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Upregulation of sodium-dependent vitamin C transporter 2 expression in adrenals increases norepinephrine production and aggravates hyperlipidemia in mice with streptozotocin-induced diabetes

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ABSTRACT

The hyperglycemia and hyperoxidation that characterize diabetes lead to reduced vitamin C (L-ascorbic acid, AA) levels in diabetic humans and animals. We examined the possibility that diabetes-induced low plasma AA levels impair AA distribution to various tissues and that these changes are closely related to the development of diabetic complications. AA levels were markedly decreased in the plasma and increased in the adrenals of mice with streptozotocin (STZ)-induced diabetes. Consistently with these results, in [1−^{14}C]AA accumulation assays, the efficiency of [1−^{14}C]AA accumulation was significantly higher in the adrenals (which had the greatest ability to accumulate [1−^{14}C]AA) of diabetic mice than in those of controls. Expression of sodium-dependent vitamin C transporter (SVCT)-2, a transporter of AA, was upregulated in diabetic adrenals. Furthermore, increased AA incorporation into the diabetic adrenals by SVCT-2 led to increased plasma norepinephrine, triglyceride and free fatty acid levels in mice with STZ-induced diabetes. Therefore, oversupplementation with AA could be deleterious in diabetic patients, because overexpression of adrenal SVCT-2 in diabetes could lead to excessive AA uptake, thus enhancing norepinephrine production and exacerbating some diabetic complications. Interestingly, however, treatment with AA dose-dependently abolished the increased expression of adrenal SVCT-2 and normalized the abovementioned plasma parameters in diabetic mice. These results suggest SVCT-2-mediated increases in AA uptake by the adrenals followed by excessive production of plasma norepinephrine may play a pivotal role in the development of diabetic complications.

1. Introduction

Vitamin C (L-ascorbic acid, AA) is an essential nutrient for antioxidation, collagen synthesis, maintenance of enzymatic activity, and α-amidation of peptide hormones [1]. Reduced AA is taken up into cells via sodium-dependent vitamin C transporter (SVCT)-1 and -2 [2–5], whereas oxidized vitamin C (dehydroascorbic acid) competes with glucose and is

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Abbreviations: AA, L-ascorbic acid; GLUT, glucose transporter; FFA, free fatty acid; NE, norepinephrine; HPLC, high performance liquid chromatography; STZ, streptozotocin; SVCT, sodium-dependent vitamin C transporter; TG, triglyceride

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transported via glucose transporters (GLUTs) [6–8]. Two SVCT isoforms, SVCT-1 and SVCT-2, have been characterized in rodents and humans [2,3,5,9,10]. Although they exhibit very similar functional properties, SVCT-1 and SVCT-2 are discretely distributed. The SVCT-1 isoform is expressed in epithelial systems, such as those of the intestine and kidney, whereas the SVCT-2 isoform is expressed in more metabolically active and specialized tissues, such as the adrenals, placenta, pancreas, and brain [2,3,9,10]. SVCT-2 expression appears to be localized to regions with a strong requirement for AA and with the ability to concentrate AA. Among the various tissues, the adrenals contain the highest levels of AA and are particularly sensitive to AA deficiency. In addition, AA functions as a cofactor for dopamine β-hydroxylase, which is required in catecholamine synthesis for conversion of dopamine into its metabolite, norepinephrine (NE), in the adrenal medulla [1]. In mice deficient in SVCT-2, the influence of the deficiency on tissue catecholamines was most prominent in the adrenals, where NE levels were decreased by 50% and epinephrine by 81% [11]. These observations suggest that AA transported by SVCT-2 is important for the production of adrenal catecholamines.

Impairment of catecholamine metabolism in diabetes has been investigated extensively, and adrenal catecholamine concentration is elevated in animals with experimentally induced diabetes [12,13]. The adrenal catecholamines have metabolic and hemodynamic effects that range from increasing blood pressure to stimulating glyconeogenesis. They enhance hepatic glucose production [14], reduce the insulin response to glucose [15], and elevate plasma concentrations of free fatty acids (FFA) and ketone bodies [16,17]. Therefore, the excessive production of catecholamines in diabetes is strongly believed to be related to various diabetic complications, including hypertension, cardiomyopathy, congestive heart failure, and hyperlipidemia, in association with elevation of plasma levels of TG, FFA, and ketone bodies [18]. In addition, because AA incorporation into the adrenals is crucial to the control of adrenal catecholamine levels [11], excessive supplementation with AA in diabetes might increase adrenal catecholamine levels and subsequently aggravate diabetic complications.

On the other hand, previous studies have indicated that AA levels are low in the plasma and various tissues of diabetic humans and animals with experimentally induced diabetes; these reduced AA levels result from the hyperglycemia and hyperoxidation that characterize diabetes [19]. The chronic hyperglycemia may cause an intracellular deficiency of AA through competitive inhibition by glucose of the membrane transport of dehydroascorbic acid, which is converted to AA in various cells. Cellular deficiency of AA has been implicated in some of the cellular pathology and complications of diabetes [6]. Studies in diabetic patients and in animal models of diabetes have shown that AA supplementation improves some diabetic complications, such as endothelium-dependent vasodilation, hyperlipidemia, and myocardial dysfunction [19–22]. Thus, AA supplementation may help to prevent the development of various diabetic complications. However, the detailed mechanisms of the pathogenesis of diabetic complications and the role of AA in preventing their development remain obscure. Furthermore, the influence of AA supplementation on AA incorporation into the adrenals and catecholamine metabolism in diabetes has not yet been investigated.

Although plasma and tissue AA levels in diabetes are decreased due to oxidative loss, increased urinary excretion and the aforementioned competitive inhibition of dehydroascorbic acid uptake [23–25], diabetes may also impair SVCTs-mediated AA incorporation into various tissues. Here, we hypothesized that the diabetes-induced low plasma AA levels and exclusion of dehydroascorbic acid from cells generally influence SVCT-2-mediated AA incorporation into the adrenal cells, which require AA and are sensitive to variations in intracellular AA concentration. In addition, we theorized that alterations in SVCT-2-mediated AA incorporation impair adrenal catecholamine metabolism and lead to various catecholamine-related complications. We further examined whether supplementation with AA would prevent the development of these complications by correcting these disorders of AA- and SVCT-2-mediated catecholamine metabolism.

2. Materials and methods

2.1. Experimental animals

Eight-week-old male ICR mice were purchased from CLEA Japan (Tokyo, Japan). Mice were housed in a room maintained at 23 ± 2 °C with 50 ± 10% humidity and a 12-h light:12-h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). In addition, they were allowed free access to water and regular rodent chow (CE2; CLEA Japan) before using experiments.

Diabetes was induced as follows; after being fasted for 20 h, mice randomly received a single i.p. injection of either 190 mg/kg streptozotocin (STZ, Wako Pure Chemicals, Osaka, Japan) freshly dissolved in 50 mM sodium citrate buffer (pH 4.5) or an equal volume of vehicle only (control group). After injection of STZ or vehicle only, regular rodent chow containing 130 mg/kg total vitamin C was switched to pelleted vitamin C-free AIN-93M diet (CLEA Japan) to avoid the likely interference of added vitamin C in the rodent chow. One week after injection, blood samples of STZ-treated mice were obtained from the tail vein under pentobarbital sodium (Nembutal Injection, Dainabot, Osaka, Japan) anesthesia for determination of plasma glucose level. STZ-treated mice with plasma glucose levels exceeding 300 mg/dL were diagnosed as diabetic and were retained as the STZ group.

Where necessary, animals were terminally anesthetized by i.p. injection of pentobarbital sodium (Nembutal Injection) except in the case of the [1−^{14}C]AA distribution assay (see Section 2.3). All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

2.2. Measurement of AA levels in plasma and tissues by high performance liquid chromatography (HPLC)

AA levels were determined as described in our previous papers [4,26]. Plasma was mixed with an equal volume of cold 10% metaphosphoric acid, whereas homogenates of various tissues were combined with four volumes of 2% metaphosphoric

acid. After centrifugation of the solutions at 13,000 × *g* for 10 min at 4 °C, the supernatants were subjected to HPLC analysis. Briefly, a liquid chromatograph (LC-10AD, Shimadzu, Kyoto, Japan) was equipped with a UV detector (SPD-10, Shimadzu) and a Chromatopac data analyzer (C-R6A, Shimadzu). Separation was achieved by isocratic elution in a Shimpack ODS column (6 mm × 150 mm), with a buffer comprising 0.1 M potassium phosphate and 0.1 M phosphoric acid (pH 3.0), at a flow rate of 0.7 mL/min. Each sample (20 μL) was analyzed at a wavelength of 243 nm, and its concentration was determined according to peak height.

2.3. [1−^{14}C]AA distribution assay

Mice 1, 4, or 8 weeks after injection of STZ or vehicle only were injected via the tail vein with 0.2 mL of [1−^{14}C]AA (0.295 MBq/μmol, Perkin-Elmer Life Sciences, Boston, MA) dissolved in normal saline to a concentration of 1 mM. Five minutes later, the mice were euthanized by CO_2 (the actual time taken for distribution was approximately 5 min and 30 s) and their blood harvested rapidly from the vena cava for separation of plasma. After various organs had been removed and weighed, 100 mg samples of each were lysed in 1 mL Soluene 350 (Packard BioScience B.V., Groningen, Netherlands), then mixed with 10 mL of Hionic Fluor (Packard BioScience B.V.); in the case of plasma, 100 μL was mixed with 4 mL Clear-sol I (Nakalai Tesque, Kyoto, Japan) for measurement of radioactivity in a liquid scintillation analyzer (Tri-Carb2100TR, Packard BioScience B.V.). Data were expressed as nmol/g.

2.4. AA treatment

Twenty-four mice from the STZ group were further allocated into three groups and provided with free access to tap water containing 0, 1, or 2 g/L AA (solutions prepared fresh daily); control mice were allowed free access to untreated tap water. After 3 weeks of treatment with or without AA, the mice were euthanized for measurement of plasma concentrations of NE, glucose, triglyceride (TG), and FFA, as well as for isolation of total RNA from the adrenals.

2.5. RNA isolation and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA from the adrenals and kidneys was prepared with TRIzol Reagent (Invitrogen, Carlsbad, CA) by the acid guanidine–phenol–chloroform method and stored at –80 °C until use. For each sample, 6 μg of RNA was reverse-transcribed by using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a 20 μL reaction mixture. After 15-fold dilution, 2 μL of reverse-transcribed template was used for real-time quantitative RT-PCR. Real-time RT-PCR was performed with a SYBR Green PCR Master Mix kit and a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) and a LightCycler system (Roche Diagnostics, Mannheim, Germany). The primers used for amplification of mSVCT-2 (GenBank accession no. AB038145) were: sense primer, 5′-GGCCCCTGCTCGAGCCATC-3′, and antisense primer, 5′-CAGCTCTGCCGTTCCATTGGC-3′. The amplification conditions for mSVCT-2 were initial denaturation at 95 °C for 15 min, followed by 35 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 9 s. The primers used for amplification of mSVCT-1 (GenBank accession no. AF058318) were: sense primer, 5′-AGATTGAGGATGTGCCACCATG-3′, and antisense primer, 5′-GGTGATACCCACACAGGTGAAGATG-3′. The amplification conditions for mSVCT-1 were: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 12 s. After PCR amplification, a melting curve was obtained by stepwise increase in the temperature from 55 to 95 °C to ensure that a single product was amplified in the reaction. As an internal control, the level of mRNA of mβ-actin (GenBank accession no. X03672) was measured; the primers for amplification were: sense primer, 5′-TTTCCAGCCTTCCTTCTTGGGT-3′, and antisense primer, 5′-TTGGCATAGAGGTCTTTACGGATG-3′. The reaction conditions were: initial denaturation at 95 °C for 15 min followed by 35 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 10 s. PCR reactions were done in duplicate, and the relative expressions of the mRNAs were normalized by dividing the mSVCT-2 values by the respective mβ-actin values.

2.6. ELISA determination of NE

Plasma NE concentrations were determined by NE enzyme immunoassay kit (IBL, Hamburg, Germany). The limitation of sensitivity for the plasma NE assay was 20 pg/mL, and the inter-assay and intra-assay coefficients of variation were 14.9 and 7.9%, respectively. The mean recoveries were 95–104.7%. Cross-reactivity with adrenaline, metanephrine, and normetanephrine was 0.46, 0.05, and 2%, respectively, and that with other substances tested was less than 0.01%.

2.7. Determination of plasma glucose, TG, and FFA concentrations

Glucose, TG, and FFA concentrations in plasma were quantified by using the Glucose C II, Triglyceride E, and NEFA C test kits, respectively (Wako Pure Chemicals). The inter-assay and intra-assay coefficients of variation were, respectively, 6.7 and 9.6% for glucose determination, 10.2 and 11.4% for TG determination, and 9.2 and 7.8% for FFA determination.

2.8. Statistics

Numerical data were presented as means ±1 S.D. from at least five mice and analyzed by Tukey's multiple comparisons test with SPSS software (Chicago, IL). Significance was assessed at the $P < 0.05$ and $P < 0.01$ levels. Experiments were performed independently at least twice; results were qualitatively identical, and representative results are shown.

3. Results

3.1. Plasma glucose levels and AA concentrations in tissues

To determine glucose and AA levels in STZ-treated mice, both the plasma used to determine glucose and AA and various

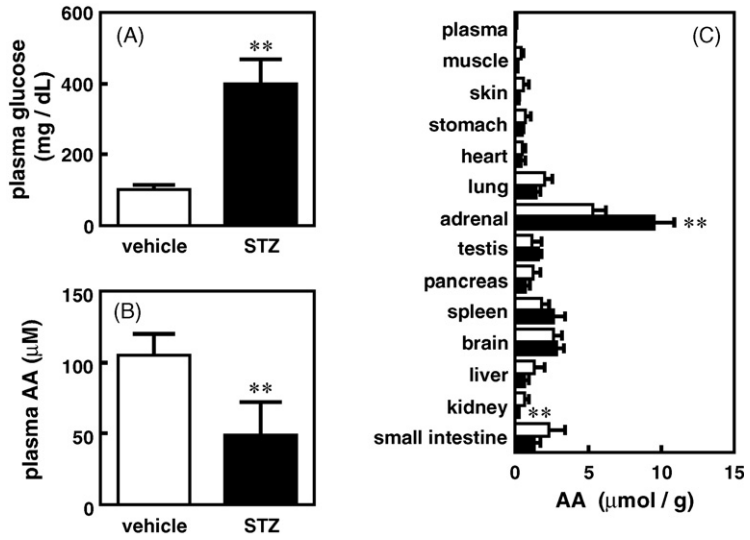

Fig. 1 – AA levels in plasma and various tissues of mice with STZ-induced diabetes. One week after i.p. administration of STZ or vehicle only, diabetic (n = 10, filled bars) and control (n = 10, open bars) mice were euthanized and plasma and various tissues from the same mice was harvested for determination of both glucose and AA levels. Plasma glucose (A) and AA concentrations (B) and the AA concentration in various tissues (C) were measured as described in Section 2. Results are expressed as means ±1 S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. vehicle-treated control mice. This experiment was performed twice independently; the results were qualitatively identical, so the results from a representative experiment are shown.

tissue samples for determination of AA levels were taken from the same animal 1 week after administration of STZ. Plasma glucose concentrations in STZ-treated diabetic mice reached 404.9 ± 62.2 mg/dL (n = 54)—approximately 380% of those of vehicle-treated mice (108.4 ± 12.5 mg/dL, $P < 0.01$; n = 32, Fig. 1A). The plasma AA concentrations of STZ-treated mice were 48.93 ± 23.33 μM (n = 10)—approximately 45% of those of vehicle-treated mice (104.8 ± 15.34 μM, $P < 0.01$; n = 10) (Fig. 1B). AA levels in the intestine, liver, brain, spleen, pancreas, testis, lung, heart, stomach, skin, and muscle were not significantly different between control and STZ-treated mice (Fig. 1C). In contrast, in the adrenals, the AA level of STZ-treated mice was 9.46 ± 1.42 μmol/g tissue—approximately 180% of that of control mice (5.30 ± 0.89 μmol/g tissue; $P < 0.01$). In the kidneys, the AA level of STZ-treated mice was 0.25 ± 0.05 μmol/g tissue, whereas that in control mice was significantly higher at 0.62 ± 0.29 μmol/g tissue ($P < 0.01$).

3.2. *Distribution of [1−^{14}C]AA in various tissues of diabetic mice*

Because AA levels were significantly higher in the adrenals of STZ-treated mice than in those of control mice but significantly lower in the kidneys of STZ-treated mice, we investigated the rate of AA transport into various tissues. We injected STZ-treated mice with [1−^{14}C]AA via the tail vein and measured AA accumulation in various tissues within 5 min and 30 s. Consistently with the results for AA level (Fig. 1C), [1−^{14}C]AA accumulation in the adrenals was significantly greater than in control mice at every test time after STZ injection (Fig. 2). Among the various other tissues tested, [1−^{14}C]AA accumulation in the stomach, lungs, and intestine was significantly greater 1 week after STZ injection than in control mice, but at 4 and 8 weeks after STZ injection, the levels in these tissues were no longer significantly greater than in the controls. In the kidneys, the level of [1−^{14}C]AA accumulation 1 week after STZ injection was not significantly different from that in the controls (Fig. 1C); however, it did show a tendency to decrease, and [1−^{14}C]AA accumulation in the kidneys of STZ mice was significantly lower than in those of control mice at 4 and 8 weeks after STZ injection (Fig. 2).

3.3. *mRNA expression of adrenal SVCT-2 and kidney SVCT-1 in diabetic mice*

Because the adrenals and kidneys were the only tissues in which the results for AA level were consistent with those for AA uptake in mice with STZ-induced diabetes, we focused on AA transport into the adrenals and kidneys. We used real-time RT-PCR to analyze the expression of the mRNAs of the AA transporters SVCT-1 and SVCT-2 in the adrenals and kidneys of mice. In our real-time RT-PCR system, expression of the mRNA of SVCT-2 in the adrenals of control mice was much higher (over 500 times) than that of SVCT-1, whereas that of SVCT-2 in the kidneys was almost undetectable (data not shown). These observations suggest that AA is transported into the adrenals mainly by SVCT-2 and into the kidneys mainly by SVCT-1. Therefore, we analyzed the expression of the mRNA of SVCT-2 in the adrenals and SVCT-1 in the kidneys of mice 4 weeks after STZ treatment. SVCT-2 mRNA expression in the adrenals of STZ-treated diabetic mice was approximately 750% of that in the control mice (Fig. 3A). On the other hand, SVCT-1 mRNA expression in the kidneys of STZ-treated diabetic mice was approximately 50% of that in the control mice (Fig. 3B).

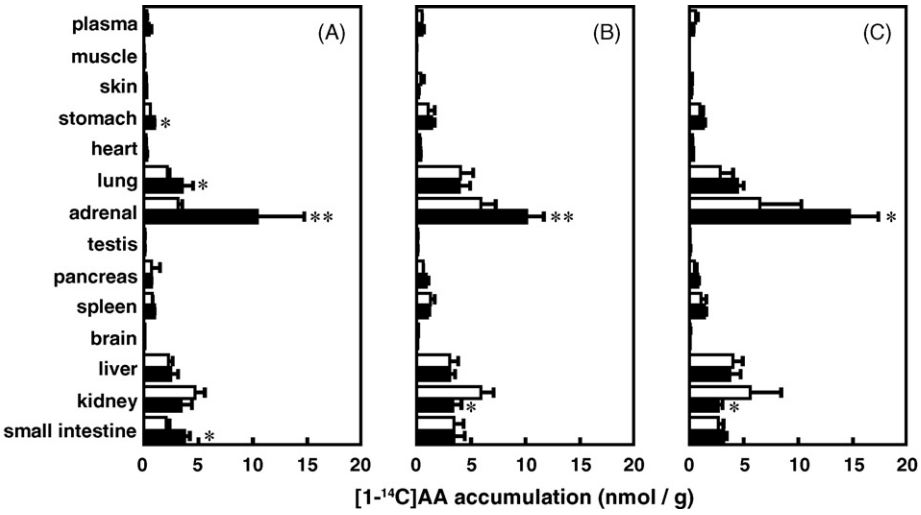

Fig. 2 – [1−^{14}C]AA accumulation in various tissues of mice with STZ-induced diabetes. One (A), 4 (B), and 8 (C) weeks after i.p. administration of STZ or vehicle only, diabetic (n = 5, filled bars) and control (n = 5, open bars) mice received [1−^{14}C]AA (50 nmol per mouse) i.v.; 5 min later, the mice were euthanized by CO_2 and the plasma and various tissues recovered. The radioactivity of a 100 mg sample of tissue or 100 μL of plasma was determined as described in Section 2. Data are expressed as means ±1 S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. vehicle-treated control mice. This experiment was performed twice independently; the results were qualitatively identical, so the results from a representative experiment are shown.

3.4. *Alteration in plasma parameters in diabetic mice*

Among the various complications of diabetes, hyperlipidemia is closely related to plasma NE levels [27,28]. Because AA functions as a cofactor of β-hydroxylase for converting dopamine to NE in the adrenals [1], the increased AA uptake due to upregulation of SVCT-2 expression in the adrenals of diabetics was expected to increase the production of NE. As expected, the concentration of plasma NE 4 weeks after STZ treatment was much higher than in control mice (Fig. 4A; $P < 0.01$). In addition, consistently with this increased plasma NE level, plasma TG and FFA levels were significantly greater in STZ-treated mice than in controls (Figs. 4B and C; both $P < 0.01$). These results suggest that hyperlipidemia might be closely related to adrenal SVCT-2 mRNA expression via the regulation of NE production.

3.5. *Effect of AA supplementation on adrenal SVCT-2 mRNA expression and plasma parameters in diabetic mice*

The upregulation of adrenal SVCT-2 mRNA expression in STZ-treated mice prompted us to examine whether supplementation with AA would increase the plasma NE level and subsequently aggravate hyperlipidemia. To gain a better understanding of the role of adrenal SVCT-2 in diabetic

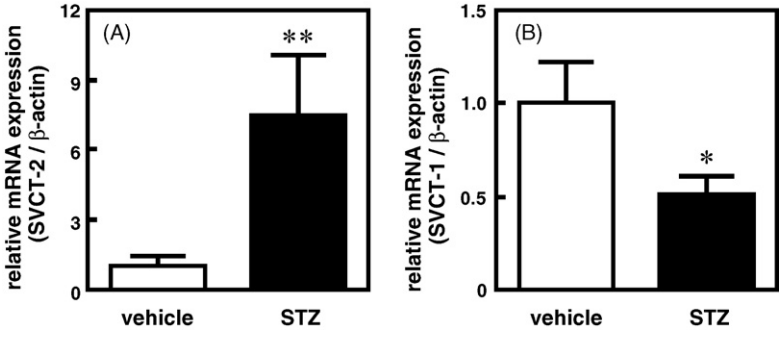

Fig. 3 – Adrenal SVCT-2 (A) and kidney SVCT-1 mRNA levels (B) in mice with STZ-induced diabetes. Four weeks after i.p. administration of administration of STZ or vehicle only, diabetic (n = 5, filled bars) and control (n = 5, open bars) mice were euthanized and the kidneys and adrenals recovered. Total RNAs from the adrenals or kidneys of STZ- or vehicle-treated control mice were prepared and used as templates for determination of adrenal SVCT-2 or renal SVCT-1 mRNA expression by real-time RT-PCR assay, as described in Section 2. These mRNA expressions were normalized relative to the expression level of mβ-actin. Data are expressed relative to mRNA levels (defined as 1) in the adrenals or kidneys of vehicle-treated mice. All results are expressed as means ±1 S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. vehicle-treated control mice. This experiment was performed twice independently; the results were qualitatively identical, so the results from a representative experiment are shown.

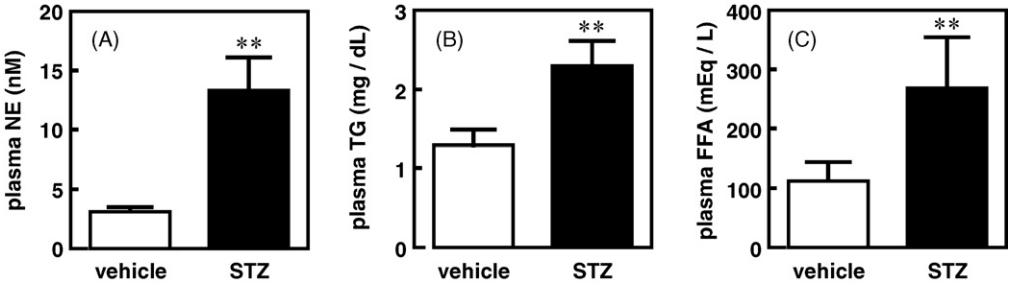

Fig. 4 – Plasma NE (A), TG (B), and FFA (C) levels in mice with STZ-induced diabetes. Four weeks after i.p. administration of STZ or vehicle only, diabetic (n = 5, filled bars) and control (n = 5, open bars) mice were euthanized and the plasma recovered. Plasma NE, TG, and FFA levels were determined as described in Section 2. All results are expressed as means ±1 S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. vehicle-treated control mice. This experiment was performed twice independently; the results were qualitatively identical, so the results from a representative experiment are shown.

hyperlipidemia, we observed the effects of AA treatment on plasma parameters in mice with STZ-induced diabetes. Contrary to our expectations, treatment of diabetic mice with AA at 2 g/L for 3 weeks, beginning 1 week after administration of STZ significantly inhibited the excessive production of plasma NE seen in diabetic mice receiving no AA (Fig. 5A; $P < 0.01$). In addition, consistently with the suppression of plasma NE production, plasma TG and FFA levels were significantly lower in mice with STZ-induced diabetes receiving AA at 2 g/L for 3 weeks than in diabetic mice receiving no AA (Fig. 5B and C; $P < 0.01$ and $P < 0.05$, respectively). Treatment of STZ-treated mice with AA at 1 or 2 g/L for 3 weeks had no obvious effect on plasma glucose levels compared with those in diabetic mice given no AA (Fig. 5D).

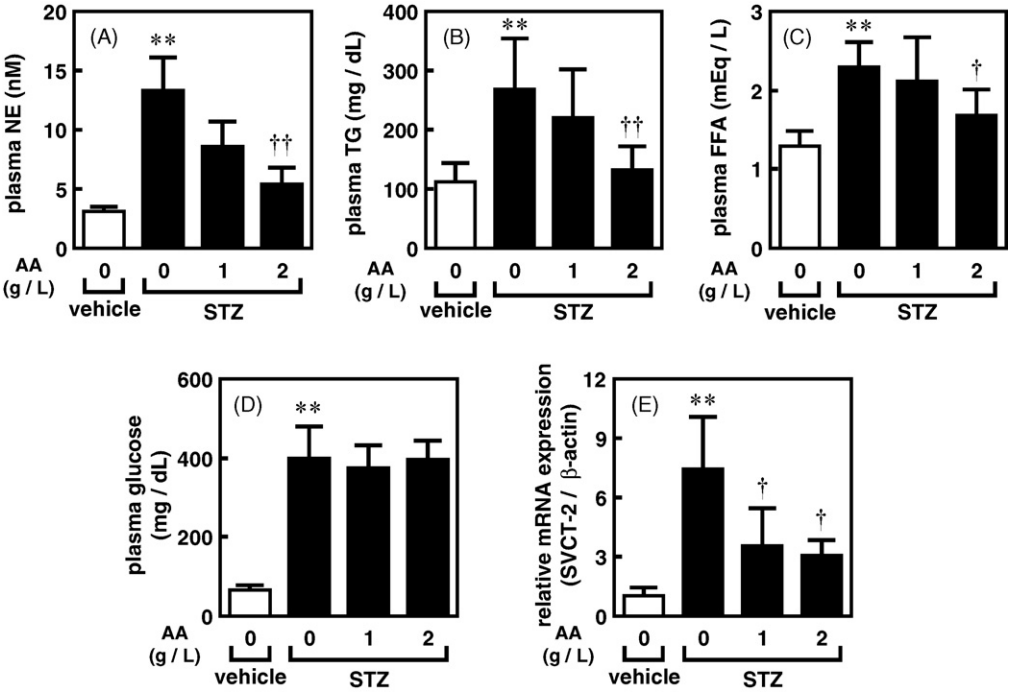

Fig. 5 – Effect of AA supplementation on adrenal SVCT-2 mRNA expression and plasma parameters in mice with STZ-induced diabetes. One week after i.p. administration of STZ, diabetic mice (filled bars) were further divided into three groups and given free access to tap water containing 0, 1, or 2 g/L AA (n = 8 for each group). Control mice (open bars, n = 10) were treated with vehicle and provided with free access to untreated water. After 3 weeks of AA treatment, plasma NE (A), TG (B), FFA (C), and glucose (D) levels were determined as described in Section 2. Total RNAs from the adrenals of STZ- and vehicle-treated mice were prepared and used as templates for determination of SVCT-2 mRNA expression by real-time RT-PCR assay, as described in Section 2. Expression of SVCT-2 mRNA (E) was normalized relative to the expression level of mβ-actin. Data are expressed relative to mRNA levels (defined as 1) in the adrenals of vehicle-treated mice. All results are expressed as means ±1 S.D. $^{**}P < 0.01$ vs. vehicle-treated control mice; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs. STZ-diabetic mice treated with AA at 0 g/L. This experiment was performed twice independently; the results were qualitatively identical, so the results from a representative experiment are shown.

We also observed the effects of AA treatment on SVCT-2 mRNA expression in mice with STZ-induced diabetes. The results indicated that treatment with AA dose-dependently and significantly inhibited overexpression of SVCT-2 mRNA in the adrenals of STZ-treated diabetic mice, as compared with vehicle treatment (Fig. 5E; $P < 0.05$). The declines in TG and FFA levels resulting from treatment with AA nearly paralleled the changes in plasma NE levels and in adrenal SVCT-2 mRNA expression. On the other hand, in non-diabetic control mice, treatment with AA at 1 or 2 g/L for 3 weeks did not result in an obvious effect on plasma levels of NE, TG or FFA, or adrenal SVCT-2 expression compared with no AA treatment (data not shown).

4. Discussion

Treatment of mice with STZ led to significantly higher AA levels in the adrenals (Fig. 1C); these findings corresponded to the results of [1−^{14}C]AA distribution analysis, in which the rate of AA transport was significantly increased in the adrenals of the diabetic mice (Fig. 2). Moreover, the increase in AA levels in the diabetic adrenals 4 weeks after STZ administration paralleled the changes in AA incorporation and in SVCT-2 mRNA expression (Figs. 2B and 3A). AA is transported via SVCT-1 and/or SVCT-2 into various cells. Previous reports [2,3], including the results of our real-time RT-PCR experiments, have shown that expression of SVCT-2 mRNA is much higher than that of SVCT-1 mRNA in the adrenals. These results suggest that upregulation of SVCT-2 mRNA expression, which was probably followed by increased production of SVCT-2 protein, contributed mainly to the observed increase in AA concentration in the diabetic adrenals.

As described previously, exposure to oxidative stress increases SVCT-2 mRNA expression in several types of cell [29,30]. In diabetes, because oxidative stress is also markedly increased and may contribute to the development of diabetic complications [31], upregulation of SVCT-2 mRNA expression in the adrenals may be due to hyperoxidation. This notion is further supported by the results of treatment with AA, an antioxidant, indicating that significant inhibition was occurred in STZ-induced SVCT-2 mRNA expression in diabetic adrenals (Fig. 5A), but not in non-diabetic adrenals (data not shown). Although future studies of the effects of other antioxidants, such as glutathione, tocopherol, and *N*-acetyl cysteine, on SVCT-2 expression are needed to elucidate the precise mechanism of regulation of adrenal SVCT-2 expression in diabetes, we infer that the hyperoxidation characteristic of diabetes may be responsible for the increases in SVCT-2 expression, AA uptake, and AA levels in the diabetic adrenals.

The decrease in AA levels in the diabetic kidney, along with the obvious reduction in AA incorporation (Fig. 1C, 2) is interesting because of its contrast to the increase in AA levels in the diabetic adrenals. Previous reports [2–4,9] and the results of our real-time RT-PCR experiment showed that SVCT-1, but not SVCT-2, is expressed in the kidney and that SVCT-1 mRNA expression is significantly decreased in the diabetic kidney (Fig. 3B). These observations indicate that downregulation of SVCT-1 expression may account for the decrease in AA concentration in the diabetic kidney. The physiological significance of this downregulated SVCT-1 expression and AA uptake in the diabetic kidney is unknown and worthy of further study in future.

Previous reports have shown that plasma and tissue catecholamine levels are increased [32–34], decreased [35], or unchanged [13] in diabetic animals, and these disparities have been explained by differences among the tissues selected or in the severity or duration of the diabetes. Under our experimental conditions, plasma NE levels were significantly increased after STZ administration (Fig. 4A), consistently with previous reports [32–34]. Our results demonstrated that upregulation of SVCT-2 expression in the adrenals nearly paralleled the increase in plasma NE level. This might have been due to the significant increase in AA incorporation and the high adrenal AA level, because AA functions as a cofactor for dopamine β-hydroxylase, which is required in catecholamine synthesis for conversion of dopamine into NE in the adrenal medulla. Thus, we infer that upregulation of SVCT-2 expression and AA uptake in diabetes, followed by excessive production of NE in the adrenals, may result in the increase in plasma NE level and lead to hypersympathetic activation [18]. Consistently with our results, in SVCT-2-null mice the influence of SVCT-2 deficiency on tissue catecholamines is most prominent in the adrenals, in which NE levels are markedly decreased [11]. In addition, AA-treatment-induced suppression of adrenal SVCT-2 expression led to a reduction in excessive production of plasma NE. These results strongly suggest that the high levels of NE in the plasma of diabetic mice most likely result from the upregulation of SVCT-2 expression and increase in AA uptake by the adrenals in the absence of AA supplementation.

In patients with pheochromocytoma, which is characterized by high plasma catecholamine levels, marked elevation in the levels of plasma glucose, TG, FFA, and total ketone bodies is observed. Pharmacological adrenergic blockade (β-blockade) normalizes plasma TG, FFA, and ketone body levels without obvious change in catecholamine concentration [36,37]. These observations suggest that levels of adrenal catecholamines (especially NE) are closely related to hyperlipidemia. Excessive sympathetic activity is also associated with some complications of diabetes, such as diabetic neuropathy, ketoacidosis, and hyperlipidemia [28,38]. This abnormality stimulates adipose tissue lipolysis and increases the lipolytic responsiveness of the adrenoceptors to catecholamines [39–42], increasing glycerol (lipolysis index) and FFA levels in plasma. Our results showed that mice with STZ-induced diabetes in the absence of AA supplementation had higher plasma levels of TG and FFA than did control mice (Fig. 4B and C). These increases may have resulted from the upregulation of SVCT-2 expression and AA uptake, followed by excessive production of NE in the adrenals. In fact, AA-treatment-induced downregulation of adrenal SVCT-2 expression was accompanied by a decrease in plasma levels of TG, FFA, and NE (Fig. 5), further supporting the notion that the AA- and SVCT-2-associated changes in lipid metabolism are mediated by NE.

Because supplemented AA is partly oxidized to dehydroascorbic acid in diabetic mice, AA supplementation may increase in concentration of plasma dehydroascorbic acid, which could compete with GLUTs and inhibit glucose uptake by various tissues, thereby increasing plasma glucose levels.

However, our results indicated that AA supplementation in STZ-induced diabetic mice markedly ameliorated hyperlipidemia, but not plasma glucose levels (Fig. 5B). This effect could not have been due to improvement in the control of insulin release, since AA supplementation hardly affects insulin release, as described previously [19,20]. However, it could be explained by the following: dehydroascorbic acid is transported by certain isoforms of GLUTs (GLUT1, GLUT3, and probably GLUT4), whereas glucose can be transported by all GLUTs [7,8]. In addition, transport affinities (Michaelis constant) of dehydroascorbic acid for GLUT1 and GLUT3 of 1.1 and 1.7 mM, respectively, are similar to, or less than those for glucose [8], the plasma dehydroascorbic acid level (~20 μM) is much lower than the plasma glucose level (~20 mM) in diabetic mice [43,44]. Thus, dehydroascorbic acid is not present at a level sufficient for it to compete with the glucose and prevent intracellular hyperglycemia. Consequently, even though dehydroascorbic acid increases in concentration after AA supplementation and competes with certain GLUT isoforms, extracellular hyperglycemia would not be markedly affected.

Although AA supplementation has been reported to ameliorate various complications of diabetes including hyperlipidemia [20–22,41,42], the mechanism behind these beneficial effects remains unclear. Our results suggests that supplementation with AA should help to prevent hyperlipidemia via the inhibition of adrenal SVCT-2 expression in diabetes. However, oversupplementation with AA could be deleterious for diabetic patients, because their adrenals could overexpress SVCT-2 and take up excessive AA to enhance NE production, with the consequence that some complications could be exacerbated. Although the benefits of AA supplementation appear to be supported by clinical observations in patients with diabetes [20,21,45,46], further investigation is needed to clarify why AA supplementation in diabetes is clinically beneficial.

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