2.3 (n = 16)	49.6± 3.8 (n = 16)**
Insulin (pg ml^{-1})	431.4±54.7 (n = 16)	249.8± 56.8 (n = 16)*
Glucagon (pg ml^{-1})	830.8±71.9 (n = 7)	372.5± 25.3 (n = 5)***

Values are the means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* *V1bR*$^{+/+}$ mice by the unpaired Student's *t* test.

Statistical analysis

Data are expressed as the means ± S.E.M. Statistical analyses were performed using the unpaired Student's *t* test and ANOVA followed by a *post hoc* comparison with Fisher's PLSD using Statview software, version 5.0 (Concepts, Inc., Berkeley, CA, USA). Differences between groups were considered statistically significant at the level of $P < 0.05$.

Results

Fasting plasma glucose, insulin and glucagon levels in *V1bR*$^{-/-}$ mice

To determine the alteration of the glucose metabolism in *V1bR*$^{-/-}$ mice, we assessed the fasting plasma glucose and insulin levels. After 18 h fasting, the plasma glucose and insulin levels of *V1bR*$^{-/-}$ mice were significantly lower than those of *V1bR*$^{+/+}$ mice. Plasma glucagon levels were also decreased in *V1bR*$^{-/-}$ mice compared with those in *V1bR*$^{+/+}$ mice (Table 1).

Intraperitoneal glucose tolerance test (GTT)

Since the fasting plasma glucose and insulin levels were lower in *V1bR*$^{-/-}$ mice, we performed a GTT to investigate whether the glucose tolerance was altered in *V1bR*$^{-/-}$ mice. After an I.P. injection with 1.5 g (kg body weight)$^{-1}$ of glucose, the plasma glucose levels were lower in *V1bR*$^{-/-}$ mice than in *V1bR*$^{+/+}$ mice, particularly at 30 and 60 min after the glucose injection (Fig. 1*A*). In addition, the plasma insulin levels were significantly lower in *V1bR*$^{-/-}$ mice than in *V1bR*$^{+/+}$ mice at all points (Fig. 1*B*). We also evaluated the difference in plasma glucose curves by two-way ANOVA. The plasma glucose curves of *V1bR*$^{-/-}$ mice tended to be lower than those of *V1bR*$^{+/+}$ mice (genotype × time, $P = 0.08$ by two-way ANOVA). The plasma insulin curves of *V1bR*$^{-/-}$ mice were significantly different from those of *V1bR*$^{+/+}$ mice (genotype × time, $P < 0.001$ by two-way ANOVA). These findings suggested

that insulin sensitivity could be enhanced in *V1bR*$^{-/-}$ mice because the plasma glucose levels were lower in *V1bR*$^{-/-}$ mice than in *V1bR*$^{+/+}$ mice despite the lower plasma insulin levels during the GTT.

Intraperitoneal insulin tolerance test (ITT) and hyperinsulinaemic–euglycaemic clamp test

To further assess *in vivo* insulin sensitivity in *V1bR*$^{-/-}$ mice, we performed an ITT and a hyperinsulinaemic–euglycaemic clamp test. Mice fasted for 4 h were used for the ITT. The plasma glucose-lowering effect by insulin administration was evaluated in mice. The plasma glucose levels after 4 h fasting were significantly lower in *V1bR*$^{-/-}$ mice than in *V1bR*$^{+/+}$ mice (Table 1). During the ITT, the decreases in the plasma glucose levels at 30 and 60 min in *V1bR*$^{-/-}$ mice were significantly greater than those in *V1bR*$^{+/+}$ mice (Fig. 2). Although the plasma glucose curves during the ITT were not significantly different between two groups (Fig. 2, genotype × time, $P = 0.17$ by two-way ANOVA), the decreases in the plasma glucose levels in *V1bR*$^{-/-}$ mice after the insulin administration tended to be greater than those in *V1bR*$^{+/+}$ mice, suggesting that the plasma glucose-lowering effect of insulin was increased in *V1bR*$^{-/-}$ mice.

Next, we evaluated whole-body glucose utilization under the hyperinsulinaemic condition. We measured the glucose infusion rate (GIR) during the hyperinsulinaemic–euglycaemic clamp test in awake *V1bR*$^{+/+}$ and *V1bR*$^{-/-}$ mice. The GIR in *V1bR*$^{-/-}$ mice was about two times higher than that in *V1b*$^{+/+}$ (67.4 ± 8.92 mg kg^{-1} min^{-1} in *V1bR*$^{-/-}$ mice, $n = 11$, *versus* 30.4 ± 5.77 mg kg^{-1} min^{-1} in *V1b*$^{+/+}$ mice, $n = 8$, $P < 0.01$) (Fig. 3). These data indicated that the insulin-stimulated whole-body glucose uptake was increased in *V1bR*$^{-/-}$ mice, which implied that insulin sensitivity was enhanced in *V1bR*$^{-/-}$ mice.

Insulin sensitivity of V1b receptor-deficient mice with a genetic background of C57BL/6J

To exclude the modification of insulin sensitivity by genetic background, we analysed fasting plasma levels of glucose and insulin and performed the ITT in male V1b receptor-deficient mice (8–10 weeks old) with a C57BL/6J genetic background (*V1bR*(*C57BL/6J*)$^{-/-}$). After 18 h fasting, the plasma insulin levels were lower in *V1bR*(*C57BL/6J*)$^{-/-}$ mice than in control mice (C57BL/6J mice) (947.5 ± 102.0 pg ml^{-1} in control mice, $n = 4$, *versus* 585.0 ± 14.4 pg ml^{-1} in *V1bR*(*C57BL/6J*)$^{-/-}$ mice, $n = 4$, $P < 0.05$). In addition, the fasting plasma glucose levels were lower in *V1bR*(*C57BL/6J*)$^{-/-}$ mice than in control C57BL/6J mice (148.9 ± 4.3 mg dl^{-1} in control mice, $n = 12$, *versus* 106.9 ± 9.8 mg dl^{-1} in *V1bR*(*C57BL/6J*)$^{-/-}$ mice, $n = 10$, $P < 0.01$). In addition, the plasma glucose levels were significantly decreased in *V1bR*(*C57BL/6J*)$^{-/-}$ mice 30 min after the insulin administration (53.3% decrease in control mice, $n = 4$, *versus* 64.2% decrease in *V1bR*(*C57BL/6J*)$^{-/-}$ mice, n = 3). These results suggested that insulin sensitivity was enhanced in *V1bR*(*C57BL/6J*)$^{-/-}$ mice.

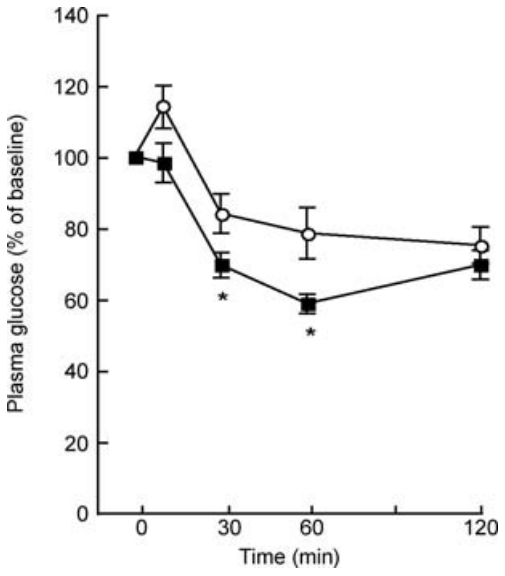

Figure 2. Intraperitoneal insulin tolerance test (ITT) for *V1bR*$^{-/-}$ mice
Relative plasma glucose levels during ITT in *V1bR*$^{+/+}$ (○, $n = 16$) and *V1bR*$^{-/-}$ (■, $n = 15$) mice. Values are the means ± S.E.M. *$P < 0.05$ *versus* *V1bR*$^{+/+}$ mice by *post hoc* comparison with Fisher's PLSD.

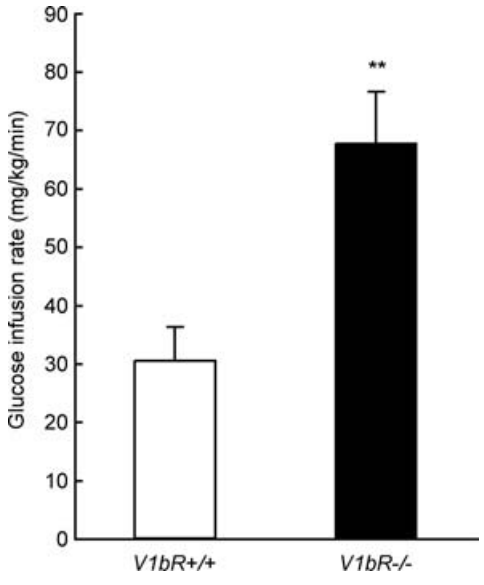

Figure 3. GIR measured by analysis with the hyperinsulinaemic–euglycaemic clamp test
Insulin-mediated whole-body glucose uptake was determined in awake *V1bR*$^{+/+}$ (open bar, $n = 8$) and *V1bR*$^{-/-}$ mice (filled bar, $n = 11$). Values are the means ± S.E.M. **$P < 0.01$ *versus* *V1bR*$^{+/+}$ mice by the unpaired Student's *t* test.

Plasma glucose and insulin response to AVP administration

In order to evaluate whether AVP was involved in insulin release *in vivo*, we measured the insulin levels after an intravenous (I.V.) administration of AVP. Fifteen minutes after AVP administration, the plasma glucose levels were increased in both $V1bR^{+/+}$ and $V1bR^{-/-}$ mice (153.1 ± 5.9 to 193.8 ± 17.1 mg dl^{-1} in control mice, $n = 3$, *versus* 146.2 ± 15.7 to 173.7 ± 33.4 mg dl^{-1} in $V1bR^{-/-}$ mice, $n = 3$). The plasma insulin levels were increased in $V1bR^{-/-}$ mice as well as in $V1bR^{+/+}$ mice (441.7 ± 28.9 to 536.7 ± 78.8 pg ml^{-1} in control mice, $n = 3$, *versus* 410.0 ± 22.9 to 461.7 ± 56.4 pg ml^{-1} in $V1bR^{-/-}$ mice, $n = 3$). However, there was no significant difference in the increase in plasma glucose and insulin levels between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice.

Expression of the AVP receptor genes in insulin-sensitive tissues

We examined whether V1b receptor mRNA was expressed in insulin-sensitive tissues such as heart, liver, muscle, WAT and BAT. In $V1bR^{+/+}$ mice, the V1b receptor transcript was detected only in WAT, not in heart, liver, muscle or BAT (Fig. 4). On the other hand, the V1b receptor transcript was not detectable in all tested tissues, including WAT, from $V1bR^{-/-}$ mice (Fig. 4). We also examined the expression levels of other AVP-receptor members such as the V1a, V2 and oxytocin receptors in the insulin-sensitive tissues. The V1a and V2 receptors were expressed in all tested tissues of $V1bR^{-/-}$ and $V1bR^{+/+}$ mice, whereas the oxytocin receptors were detected in all tissues except the liver. The expression levels of these receptors were not altered in insulin-sensitive tissues of $V1bR^{-/-}$ mice, suggesting that there was no obvious up-regulation of other receptors in $V1bR^{-/-}$ mice. In addition, we analysed the expression levels of the insulin receptor in these tissues but found that there was no difference in the mRNA expression levels of $V1bR^{-/-}$ and $V1bR^{+/+}$ mice (data not shown).

Insulin-stimulated phosphorylation of Akt in adipocytes of $V1bR^{-/-}$ mice

In order to address underlying mechanisms of enhanced insulin sensitivity, we analysed insulin signalling in adipocytes isolated from WAT, an insulin-sensitive tissue, of $V1bR^{-/-}$ mice. As Akt is implicated in insulin signalling and is phsophorylated in response to insulin, we investigated the phosphorylation of Akt with the adipocytes. In Oil-Red O staining, there was no remarkable difference between the adipocytes of $V1bR^{+/+}$ and $V1bR^{-/-}$ mice. After insulin stimulation, while the total amount of Akt detected by Western blotting with the anti-Akt antibody did not differ between $V1bR^{-/-}$ and $V1bR^{+/+}$ mice, insulin-stimulated phosphorylation of Akt was higher in $V1bR^{-/-}$ mice than in $V1bR^{+/+}$ mice (Fig. 5).

Discussion

Several *ex vivo* or *in vitro* studies have shown that AVP stimulates insulin or glucagon secretion depending on the glucose concentrations in mouse islets and rat pancreas (Gao *et al.* 1990, 1992; Abu-Basha *et al.* 2002). In our previous study, we also found that AVP stimulates insulin release from mouse pancreatic islet cells and that AVP-induced insulin release from pancreatic islet cells

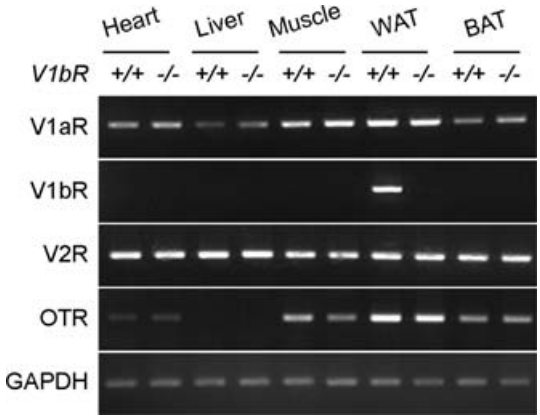

Figure 4. Expression of the vasopressin receptor family
Total RNAs were purified from the heart, liver, muscle, WAT and BAT. RT-PCR was then performed to detect vasopressin receptor gene expression using specific primer sets as described in Methods. The RT-PCR for GAPDH was performed as a control. $^{+/+}$ and $^{-/-}$ show $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, respectively. OTR, oxytocin receptor.

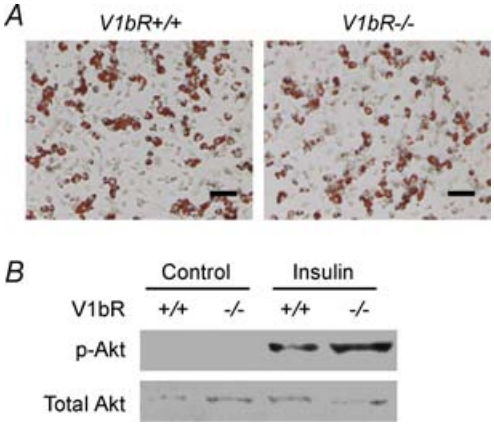

Figure 5. Effect on phosphorylation of Akt by insulin stimulation
Phosphorylation of Akt was detected using isolated and derived adipocytes from WAT in the absence and presence of insulin. *A*, adipocytes stained with Oil-Red O. The bars indicate 100 μm. *B*, immunoblots of the phosphorylation of Akt and total Akt. $^{+/+}$ and $^{-/-}$ show $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, respectively. p-Akt, phosphorylated Akt; Akt, total Akt.

was blunted in V1b receptor-deficient mice (Oshikawa *et al.* 2004), suggesting that AVP/V1b signalling could be involved in regulating glucose homeostasis by affecting *in vivo* insulin release from mouse pancreatic islet cells. Therefore, in this study, we investigated the effect of AVP on regulating blood glucose levels in V1b receptor-deficient mice. *V1bR*$^{-/-}$ mice showed lower plasma glucose levels accompanied by decreased plasma insulin levels under the fasting condition. Using studies with the GTT, ITT and hyperinsulinaemic–euglycaemic clamp test, we found that insulin sensitivity was increased at the *in vivo* level in *V1bR*$^{-/-}$ mice. In addition, Akt phosphorylation by insulin stimulation was enhanced in cultured adipocytes of *V1bR*$^{-/-}$ mice, suggesting that enhanced insulin signalling could contribute to increased insulin sensitivity. This phenotype of altered insulin sensitivity was also observed in V1b receptor-deficient mice with the C57BL/6J genetic background (*V1bR*(*C57BL/6J*)$^{-/-}$) as well as in mutant mice with the mixed genetic background of 129Sv and C57BL/6J, indicating that a deficiency of the V1b receptor caused insulin hypersensitivity and this phenotype was not influenced by genetic background.

Since *in vitro* AVP-induced insulin release was impaired in *V1bR*$^{-/-}$ mice, we had expected that these mice would show lower plasma insulin and higher plasma glucose levels. As demonstrated in the present study, the lower plasma insulin levels in *V1bR*$^{-/-}$ mice were concordant with the finding of our previous *in vitro* study (Oshikawa *et al.* 2004). However, *V1bR*$^{-/-}$ mice unexpectedly exhibited lower plasma glucose levels under the fasting condition, even after glucose loading. The following are possible explanations for the lower plasma glucose in *V1bR*$^{-/-}$ mice. First, the lower levels of plasma glucose in *V1bR*$^{-/-}$ mice observed under the fasting condition and after glucose loading could have been caused by increased insulin sensitivity. Indeed, other genetically modified mice in which insulin hypersensitivity was observed also had lower levels of plasma glucose (Terauchi *et al.* 1999; Ueki *et al.* 2002; Duttaroy *et al.* 2004). Second, reduced glucagon secretion may have contributed to lower levels of plasma glucose, particularly under the fasting condition. AVP was reported to stimulate glucagon release *in vitro* (Gao *et al.* 1992; Yibchok-anun & Hsu, 1998; Folny *et al.* 2003), and we recently reported that AVP stimulated glucagon release via the V1b and oxytocin receptors *in vitro* (Fujiwara *et al.* 2007). However, the oxytocin receptor can contribute to AVP-induced glucagon release only at a high ligand concentration, e.g. 10^{-8} M, which is over the physiological range, and AVP-induced glucagon release was mainly mediated through the V1b receptors in the physiological range of AVP *in vivo* (Yibchok-Anun *et al.* 1999; Fujiwara *et al.* 2007). Therefore, the V1b receptor deficiency could have led to reduced glucagon secretion, resulting in reduced plasma glucose levels. In addition to the two above-mentioned possible explanations for insulin hypersensitivity and reduced glucagon secretion, glucocorticoid hormones could have affected glucose homeostasis in *V1bR*$^{-/-}$ mice. In our previous study, we found that plasma ACTH and corticosterone levels were lower in *V1bR*$^{-/-}$ mice than in *V1bR*$^{+/+}$ mice (Tanoue *et al.* 2004). In rodents, corticosterone is known as a key hormone that causes gluconeogenesis in the liver and increases plasma glucose levels (Golf *et al.* 1984; Sistare & Haynes, 1985). Several studies have shown that plasma glucose levels are decreased in adrenalectomized animals or patients with chronic adrenal insufficiency (Addison's disease) (Serrano-Rios *et al.* 1974; Haluzik *et al.* 2002; Long *et al.* 2003). Thus, at least in part, decreased corticosterone levels could have caused lower plasma glucose levels in the *V1bR*$^{-/-}$ mice, although the reduction in the basal corticosterone levels was somewhat trivial in the *V1bR*$^{-/-}$ mice. The reduced plasma glucose levels observed in *V1bR*$^{-/-}$ mice could have been due to enhanced insulin sensitivity, reduced glucagon and/or reduced corticosterone levels.

As described above, plasma insulin levels were lower in *V1bR*$^{-/-}$ mice than in *V1bR*$^{+/+}$ mice under the fasting condition as well as during glucose loading. Insulin secretion from pancreatic islets is mainly regulated by glucose, which stimulates an increase in the intracellular Ca^{2+} concentrations in pancreatic β-cells and causes insulin secretion (Henquin, 2004). AVP can work as a positive modulator for glucose-stimulated insulin release by acting on this signal (Gao *et al.* 1990; Lu *et al.* 1993). There are two possible explanations for the lower plasma insulin levels in *V1bR*$^{-/-}$ mice: (i) a primary disturbed effect on modulating insulin release by deficient AVP/V1b-receptor signalling, leading to impaired insulin secretion from pancreatic β-cells, and (ii) secondary effects of decreased plasma glucose levels. Our *in vitro* study with *V1bR*$^{-/-}$ mice clearly demonstrated that AVP can stimulate insulin release from pancreatic β-cells via the V1b receptor (Oshikawa *et al.* 2004), but the effect of AVP on plasma insulin levels remains unclear. It was reported that the I.V. administration of AVP increased the plasma insulin and glucagon levels in rat and sheep, suggesting that AVP may regulate glucose homeostasis by influencing pancreatic hormone secretion *in vivo* as well as *in vitro* (Dunning *et al.* 1984*a*; Mineo *et al.* 1997). In contrast, I.V. administration of AVP does not affect human plasma insulin levels (Spruce *et al.* 1985). In order to evaluate whether AVP could primarily affect insulin release *in vivo*, we measured the glucose and insulin levels after the I.V. administration of AVP in *V1bR*$^{+/+}$ and *V1bR*$^{-/-}$ mice. In our present experiments, 100 ng kg^{-1} of AVP administration increased plasma glucose and insulin levels in both *V1bR*$^{+/+}$ and *V1bR*$^{-/-}$ mice. The alteration of the plasma glucose levels by AVP administration may result from direct stimulation of the liver following gluconeogenesis and glycogenolysis (Hems & Whitton, 1973). As there was no significant difference in the increase

in plasma insulin levels between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice, the insulin response could have been due to the secondary stimulus effect of increased plasma glucose levels rather than the primary stimulus effect of AVP on the V1b receptors in pancreatic β-cells. Thus, as the secondary effect, it is possible that the lower plasma glucose levels in $V1bR^{-/-}$ mice led to the suppression of glucose-dependent insulin secretion, resulting in reduced plasma insulin levels.

The mechanisms of increased insulin sensitivity in $V1bR^{-/-}$ mice are not fully explained by the results of our study. The V1b receptor was expressed in WAT, where the phosphorylation of Akt involved in insulin signalling was increased in $V1bR^{-/-}$ mice. We hypothesized that not only the glucose metabolism but also adipose tissue lipolysis could be altered in $V1bR^{-/-}$ mice, which would result in increased insulin signalling in WAT. Therefore, we examined the serum glycerol level to evaluate adipose tissue lipolysis in $V1bR^{-/-}$ mice, but we found that there was no significant difference between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice (serum glycerol, $155.5 \pm 17.0\ \mu\text{mol l}^{-1}$ in $V1bR^{+/+}$ mice, $n = 5$, and $154.5 \pm 7.4\ \mu\text{mol l}^{-1}$ in $V1bR^{-/-}$ mice, $n = 5$, $P = 0.96$ by Student's t test). This result suggests that there is no difference in adipose tissue lipolysis between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice. Thus, the glucose metabolism, but not the lipid metabolism, was altered in $V1bR^{-/-}$ mice, and enhanced insulin signalling in WAT could lead to increased glucose uptake, resulting in insulin hypersensitivity. In addition to the direct modification of insulin sensitivity in adipose tissue, insulin sensitivity could be altered in other insulin-sensitive tissues, such as liver, heart and muscle, because the GIR assessed by the clamp test was two times higher in $V1bR^{-/-}$ mice than in $V1bR^{+/+}$ mice. Especially, muscle is one of the major tissues for the uptake and utilization of glucose in response to insulin stimulation (Klip & Paquet, 1990). However, several reports have indicated that the altered glucose metabolism in both muscle and adipose tissue could affect the whole-body glucose metabolism and insulin sensitivity (Shepherd *et al.* 1993; Abel *et al.* 2001; Carvalho *et al.* 2005). For instance, the whole-body glucose metabolism and insulin sensitivity are altered in mice in which glucose transporter 4 is modified by an adipose tissue-specific-transgenic or knockout technique (Shepherd *et al.* 1993; Abel *et al.* 2001). Similarly, our finding suggests that the alteration of the adipose tissue glucose metabolism could contribute to the whole-body glucose metabolism and insulin sensitivity in $V1bR^{-/-}$ mice.

Since the V1b receptor is not detected in muscle, heart or liver, the modification of insulin sensitivity does not appear to depend directly on the V1b receptor but could be an indirect effect of it. Similar findings were observed in M_3 muscarinic acetylcholine receptor-deficient mice (Duttaroy *et al.* 2004). In these mice, muscarinic stimulation of pancreatic insulin and glucagon release was abolished. These findings imply increased insulin sensitivity in M_3 receptor-deficient mice, although the M_3 receptor has no direct effect on insulin sensitivity (Duttaroy *et al.* 2004). One of the reasons for hypersensitivity to insulin is the considerable negative feedback of the insulin receptor. Several reports have suggested that insulin sensitivity is controlled by downstream signalling molecules of the insulin receptor, such as the insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K) and protein kinase B/Akt (PKB/Akt) (Summers *et al.* 1999; Watson *et al.* 2004). Usually, insulin activates the insulin receptor and promotes the entry of glucose into tissues; however, a high density of insulin down-regulates the activated insulin receptor by the negative feedback of activated PI3K and PKB/Akt (Hirashima *et al.* 2003). In addition, reciprocal feedback regulation causes the dissociation of the insulin receptor and IRS with PI3K in primary adipocytes (Hers *et al.* 2002). Thus, it is possible that the low plasma insulin level in $V1bR^{-/-}$ mice may reduce the negative feedback of the insulin receptor, probably resulting in hypersensitivity to insulin.

In a recent study with V1b vasopressin receptor-deficient mice, Lolait *et al.* (2007) reported that there was no significant difference in the glucose-lowering effect of insulin administration in control and mutant mice, although the plasma glucose levels after the insulin administration tended to be lower in the mutant mice. The different results in the study of Lolait *et al.* and ours in terms of the glucose-lowering effect by insulin with V1b receptor-deficient mice could be in part due to different experimental protocols, such as fasting periods. While we performed the ITT with insulin at a concentration of $1\ \text{U kg}^{-1}$ after short-term (4 h) fasting, Lolait *et al.* performed the ITT with insulin at a concentration of 0.75 or $3\ \text{U kg}^{-1}$ after long-term (12 h) fasting. It is well known that the fasting period is critical to the assessment of the hypoglycaemic effect of insulin (Randle, 1998). In fact, we observed no significant difference in the glucose-lowering effect between $V1bR^{+/+}$ and $V1bR^{-/-}$ mice evaluated by ITT at all measuring points under the same fasting condition as that used in the GTT experiment (18 h fasting) (data not shown). Thus, increased insulin sensitivity was observed during the ITT with $V1bR^{-/-}$ mice after only 4 h fasting, and this phenotype was further confirmed by the clamp test. Furthermore, another study with V1b receptor-deficient mice congenic for C57BL/6J mice for the glucose-lowering effect by insulin showed that a deficiency of the V1b receptor resulted in increased insulin sensitivity. These results indicated that insulin hypersensitivity by V1b-receptor deficiency was not influenced by genetic background.

In conclusion, our study with V1b receptor-deficient mice revealed that a deficiency of the V1b receptor resulted in a phenotype with lower plasma insulin and

glucagon concentrations and hypersensitivity to insulin. We found that the glucose metabolism in the adipose tissue was altered in *V1bR*$^{-/-}$ mice, while lipolysis was not altered. The altered adipose tissue glucose metabolism could contribute to whole-body insulin sensitivity. Thus, AVP/V1b receptor signalling is an important pathway to modulate insulin sensitivity. These findings indicate that the AVP/V1b receptor pathway plays a crucial role in regulating glucose homeostasis by affecting insulin and glucagon secretion and that long-term antagonism for the V1b receptor could result in increased insulin sensitivity.

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