ORIGINAL RESEARCH

V-ATPase blockade reduces renal gluconeogenesis and improves insulin secretion in type 2 diabetic rats

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Running title: V-ATPase blockade in type 2 diabetes

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Abstract

Vacuolar H^+-adenosine triphosphatase (V-ATPase) stimulates vesicular acidification that may activate cytoplasmic enzymes, hormone secretion and membrane recycling of transporters. We investigated the effect of blockade of V-ATPase by bafilomycin B1 on renal gluconeogenesis, mitochondria enzymes and insulin secretion in type 2 diabetic rats. Spontaneous type 2 diabetic Torii rats were treated with intraperitoneal injection of bafilomycin B1 for 1 week, and the kidneys were examined after 24-h starvation in metabolic cages. The renal expression and activity of V-ATPase were increased in the brush border membrane of the proximal tubules in diabetic rat. The blockade of V-ATPase by bafilomycin B1 reduced the renal V-ATPase activity and urinary ammonium in diabetic rats. Treatment with bafilomycin suppressed the enhanced renal gluconeogenesis enzymes and mitochondria electron transport enzymes in type 2 diabetic rats and reduced the renal cytoplasmic glucose levels. Insulinogenic index and pancreatic insulin granules were decreased in diabetic rats with increased V-ATPase expression in the islet cells, and treatment with bafilomycin B1 reversed these changes and increased the insulin secretion index. Hepatosteatosis in type 2 diabetic rats was

ameliorated by bafilomycin treatment. As a consequence, treatment with bafilomycin B1 significantly decreased the plasma glucose level after 24-hour starvation in diabetic rats.

In conclusion, V-ATPase inhibitor improved plasma glucose level in type 2 diabetes by inhibiting renal mitochondria gluconeogenesis and improving insulin secretion.

Keywords: H^+-ATPase, diabetes, gluconeogenesis, mitochondria, ammoniagenesis, insulin

Introduction

Vacuolar type H^+-ATPase (V-ATPase) has various important roles in the acid-base balance, activation of endocytosis and lysosomal enzymes, membrane recycling of receptors and channels [1-3], osteoclast activation and cancer metastasis [4, 5]. We recently reported that the blockade of V-ATPase by bafilomycin B1 ameliorated plasma glucose levels under the starvation conditions in streptozotocin-induced type 1 diabetic rats [6]. Renal gluconeogenesis is enhanced by acidosis and starvation, whereas hepatic gluconeogenesis is enhanced by glucagon, cortisol and beta-adrenergic receptors and inhibited by insulin [7-11]. Thus, the kidney has an important role to maintain plasma glucose level under starvation condition. To demonstrate the greatest effect of bafilomycin on the renal gluconeogenesis, studies were performed under 24-hour starvation.

As phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting enzyme of renal gluconeogenesis, is activated in the acidic environment [12], V-ATPase inhibition by bafilomycin was shown to reduce the PEPCK expression and activity in the proximal tubule and reduced the renal cytoplasmic glucose and plasma glucose levels under

starvation conditions [6].

V-ATPase plays a role in the processing of pre-hormones in the vesicles and their excretion into the blood. Indeed, insulin release has been shown to require V-ATPase activity [13-15].

In the present study we invested the effects of bafilomycin in a type 2 diabetic rat model and found that the blockade of V-ATPase ameliorates plasma glucose not only via the inhibition of renal gluconeogenesis but also through the influence of insulin secretion and insulin sensitivity.

Materials and Methods

Animal experiments

Eighteen male Spontaneously Diabetic Torii (SDT) rats (CREA Japan, Inc., Tokyo, Japan) had *ad libitum* access to tap water and standard rat chow. At 18-24 weeks of age, the SDT rats developed type 2 diabetes mellitus with plasma glucose levels exceeding 200 mg/dL (T2DM rats), and a group of the DM rats was treated with bafilomycin B1 (100 nmol/kg/day intraperitoneally; Enzo Life Sciences, Ann Arbor, MI, USA) for 1

week (T2DM+BFM group, n=9). The dose of bafilomycin has been determined from our previous studies to an amount that can reduce plasma glucose levels without complete loss of appetite or hypoglycemia [6]. Age-matched Sprague-Dawley rats served as controls (Control group, n=9). Twenty-four-hour urine and blood samples were collected using a metabolic cage at day 0 (D0) and day 6 (D6) after starting BFM injection under feeding conditions with free access to water and food and then at day 7 (D7) under 24-hour starvation conditions [6]. After 24-hour starvation in the metabolic cages, the rats were anesthetized with 1.5% isoflurane inhalation solution (Mylan, Osaka, Japan) with a BRTK-100A anesthesia machine (Bio Research Center, Tokyo, Japan) and their blood pressure was measured through a catheter inserted into the internal carotid artery using a transducer. After sampling blood from the jugular vein catheter, intravenous insulin-glucose tolerance tests (ITT) were performed by the intravenous injection of glucose (0.7 g/kg body weight) and rapid insulin (0.175 unit/kg body weight) [16], and the K index of the ITT was used to evaluate the insulin resistance [6, 17, 18]. The rats were then euthanized with 10% isoflurane inhalation

solution, and the kidneys, liver and pancreas were removed for a protein expression analysis and morphological studies.

All of the procedures were conducted in accordance with the Guidelines for Animal Experimentation in Dokkyo Medical University and approved by the Medical Experimental Animal Ethics Committee of Dokkyo Medical (17-918) and the University of Tokyo (15-P-134).

The proteomics analysis of glucose metabolism

The whole kidneys from each group were homogenized, and the isobaric tags for relative and absolute quantification (iTRAQ) methods was used [19]. The tag-labeled peptides in proteins were pooled and analyzed using a Time-of-Flight Mass (TOF-MS) spectrometer (Filgen, Nagoya, Japan).

The enzymes of the glucose metabolism were selected from a list of about 2800 proteins in the kidneys, and the relative expression ratio of each enzyme, T2DM/control and T2DM+BFM/T2DM, was demonstrated as 5 gradient colors, thus demonstrating both an increased and a decreased expression.

Western blotting

As described previously [6], half of the whole kidney or a part of liver were homogenized in a 5-fold volume of 20 mmol/L Tris buffer with proteinase inhibitors, and the cytosolic and membrane fractions were obtained by centrifugation at 5,000 *g* for 15 minutes followed by centrifugation of the supernatant at 48,000 *g* for 60 minutes. The samples containing 50 μg of kidney homogenate were applied to a 4-12% Bis-Tris plus gel (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA) and electroblotted onto polyvinylidene fluoride membranes. The specific protein bands were identified using mouse monoclonal antibodies against V-ATPase B2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal antibodies for glucose-6-phosphatase (G6Pase) (Abcam, Tokyo, Japan) or PEPCK (Abcam, Tokyo, Japan) at 1:200 dilution followed by incubation with a horseradish peroxidase (HRP)–conjugated secondary antibody against mouse Ig or rabbit Ig (Dako, Glostrup, Denmark) at 1:2000 dilution and diaminobenzidine (DAB, 8 mmol/l) reaction. The density of the bands was analyzed using the National Institutes of Health Image software program ImageJ (https://imagej.nih.gov/ij/).

Immunohistochemistry

Wax-embedded tissues were processed for immunohistochemistry, as previously described [6]. Sections (2-μm-thick) were incubated with monoclonal antibodies against V-ATPase B2 (Santa Cruz Biotechnology), or rabbit polyclonal antibodies for glucose-6-phosphatase (G6Pase, Abcam, Tokyo, Japan), or rabbit polyclonal antibodies for cytochrome c oxidase IV (COXIV, Proteintech, IL, USA) at 1:200 dilution followed by incubation with HRP-conjugated anti-mouse Ig antibody (Dako) or rabbit Ig (Dako) at 1:100 dilution, and immunoreactivity was detected by a DAB reaction.

Electron microscopy (EM)

A part of the cortex of kidney and pancreas were fixed with 2.5% glutaraldehyde (TAAB, Berks, England) in 0.2 M cacodylate buffer solution (Wako, Osaka, Japan), and 2 mm^3 blocks were cut and post-fixed with osmium tetroxide, and embedded in epoxy resin (TAAB), as previously described [20]. The ultrathin sections were cut with an ultramicrotome and counterstained with uranium acetate and lead citrate and observed by transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan).

Twenty to 30 pictures with a magnification of x5,000 from each group were used to count insulin granules in the beta cells.

The measurement of the glucose, HbA1c, rat insulin and ammonium

The glucose levels in the blood, urine and homogenates of the renal cytosolic fraction were measured using the Glutest Pro R device (Arkray Factory, Shiga, Japan). The HbA1c level was measured using a DCA 2000 plus system (Bayer Medical, Tokyo, Japan). Rat insulin was measured using rat insulin assay kit (Morinaga, Yokohama, Japan), and insulinogenic index was calculated as Δ insulin/Δ glucose after 30min of glucose injection. Ammonium was measured using a Pocket Chem BA PA-4140 (Arkray Factory). Urinary ammonia and glucose were calculated as daily excretion.

Statistical analyses

The data are expressed as the means ± standard deviation. A Kruskal-Wallis analysis with a Steel-Dwass post-hoc test was used for the comparison among groups. Plasma glucose level in intravenous ITT was analyzed by a repeated-measures analysis of variance. *P* values of < 0.05 were considered to indicate statistical significance.

Results

Physiological data under feeding conditions and after 24-h starvation

Under feeding conditions, the body weights were insignificantly reduced in the T2DM+BFM rats compared with the T2DM rats even the though the food intake and urine volume were significantly reduced in T2DM+BFM rats (Table 1). Plasma glucose levels at D6 were not significantly different between the T2DM rats and T2DM+BFM rats under feeding conditions. However, after 24-h starvation condition at D7, the plasma glucose level was significantly decreased in the T2DM+BFM rats compared with the T2DM rats (Table 1), indicating that effect of BFM on the plasma glucose level becomes obvious when renal gluconeogenesis is activated by 24-h starvation. Although HbA1c represents monthly changes in plasma glucose levels, changes in glucose levels also affect weekly changes in HbA1c, confirming the effect of BFM on T2DM glucose levels (+1.4 in T2DM vs. +0.5 in T2DM+BFM). Blood pressure did not change significantly among three groups.

Localization of V-ATPase in the kidney and its roles in ammonia excretion

The renal expression of V-ATPase localized in the brush border membrane of proximal

tubules was increased in the T2DM rats compared with the control rats (Figure 1). The proximal tubule is a site of ammoniagenesis, and glutamine is converted to alpha-ketoglutarate and two ammonia molecules, which diffuse through apical membrane of the proximal tubule and are converted to ammonium ion via proton produced by V-ATPase and Na^+/H^+ exchanger. Bafilomycin B1 treatment reduced the expression of V-ATPase in the kidney and reduced the urinary ammonium excretion, whereas the plasma ammonia levels did not show significant changes (Figure 1).

The renal glucose metabolomics and effects of V-ATPase blockade

The renal metabolomics analysis by a TOF-MS analysis revealed that the levels of ammoniagenesis enzymes and consequent gluconeogenesis enzymes were increased in T2DM kidneys compared to control kidneys after 24-h starvation. In particular, the levels of α-ketoglutarate dehydrogenase in the TCA cycle and glucose-6-phosphatase for gluconeogenesis were about 2-fold higher in the kidney of T2DM rats than in control rats (Figure 2). Renal tubule G6Pase immunoreactivity and electron microscopic apical vesicles in which G6Pase is located increased in T2DM rats compared to control rats (Figure 3). V-ATPase blockade with bafilomycin B1 decreased most of the TCA

cycle and gluconeogenesis enzymes in diabetic kidneys (Figure 2) with reduced G6Pase and apical vesicles in the proximal tubules (Figure 3). The level of glycolysis-specific enzymes, including ATP-dependent phosphofructokinase and pyruvate kinase, were decreased in the kidney of T2DM rats, and bafilomycin treatment reversed them (Figure 2). Interestingly, the levels of enzymes involved in the mitochondria electron transport chain were increased in the kidney of T2DM rats (Figure 2), indicating enhanced ATP synthesis. V-ATPase blockade with bafilomycin reduced the renal expression of mitochondria electron transport chain enzymes (Figure 2). Renal tubular mitochondria complex IV immunoreactivity and mitochondria size observed by EM increased in T2DM rats compared to control rats, and bafilomycin decreased them (Figure 3).

The accumulation of hepatic lipid and hepatic gluconeogenesis

The liver in T2DM rats showed increased amounts of PAS-positive material and lipid accumulation on oil red staining (Figure 4). Treatment with bafilomycin reduced the hepatic lipid accumulation to the control level (Figure 4). The levels of hepatic PEPCK and G6Pase did not change significantly in T2DM rat compared to control rat after 24-starvation, or BFM treatment (Figure 4c, d).

Effect of bafilomycin on pancreatic insulin granules and insulin release and sensitivity

The islet cells of Langerhans expressed V-ATPase and its immunoreactivity is stronger in T2DM rats compared with those in control (Figure 5). The number of insulin granules decreased in T2DM rats compared with control rats (Figure 4B-C). Treatment with bafilomycin decreased V-ATPase immunoreactivity in the islet cells, and the insulin granules were preserved (Figure 5A-C). The insulin secretion after glucose injection was calculated as the insulinogenic index. Interestingly, the decreased insulin secretion in type 2 diabetic rats was increased in T2DM rats treated with bafilomycin (Figure 5D). After 30 minutes of intravenous ITT, the plasma glucose levels remained in high in T2DM rats, whereas treatment with bafilomycin reduced the plasma glucose levels near the control levels (Figure 6A). The insulin sensitivity as indicated by the KITT values calculated from the half-time of the intravenous ITT was reduced in T2DM rats compared with control rats, and treatment with bafilomycin restored this reduced insulin sensitivity (Figure 6B).

Discussion

The present findings demonstrated that V-ATPase blocking via bafilomycin B1 inhibited renal ammoniagenesis and gluconeogenesis enzymes as well as mitochondria TCA cycle and electron transport system enzymes in T2DM rats, and reduced the plasma glucose level, especially after 24-h starvation. In addition, we also showed that the insulin secretion and sensitivity were restored by bafilomycin treatment.

The kidneys produce ammonia from glutamine and glutamate, and ammonia is converted to ammonium ions reacted with proton via V-ATPase and Na^+/H^+ exchanger in the apical membrane of the proximal tubules. We showed that the levels of ammoniagenesis enzymes and V-ATPase in the proximal tubule were increased in T2DM rats. V-ATPase blockade by bafilomycin significantly reduced the urinary ammonia excretion in T2DM rats; however, the plasma ammonia levels did no change as any remaining ammonia can be converted to urea in the liver.

We recently reported that renal gluconeogenesis was increased after 24-h starvation in type 1 diabetes mellitus (T1DM) rat, and BFM treatment suppressed renal gluconeogenesis [6]. In the T2DM rats used in the present study, we confirmed

increased renal gluconeogenesis enzymes including glucose-6-phosphatase (Figure 2, 3). BFM treatment reduced the levels of gluconeogenesis enzymes. Seven of the 10 glycolysis enzymes are common to gluconeogenesis, and the levels of glycolysis specific enzymes are decreased in T2DM rats, but enhanced by BFM treatment.

Our results of TCA cycle enzyme inhibition by bafilomycin B1 is consistent with the levels of metabolites from the TCA cycle, including citrate, cis-aconate, isocitrate and α-ketoglutarate were reduced by bafilomycin A1 in the neurons [21]. The mechanism of inhibition of mitochondrial TCA cycle and respiratory electron transport chain reaction by bafilomycin A1 is due to intracellular acidification and elevated cytosolic Ca^{2+}, which open permeability transition pores (PTPs) [22]. It was recently shown that mitochondria F-ATP synthase transformed to a PTP via Ca^{2+} binding to beta-subunit of F-ATP synthase [23]. Furthermore, it was reported that V-ATPase depletion affects mitochondrial ATPase function in trypanosomes, indicating direct communications between these ATPase to transport proton [24]. In this manner, an inhibition of V-ATPase with bafilomycin may inhibit mitochondrial ATP synthesis, thus contributing to reduce renal gluconeogenesis and tubular glucose metabolism.

The levels of hepatic PEPCK and G6Pase were not increased after 24-h starvation in T2DM rats or BFM treatment, as hepatic gluconeogenesis is increased by glucagon and cortisol and inhibited by insulin, but not regulated by starvation or acidosis [7, 8, 11, 25]. Interestingly, fatty liver changes in T2DM rats were markedly improved by BFM treatment, although the underlying mechanism is not clear. Similarly, anti-thiocyamine, a large group of polyphenols, inhibited the hepatic accumulation of lipids by inhibiting the translocation of the β-subunit of mitochondria F0F1-ATPase and reducing the enzyme activity of mitochondria acyl-CoA:glycerol-sn-phosphate acylglycerase 1 (mtGPAT1) [26]. N-ethylmaleimide (NEM), which inhibits V-ATPase at low concentrations but not F-type ATPase, also inhibited GPAT1 and fatty acid accumulation [27]. Antisense oligonucleotides to the prorenin receptor, which regulates V-ATPase integration, inhibit hepatosteatosis via the suppression of pyruvate dehydrogenase to reduce acetyl-CoA production and via the suppression of acetyl-CoA carboxylase to reduce triglyceride production [28]. We also noted that the enhanced levels of pyruvate dehydrogenase in T2DM rats were suppressed by bafilomycin (Figure 2), resulting the reduction in acetyl-CoA production and improving

hepatosteatosis.

In our study, pancreatic V-ATPase were increased in T2DM rats and the electron microscopic observation of beta cells revealed many empty vesicles in the islets of T2DM rats. V-ATPase is located in the islet cells and plays an essential role in insulin secretion [13]. The genetic knockout of the a3 subunit of V-ATPase has been shown to reduce the insulin secretion [14]. We explain that the depletion of insulin after its over-release of insulin via enhanced V-ATPase in T2DM rat, and bafilomycin treatment restored the numbers of insulin granules by inhibiting the basal secretion in the starvation condition via inhibition of V-ATPase. When glucose was challenged with the intravenous ITT examination, these restored insulin granules in starvation condition were released, improving insulin secretion in the T2DM rats treated with bafilomycin. These findings are consistent with a previous report showing that impaired insulin granule acidification following knockdown of ATP6ap2, a subunit of V-ATPase, paradoxically increased the proinsulin secretion [15].

The effect of BFM on the plasma glucose level became obvious after 24-hour starvation condition rather than feeding condition in this study, indicating that BFM

inhibited renal gluconeogenesis, which contributes to about 40% of total gluconeogenesis under starvation conditions [7, 8, 25]. Enhanced enzymes in the TCA cycle and mitochondria electron transport chains in T2DM rats were blocked by BFM in our study. Bafilomycin mediates the uncoupling of the mitochondria electron transport chain, which partially depolarizes mitochondria and induces HIF-dependent signaling, and thereby increasing the expression of GLUT1 in colon cancer cell [29]. Further studies are necessary to elucidate the mechanisms of bafilomycin on glucose uptake in the muscle and the intracellular signaling.

This study has some limitations, including proteomic analysis and long-term side effects of bafilomycin B1. TOF-MS analysis was just performed in one kidney of each group because of high costs. Since the dose of bafilomycin B1 was determined based on previous experiments in streptozotocin-induced type 1 diabetic rats [6], it may be too much in less severe type 2 diabetic rats in this study. And it is possible that the adverse effect of bafilomycin caused a decrease in food intake and affected in the metabolic changes observed in this study. Intraperitoneal injection of bafilomycin can damage the intestine or act directly on the intestine, causing a reduction in food intake.

The long-term treatment with bafilomycin reduced food intake and showed hypoglycemia, thus, we could not treat rats more than one week. However, the findings such as inhibition of renal gluconeogenesis enzymes and mitochondrial TCA cycle enzymes are unlikely to be caused by reduced food intake. These effects of bafilomycin may be involved in the hypoglycemic effect of bafilomycin, and worthwhile for further studies investigating the role of V-ATPase in glucose metabolism, especially to therapeutically target kidney- or pancreas-specific blockade of V-ATPase.

In conclusion, blockade of V-ATPase with bafilomycin B1 inhibited renal gluconeogenesis enzymes and also improves insulin secretion, resulting reduction of plasma glucose level after 24-h starvation.

Conflict of Interest

The authors declare no conflicts of interest associated with this work.

Acknowledgments

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Table 1. Body weight, food intake, plasma and urinary glucose, HbA1c values, KITT and blood pressure under feeding conditions at day 0 (D0) and 6 (D6) and after 24-h starvation on day 7(D7).

		Control (n = 9)	T2DM (n = 9)	T2DM+BFM (n = 9)
Body weight (g)	D0	556±182	469±72	497±50
	D6	566±164	466±45	431±41
	D7	538±149	431±41	436±51
Food intake (g)	D0	25±3	37±8 **	31±8
	D6	25±3	39±8 **	17±3 ††
	D7	0	0	0
Urinary volume (mL/day)	D0	33±10	148±62 **	121±67 **
	D6	33±12	172±44 **	47±68 †
	D7	27±6	37±14	21±12 †
Urinary glucose excretion (mg/day)	D0	18±6	14005±6712 **	10567±6618 **
	D6	19±7	15900±2893 **	2049±5849 †
	D7	14±6	3162±5616	78±158
Plasma glucose (mg/dL)	D0	105±22	505±75 **	500±67 **
	D6	102±16	467±106 **	273±160 **
	D7	83±6	269±31 ***	168±19 **†
HbA1c %	Pre	3.4±0.3	7.4±1.3 ***	7.3±2.0 ***
	D7	3.4±0.2	8.8±1.1 ***	7.8±1.7***
KITT value	D7	7.1±3.2	0.7±0.8 **	3.2±1.5 *†††
SBP (mmHg)	D7	106±24	103±18	116±31
DBP (mmHg)	D7	79±21	77±12	88±22

Data are expressed as mean ± standard deviation. *p<0.05, **p<0.01, ***p<0.001 vs. control, †p<0.05, †††p<0.001 vs. DM. BFM = bafilomycin; D0 = day 0, D7 = day 7, DM = diabetes mellitus; HbA1c = hemoglobin A1c.

Figure legends

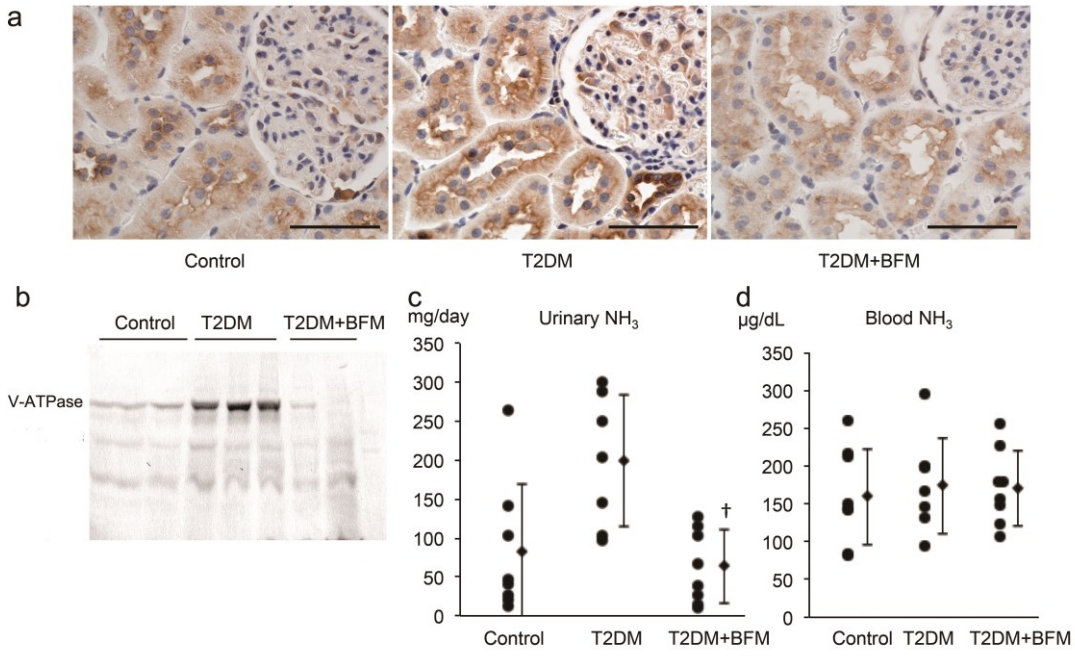

Figure 1. Immunohistochemistry (a) and Western blotting (b) for V-ATPase, and urinary NH_3 excretion (c) and plasma NH_3 levels (d) in control rats, Spontaneously Diabetic Torii type 2 diabetes mellitus (T2DM) rats, and T2DM rats treated with bafilomycin B1 (BFM; 100 nmol/kg BW). † $p<0.05$ vs. T2DM. The bars indicate 50 μm.

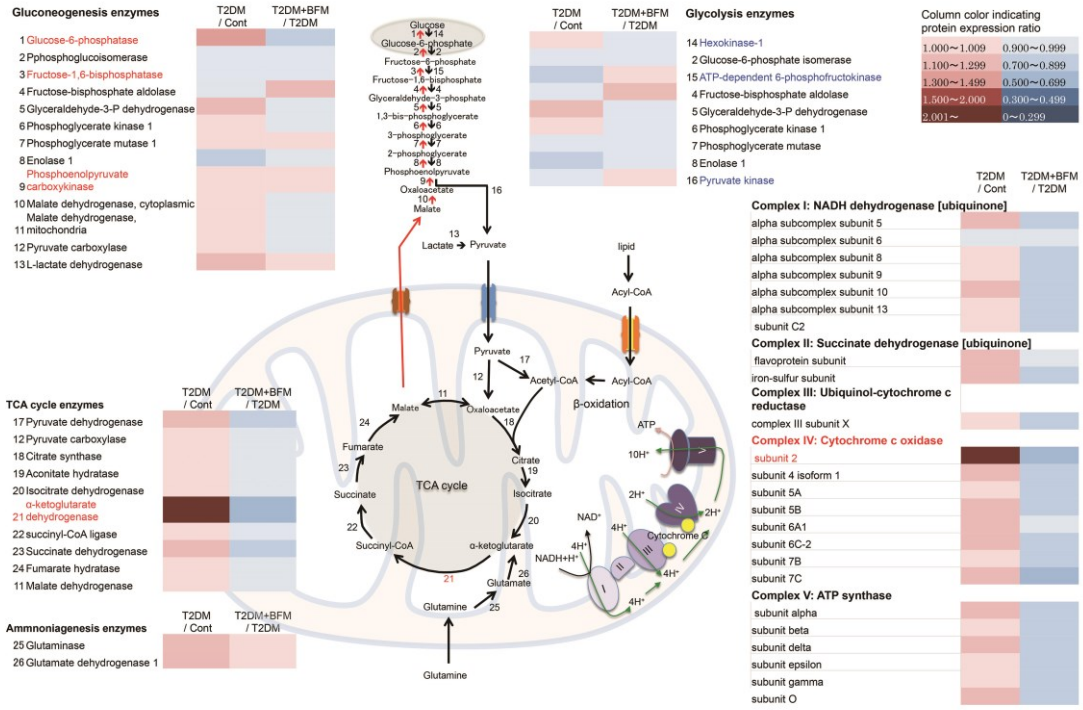

Figure 2. Relative expression ratio of renal enzymes for gluconeogenesis, glycolysis, TCA cycle, ammoniagenesis, and mitochondrial electron transport chain between type 2 diabetes (T2DM) and control, or between T2DM and T2DM treated with bafilomycin B1 (T2DM+BFM). The red column shows the color gradient when the ratio is increased, and the blue column indicates that the ratio is decreased.

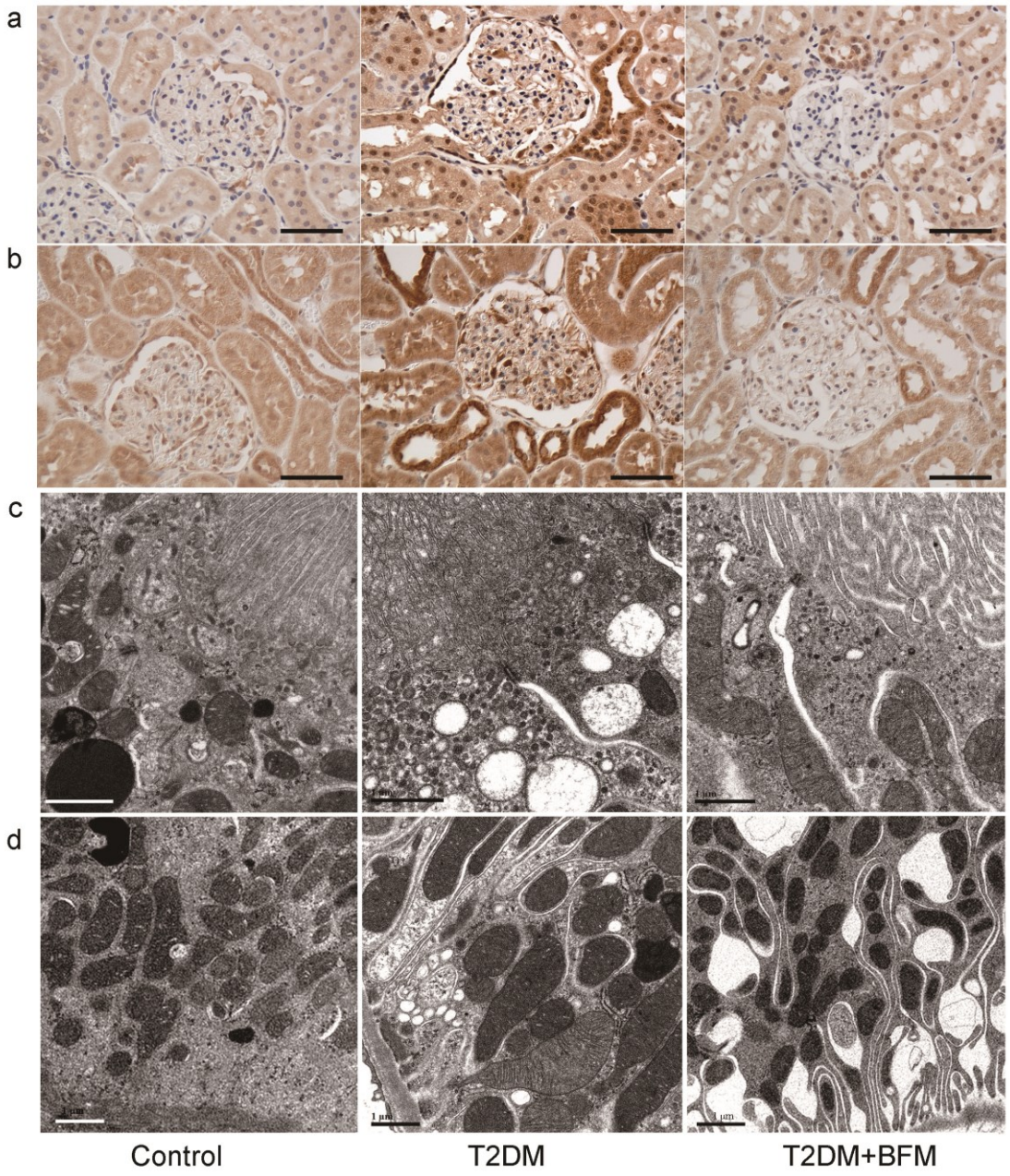

Figure 3. Immunohistochemistry for glucose-6-phosphatase (a) and mitochondrial electron transport chain complex IV (b), and electron microscopy of proximal tubular apical membrane (c) and basal membrane (d) in control rat, type 2 diabetes (T2DM) and T2DM treated with bafilomycin B1 (T2DM+BFM). The bars indicate 50 μm (a,b) and 1

μm (c, d).

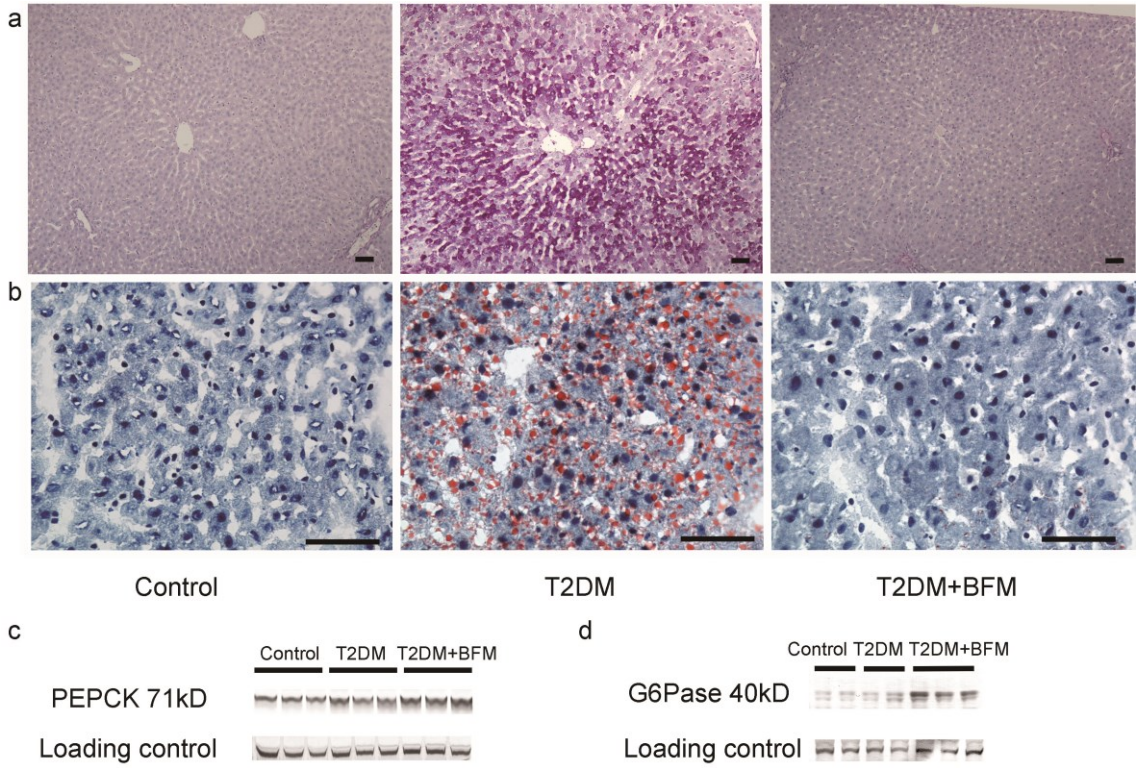

Figure 4. Periodic acid Schiff (PAS) staining (a) and oil red staining (b) of the liver and Western blotting for hepatic cytoplasmic phosphoenol pyruvate carboxykinase (c) and membrane glucose-6-phosphatase (d) in the control rats, type 2 diabetes mellitus (T2DM) rats and T2DM rats treated with bafilomycin B1(T2DM+BFM). The bars indicate 50 μm.

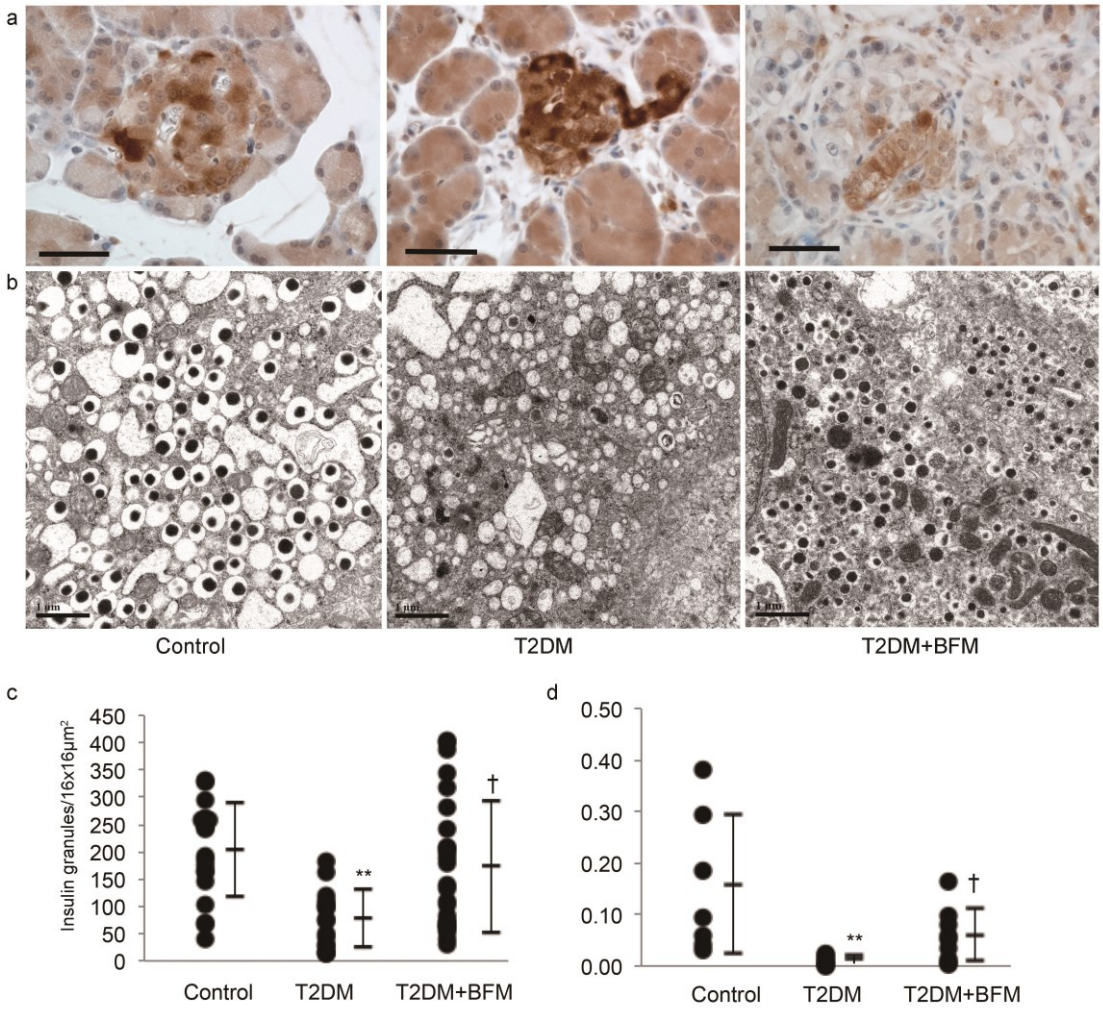

Figure 5. Immunostaining for V-ATPase in the islets of the pancreas (a), electron microscopy of insulin granules in beta cells (b) and the number of insulin granules (c) and insulinogenic index (d) in control rats, type 2 diabetes mellitus (T2DM) rats and T2DM rats treated with bafilomycin B1 (T2DM+BFM). The bar indicates 50μm (a) and 1μm (b). ** $p<0.01$ vs. Control, † $p<0.05$ vs. T2DM.

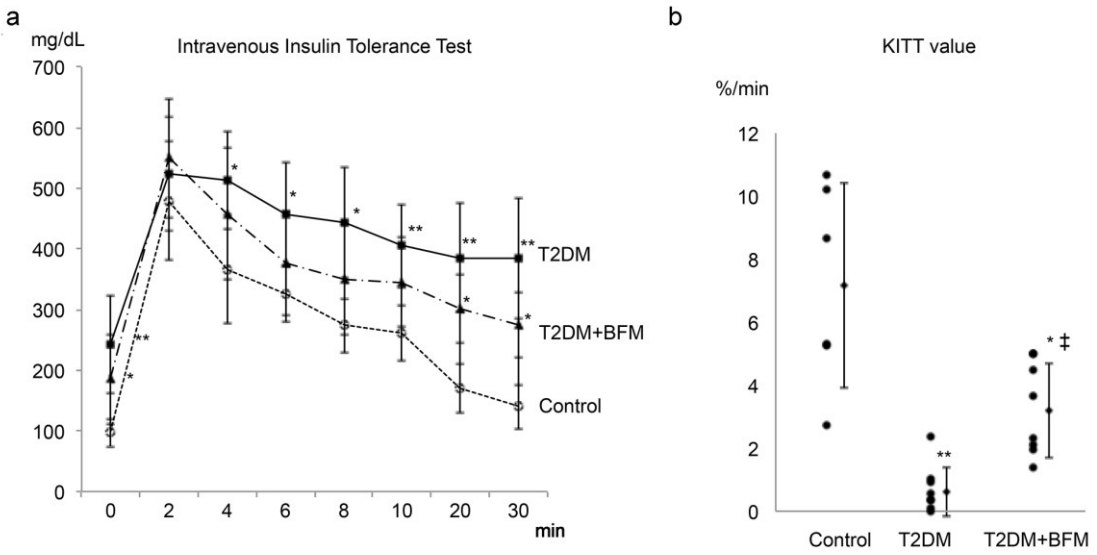

Figure 6. Intravenous insulin tolerance test (a) and KITT values of insulin sensitivity (b) in control rats (n=8), type 2 diabetes mellitus (T2DM) rats (n=8) and T2DM rats treated with bafilomycin B1 (T2DM+BFM, n=6). *p<0.05, **p<0.01 vs. Control, †p<0.05, ‡p<0.01 vs. T2DM.