Elevated Urinary Cr Loss Induces a Reduction in Renal Cr Concentration and the Negative Cr Balance in Streptozotocin-Induced Diabetic Mice

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Summary Chromium (Cr) is an essential trace element and is important for normal carbohydrate metabolism, and its deficiency in animals can cause a diabetic-like state. Human and experimental animal studies suggest that urinary Cr excretion is increased in diabetic populations. To investigate whether hyperglycemia-induced elevation of urinary Cr excretion reduces tissue Cr storage conditions, we assessed total Cr balance and Cr distribution in streptozotocin (STZ)-induced diabetic mice. Male C57BL mice were randomly assigned to STZ or control groups and their urine was collected 7, 14, 21 and 28 d after STZ injection. An inductively coupled plasma mass spectrometry instrument equipped with a dynamic reaction cell was used for determination of Cr in urine, plasma and tissues samples. The urinary excretions of Cr were 15.4±3.0 and 356±62 ng/d, and the renal Cr concentrations were 0.85±0.12 and 0.17±0.03 ng/mg for the control and diabetes groups, respectively (p<0.01), after 28 d. The Cr balance in STZ-treated mice was distinctly negative due to the increase in urinary Cr loss (p<0.01). These results suggest that in mice, STZ induces a reduction in renal Cr concentration and total negative Cr balance caused by an increase in urinary Cr output.

Key Words chromium, chromium balance, diabetes, ICP-DRC-MS, hyperglycemia

Diabetes mellitus is a serious and chronic metabolic disorder. Previous research has suggested diabetes is associated with abnormalities in the metabolism of trace elements, including chromium (Cr), selenium, zinc, copper and others. In particular, Cr has been shown to directly modulate glucose and lipid homeostasis (1). Furthermore, a Cr deficiency in humans and in laboratory animals can cause impaired glucose tolerance, which can be reversed with Cr supplementation (2).

Our previous study (3) revealed that dietary Cr supplementation improves several indices related to diabetic nephropathy, such as photomicrographs of renal uriniferous tubules, urinary albumin level, creatinine clearance and BUN/creatinine in KK-Ay diabetic mice. Simultaneous measurement of renal Cr concentration showed a significant reduction in KK-Ay diabetic mice compared to C57BL non-diabetic mice, and this reduction in renal Cr levels was ameliorated to the normal level by dietary Cr supplementation.

It is unknown whether a reduction in renal Cr concentration partly triggers diabetic nephropathy. Although continuous diabetic hyperglycemia causes diabetic nephropathy, the effect of hyperglycemia on renal Cr concentration is not well understood. In our previous report (3), urinary Cr excretion in diabetic mice was more than three times the standard Cr excretion in non-diabetic mice, which was coincident with the reduction in renal Cr concentration.

Increased urinary Cr excretion has been induced in several diabetic models. Morris et al. (4–6) have reported that urine Cr concentrations of adult-onset diabetics are approximately twice as high as those of healthy subjects. Our previous study (3) suggested that urinary Cr excretion is increased in type 2 diabetic mice compared with normal mice. In healthy individuals, increased urinary Cr output has been observed in response to increases in serum glucose or insulin (7–12). Therefore, diabetes mellitus may disrupt the balance between the absorption and excretion of Cr. Nevertheless, information concerning the influence of diabetes mellitus on the metabolism of Cr is still limited.

These previous studies revealed elevation of plasma Cr levels caused by hyperglycemia; however, the tissue Cr levels were not investigated. Precise determination of Cr in tissue or plasma requires a skilled technician, as the concentrations of Cr in blood, urine and tissue are close to the limit of determination by atomic absorption spectrophotometry (13–16). In the present study, the dynamic reaction cell (DRC) and/or collision cell technique proved to be effective methods for alleviating...
such spectroscopic interferences in inductively coupled plasma mass spectrometry (ICP-DRC-MS, ELAN DRC II; PerkinElmer, Wellesley, MA, USA).

In the present study, we aimed to clarify whether elevation of urinary Cr is caused by diabetic renal dysfunction, since the major cause of reduction in Cr storage would be the elevation of urinary Cr excretion. Treatment with streptozotocin (STZ) induces an abrupt hyperglycemia without renal dysfunction and the continuous hyperglycemia gradually leads to the onset of renal dysfunction. Sequential observation of urinary Cr excretion and renal function was used to demonstrate the effect of hyperglycemia and renal dysfunction on the elevation of renal Cr excretion. We also investigated the balance of Cr and its tissue retention in STZ-treated mice. Apart from the relationship between renal and urinary Cr concentration and diabetic nephropathy, it is apparent that increased urinary Cr loss may deplete stores of Cr in diabetes.

MATERIALS AND METHODS

Animals. All procedures were performed in accordance with the animal experimentation guidelines of Sugiyama Jogakuen University. Male 5-wk-old C57BL/6Cr mice (CLEA Japan, Inc., Tokyo, Japan) weighing 14–20 g were used. Animals were housed individually in plastic cages under a controlled atmosphere (temperature 24.5 ± 1˚C, humidity 55 ± 5%, light from 08:00 to 20:00 h). MilliQ water (Millipore, Bedford, MA) and a commercial diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) of the same lot number were available ad libitum. Food intake and body weights were recorded daily. The concentration of Cr in the CRF-1 diets was 46.5 ± 5.5 μg of Cr per kg diet as determined by ICP-DRC-MS.

After a 3-d acclimation period, the mice were randomly divided into either STZ-treated (n=8) or normal control (n=5) groups. Eight mice were injected intraperitoneally with two doses of 0.1 mg/g body wt of STZ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 0.05 M citrate acid buffer (pH 4.5; Kanto Chemical, Tokyo, Japan), once after 24 h food deprivation and again at a similar time of the day two days later. Control mice were injected with equal volumes of citric acid buffer at the same times as the STZ injections. The day of the last STZ or buffer injection was defined as day 0. At day 3 (72 h after the last injection), the fasting plasma glucose concentration was measured using tail vein blood samples and STZ mice were confirmed to be hyperglycemic (plasma glucose level >240 mg/dL). The mice had free access to food and water for 4 wk from day 0. During this period, blood, urine and feces were collected from all mice once a week, on days 7, 14, 21 and 28. The mice were housed in individual stainless steel mesh cages for urine and feces collection.

On the final day of the experiment, day 28, the mice were killed and blood samples collected from the femoral artery. The blood samples were centrifuged at 12,000 × g for 30 min to obtain plasma and the pancreas, kidneys, gastrocnemius muscles and liver were quickly removed and stored at −80˚C.

Analyses of plasma for biological parameters. During the experimental period, blood was obtained on days 7, 14, 21 and 28 from each mouse by a retro-orbital plexus bleed at approximately the same time of day for each collection. Collected blood was stored in heparinized centrifuge tubes on ice and then centrifuged at 12,000 × g for 30 min.

Commercial kits purchased from Wako Pure Chemical Industries, Ltd. were used for measuring the plasma concentrations of glucose (Glucose-CII Test Wako), blood urea nitrogen (BUN; BUN B-Test Wako) and creatinine (Creatinine-Test Wako). Insulin concentration was also analyzed using a commercial kit (Mouse Insulin Assay Kit, Morinaga Institute of Biological Science, Yokohama, Japan).

Urine and feces collection and analyses. Urine samples excreted over a 67-h period by the control and STZ mice were collected on days 7, 14, 21 and 28. At the end of each collection period, the urine volume was measured and the urine centrifuged at 2,500 × g for 10 min and stored at −80˚C. Feces samples excreted over a 67-h period by the control and STZ mice were collected on days 7, 14, 21 and 28 and stored at −80˚C. Commercial kits were used to measure urine concentrations of creatinine (Creatinine-Test Wako, Wako Pure Chemical Industries), total protein (Micro TP-Test Wako, Wako Pure Chemical Industries) and albumin (Levis Urea Albumin; Shibayagi Co., Shibukawa, Japan).

Chromium concentrations in plasma, urine, feces and tissue. Adequate amounts of plasma, urine, feces, tissue samples and diet were digested by high-pressure microwave digestion (Multiwave, PerkinElmer) in 70% HNO3 for ultratrace analysis (Wako Pure Chemical Industries). The resultant clear solution was transferred to 50-mL PP volumetric tubes, resulting in a 1 : 10 dilution and then 1.000 g of a 0.1 μg/g ytrrium (Y) standard solution in 5% HNO3 was added as an internal standard to give a concentration of 10 ng/g Y. Chromium concentrations were determined using ICP-DRC-MS. The mass spectrometer settings and plasma conditions were optimized with a solution of 10 μg indium/L and the instrument operating conditions were as follows: ratio frequency power, 1.100 W; plasma gas flow, 15 L/min; auxiliary gas flow, 1.2 L/min; and nebulizer gas flow, 0.91 L/min. Data collection variables were as follows: total replicates per integration, 20; signal integration time per replicate, 50 s; dwell time per sweep, 20 s; scanning mode, peak hopping at three points per peak sample. The determination limit of Cr was estimated to be 0.1 ng/g in 5% HNO3. To test the reliability of these data, the recovery of data was calculated for the data. Addition of known concentrations of Cr standards to samples (standard addition) showed an 84.9 ± 2.1% recovery of Cr by this method in this investigation.

Calculations. Relative apparent absorption of Cr was calculated according to the following equation:

Relative apparent absorption (%) = 100 × [(Cr intake − Cr fecal excretion)/(Cr intake)]

Conventional chemical balance was determined as
follows:

- **Cr balance=Cr intake from diet and water—** (Cr excretion in feces and urine)

**Statistical analyses.** Data are presented as mean±SE. All statistical analyses were performed using GraphPad Prism 4.03 software (GraphPad Software Inc., San Diego, CA, USA). Two-way analysis of variance (ANOVA) was used to determine the main effect of diabetes, the time-course and the interactions with chromium. Control and STZ mice were compared using an unpaired two-tailed Student’s t-test. Differences were considered significant at p<0.05.

**RESULTS**

**Body weight, food consumption and tissue weights**

Body weight was significantly lower in the STZ mice than in the control mice (p<0.05, Table 1). Cumulative feed intake was significantly higher in the STZ mice than in the control mice (p<0.001), whereas feed efficiency was significantly lower in the STZ mice than in the control mice during the 4 wk of the experimental period (p<0.001, Table 1).

The STZ mice had lower organ weights for pancreas, liver and gastrocnemius muscles (p<0.05, Table 1). No significant differences were detected in the weights of the kidneys between the two groups at day 28 (p>0.05, Table 1).

**Plasma glucose and insulin**

STZ mice exhibited hyperglycemia by day 3 after STZ-treatment (p<0.001, data not shown). The STZ mice were characterized by hyperglycemia and hypoinsulinemia (Table 2).

**Analyses of renal function**

Urinary albumin and protein excretions at day 28 were significantly higher in the STZ mice than in the control mice (p<0.05 and p<0.01, respectively, Fig. 1). The plasma BUN and creatinine concentrations increased significantly in STZ mice from day 0 to day 28 (p<0.001 and p<0.05, respectively, Table 2). The creatinine clearance rate on day 28 was significantly higher in the STZ mice than in the control mice (p<0.05, Table 2).

**Tissue retention of Cr**

Table 3 summarizes the Cr concentration in tissues.

The Cr concentration in the kidney decreased significantly in STZ mice compared with control mice (p<0.001), while in gastrocnemius muscle, the concentration of Cr was significantly higher in the STZ mice than in the control mice (p<0.01). The Cr concentration in pancreas and liver did not change with STZ treatment (p>0.05).

The Cr concentration in the plasma was significantly higher in the STZ mice (149±22.4 ng/mL) than in the control mice on day 28 (10.5±2.29 ng/mL, p<0.001).

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**Table 1. Body weight gain, feed intake and organ weight in control and STZ mice after 28 d.**

|                      | Control | STZ   |
|----------------------|---------|-------|
| **Final body weight, g** | 25.3±0.4 | 18.7±0.8* |
| **Body weight gain, g**   | 3.4±0.3  | 1.7±0.5  |
| **Feed intake, g**       | 131±1.6  | 210±9.9*** |
| **Feed efficiency**      | 2.6±0.3  | 0.8±0.3*** |
| **Fresh organ weight, g** |         |       |
| Pancreas               | 0.18±0.02 | 0.12±0.01* |
| Kidney                 | 0.33±0.01 | 0.31±0.01  |
| M. gastrocnemius       | 0.33±0.04 | 0.21±0.03* |
| Liver                  | 1.00±0.02 | 0.89±0.03* |

1 Values are mean±SE; n=5 for Control and 8 for STZ groups. *p<0.05, ***p<0.001, STZ vs Control.

**Table 2. Plasma glucose, insulin, BUN, creatinine concentration and creatinine clearance rate in Control and STZ mice at day 28.**

|                   | Control   | STZ       |
|-------------------|-----------|-----------|
| Glucose, mg/dL    | 170±13.4  | 356±44.8** |
| Insulin, ng/mL    | 0.48±0.03 | 0.33±0.02** |
| BUN, mg/dL        | 24.2±0.79 | 44.4±2.94*** |
| Creatinine, mg/dL | 0.33±0.06 | 0.59±0.08*  |
| Creatinine clearance rate | 0.85±0.36 | 5.00±1.12* |

1 Values are mean±SE; n=5 for Control and 8 for STZ groups. *p<0.05, **p<0.01, ***p<0.001, STZ vs Control.

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**Fig. 1.** Change in urinary albumin (A) and urinary protein (B) in control (○) and STZ (●) mice. The number of animals in each group is as follows: n=5 for control, n=8 for STZ. Values are means±SE. *p<0.05, **p<0.01, ***p<0.001, control vs STZ.
Intestinal absorption and balance of Cr

Chromium concentration, ng Cr/mg fresh organ weight

| Chromium concentration, ng Cr/mg fresh organ weight | Control | STZ |
|-----------------------------------------------------|---------|-----|
| Pancreas                                            | 0.41±0.07 | 0.44±0.10 |
| Kidney                                              | 0.85±0.12 | 0.17±0.03*** |
| M. gastrocnemius                                    | 0.16±0.01 | 0.91±0.18** |
| Liver                                               | 0.16±0.04 | 0.15±0.01 |

Values are mean±SE; n=5 for Control and 8 for STZ groups. Tissues were collected 28 d after STZ injection. **p<0.01, ***p<0.001, STZ vs Control.

DISCUSSION

This study demonstrated that insulin-deficient diabetic mice induce a significant reduction in renal Cr levels and an elevation of urinary Cr excretion, which induced a negative Cr balance in STZ-treated mice at 28 d after treatment. The initial observations revealed that the elevation of urinary Cr excretion occurred within a week of STZ treatment, distinct from renal dysfunction. The longer-term observation suggested little contribution of renal dysfunction to the elevation of urinary Cr excretion.

High levels of urinary Cr have previously been reported in diabetic humans (17, 18), type 2 diabetic mice (3) and alloxan or STZ-diabetic rats (19–21). Anderson et al. (17, 18) have also observed that diabetic subjects excrete significantly more chromium in urine than normal subjects. An excessive urinary loss of Cr is not limited to the diabetic state, but is also associated with various physiological and pathophysiological stresses, including exercise (7, 22) and the consumption of high-sugar diets (9). However, total Cr balance at the whole body level was not discussed in these previous studies.

In STZ-induced diabetic rats, the effect of hyperglycemia can be observed at relatively early stages of the chromium nutritional status. The present results showed that STZ-induced diabetes in mice produced a negative Cr balance in addition to the increase in urinary Cr loss. Using a 50Cr stable isotope, Feng et al. (19) found that diabetic rats reduce the bioavailability of Cr more than normal rats. Although there have been only a few studies in humans without stable isotopes, Offenbacher et al. (23) suggested that the Cr balance in two normal males, aged 62 and 66, were positive, indicating equilibrium. These studies led to the speculation that diabetes may be due to Cr deficit status caused by the lower availability of ingested Cr and higher urinary Cr loss.

The present study suggested that increased Cr excretion into urine was induced by hyperglycemia in diabetic mice. A previous study found that urinary Cr losses in humans are stimulated by diets high in simple sugars (9). Anderson et al. (8, 24) suggested that an increase in urinary Cr concentration occurs in response to glucose challenge. Earle et al. (25) found that lean type 1 diabetes subjects were excreting more Cr than both the lean and obese control subjects in the basal and post-glucose states. Anderson et al. (11) also demonstrated that urinary Cr losses are related to the insulinogenic properties of carbohydrates. In the present study, increased urinary Cr loss was confirmed very quickly after STZ injection, i.e., on day 7 (Fig. 2), and thus may have altered Cr transport.

Although we observed renal dysfunction in the STZ-treated mice, it is unlikely that increasing Cr excretion into urine is induced by renal dysfunction in diabetes. Donaldson and Rennert (26) suggested that increased

Table 3. Chromium concentration of tissues from Control and STZ mice for 28 d.

| Chromium concentration, ng Cr/mg fresh organ weight | Control | STZ |
|-----------------------------------------------------|---------|-----|
| Pancreas                                            | 0.41±0.07 | 0.44±0.10 |
| Kidney                                              | 0.85±0.12 | 0.17±0.03*** |
| M. gastrocnemius                                    | 0.16±0.01 | 0.91±0.18** |
| Liver                                               | 0.16±0.04 | 0.15±0.01 |

Values are mean±SE; n=5 for Control and 8 for STZ groups. Tissues were collected 28 d after STZ injection. **p<0.01, ***p<0.001, STZ vs Control.

Table 4. The apparent intestinal absorption and chemical balance of Cr in Control and STZ mice after 4 wk free access to diet.

| Food consumption, g/d | Control | STZ |
|-----------------------|---------|-----|
| 4.72±0.42             | 4.94±0.26 |
| Cr intake, ng/d       | 219±19.5 | 229±12.3 |
| Cr fecal excretion, ng/d | 132±23.3 | 363±81.2 |
| Relative fecal excretion of Cr, % | 61.8±12.6 | 154±28.4 |
| Apparent absorption of Cr, ng/d | 87.6±33.8 | -133±74.7 |
| Apparent absorption of Cr, % | 38.2±12.6 | -54.2±28.4 |
| Cr urinary excretion, ng/d | 15.4±2.95 | 356±62.0** |
| Cr balance, ng/d     | 72.3±33.6 | -489±101** |

**p<0.01, ***p<0.001, STZ vs Control.
Cr excretion may result from a glucose challenge or from a volume diuresis. However, previous studies have suggested that treatment with chromium picolinate improves renal function in diabetic mice (3) or rats (27) and uninephrectomized rats (9, 17). Those results suggest that the renal dysfunction in diabetes may be related to increased Cr excretion into urine, although the precise mechanisms of urinary Cr excretion remain to be elucidated.

The increased Cr output into urine and feces of STZ-treated mice may be reflected by a difference in the absorption and distribution of chromium in diabetes or hyperglycemia. Our results show that the Cr excretion into urine and feces in STZ-treated mice was more than that in normal mice (Table 4). However, whether it is a cause or result of hyperglycemia is still unknown. More detailed studies are needed to reveal more precisely the mechanism of Cr absorption.

Hyperglycemia can induce a temporary increase in the plasma Cr concentration, regardless of diabetes type. In the present study, the STZ mice had a higher plasma concentration of Cr compared to controls. Our previous study (3) found that both urinary Cr excretion and the plasma concentration of Cr were higher in type 2 diabetic mice compared with normal mice. In addition, Feng et al. (19) also reported that mean levels of blood Cr were higher in insulin-dependent diabetic rats compared with normal rats. In contrast, significantly lower plasma Cr levels were observed in diabetic human patients compared with non-diabetic healthy controls (1, 6, 16, 28, 29). One possible explanation for these discrepancies in the plasma Cr level is that hyperglycemia in the diabetic state recruits intracellular Cr storage to the plasma low-molecular-weight chromium-binding substance form. Continuous hyperglycemia then leads to increased excretion of urinary Cr and causes a Cr-depleted state in plasma and tissue throughout the whole body.

A decrease in renal Cr concentration in diabetes could lead to an increase in urinary Cr loss. We found a significant reduction in the renal Cr level in the STZ-treated mice. Similar alterations concerning Cr levels, such as elevation in plasma and reduction in kidney Cr levels, have been observed in type-2 diabetic mice (3). Feng et al. have also reported low levels of renal Cr in type-1 diabetic rats 7 d after administration of the diabetogenic drug, alloxan (19, 20). Thus, a decrease in renal Cr concentration may be an initial criterion for the diagnosis of Cr deficiency. In addition to this, further studies are required to investigate the relationship between diabetic nephropathy and renal Cr concentration.

Our study on Cr distribution in organs and tissues in the STZ-induced mice may indicate that Cr metabolism in diabetes is different from that in normal subjects. The concentration of Cr in the gastrocnemius muscle was elevated in our diabetic mice. Clodfelder et al. (21) also reported that diabetic rats accumulate more Cr from Cr-picolinate in the muscle than healthy rats, while in another study by the same group, the muscle of the diabetic rats was found to contain 7-fold more Cr after 2 h than the tissue in healthy rats (19). While diabetes affected Cr absorption, greatly complicating any comparisons, the results are consistent with those of the current study in that chromium transport in STZ-treated rats was confirmed. This was expected because the Cr level in skeletal muscle enhances glucose disposal. Indeed, oral chromium enhances the skeletal muscle insulin signaling pathway in diabetic mice and rats (3, 30, 31).

In conclusion, our results clearly indicate that a reduction of renal Cr concentration and a total negative Cr balance in STZ-treated mice is caused by an increase in urinary Cr output. Furthermore, our study provides evidence that hyperglycemia itself may induce urinary Cr loss prior to renal dysfunction. More research is required to understand the biological roles of Cr in diabetes and therefore to aid in the determination of the appropriate levels of Cr supplementation in diabetic patients.

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