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Citation	Biochimica et Biophysica Acta (BBA) General subjects, 1861(3), 559-566 https://doi.org/10.1016/j.bbagen.2016.11.042
Issue Date	2017-03
Doc URL	http://hdl.handle.net/2115/68654
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Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	WoS_77643_kobayashi.pdf

Fructose suppresses uric acid excretion to the intestinal lumen as a result of the induction of oxidative stress by NADPH oxidase activation

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Abstract

Background: A high intake of fructose increases the risk for hyperuricemia. It has been reported that long-term fructose consumption suppressed renal uric acid excretion and increased serum uric acid level. However, the effect of single administration of fructose on excretion of uric acid has not been clarified.

Methods: We used male Wistar rats, which were orally administered fructose (5 g/kg). Those rats were used in each experiment at 12 h after administration.

Results: Single administration of fructose suppressed the function of ileal uric acid excretion and had no effect on the function of renal uric acid excretion. Breast cancer resistance protein (BCRP) predominantly contributes to intestinal excretion of uric acid as an active homodimer. Single administration of fructose decreased BCRP homodimer level in the ileum. Moreover, diphenyleneiodonium (DPI), an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), recovered the suppression of the function of ileal uric acid excretion and the Bcrp homodimer level in the ileum of rats that received single administration of fructose.

Conclusions: Single administration of fructose decreases in BCRP homodimer level, resulting in the suppression the function of ileal uric acid excretion. The suppression of the function of ileal uric acid excretion by single administration of fructose is caused by the activation of Nox. The results of our study provide a new insight into the mechanism of fructose-induced hyperuricemia.

stress, NADPH oxidase

1. Introduction

Since various soft drinks and snacks contain fructose as a sweetener, we intake a large amount of fructose from daily foods. It is known that intake of a large amount of fructose increases the risk for development of several diseases or disorders including metabolic syndrome, chronic kidney disease and cardiovascular disease [1-3]. The development of diseases or disorders induced by fructose may be associated with the activation of dinucleotide phosphate (NADPH) oxidase (Nox), because Nox activation induces oxidative stress, which leads to the development of several diseases or disorders including metabolic syndrome, chronic kidney disease and cardiovascular disease [4-6].

A high serum concentration of uric acid is one of the causes of metabolic syndrome. It is known that a high intake of fructose activates AMP deaminase, resulting in an enhancement of the rate of purine degradation. And then, serum uric acid level is increased because uric acid is a final product of purine metabolism in human [7]. Indeed, a high intake of fructose is known to be a risk factor of hyperuricemia. Hyperuricemia is classified into three types: overproduction type, underexcretion type and combined type. An induction of uric acid production by the stimulatory effect of fructose on purine degradation causes overproduction of uric acid. Moreover, it has been reported that long-term fructose administration suppressed renal excretion of uric acid, resulting in elevation of serum uric acid level [8]. However, the effect of single administration of fructose on excretion of uric acid is not known. Reducing serum uric acid level in the primary stage of hyperuricemia can prevent the development of other diseases or disorders, such as metabolic

3

syndrome, chronic kidney disease and cardiovascular disease. Therefore, it is important to reveal the details of the effect of fructose consumption on the uric acid excretion.

It is known that two-thirds of uric acid is excreted from the kidney into urine and the remaining one-third is excreted from the intestine into feces. Renal uric acid excretion is regulated by several transporters. Urate transporter 1 (URAT1, also known as SLC22A12) and glucose transporter 9 (GLUT9, also known as SLC2A9) mediate renal uric acid reabsorption [9, 10]. Sodium phosphate transporter (NPT1, also known as SLC17A1) mediates renal uric acid secretion [11, 12]. Intestinal uric acid excretion is also regulated by transporters. Breast cancer resistance protein (BCRP, also known as ABCG2), which belongs to the ATP-binding cassette (ABC) transporter superfamily, predominantly contributes to intestinal excretion of uric acid [13-16]. ABC transporters, such as P-glycoprotein (P-gp) and multidrug resistance proteins (MRPs), have an internally duplicated structure with two ATP-binding domains. On the other hand, BCRP has only one N-terminal ATP-binding domain [17]. Therefore, BCRP has to form a homodimer, bridged by a disulfide bond, to efflux BCRP substrates [18-20]. We previously reported that dimerization of BCRP was influenced by oxidative stress [21, 22]. Since fructose consumption induces oxidative stress by activation of Nox, it is speculated that fructose consumption may also affect the excretion of uric acid to the intestinal lumen.

In this study, to elucidate the effect of single administration of fructose on the function of uric acid excretion, we compared the function of uric acid excretion in rats that received single administration of fructose with that in sham-operated rats. We found that single administration of

fructose suppressed the function of uric acid excretion to the intestinal lumen. We also found that single administration of fructose induced the suppression of Bcrp dimerization as a result of the induction of oxidative stress by Nox activation.

2. Materials and Methods

2.1. Chemicals

Fructose was purchased from KANTO KAGAKU (Tokyo, Japan). Diphenyleneiodonium chloride (DPI) was purchased from Cayman Chemical (Ann Arbor, MI). D-[^{3}H]-mannitol was obtained from PerkinElmer (Waltham, MA). All other reagents were of the highest grade available and used without further purification.

2.2. Animals

Male Wistar rats, aged 6 weeks, were obtained from JLA (Saitama, Japan). For the purpose of acclimation, the rats were allowed free access to food (Rodent laboratory diet$^®$ EQ 5L37) and water for at least 1 week. The housing conditions were the same as those described previously [21]. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals".

2.3. Drug administration

The condition of fructose single administration was the same as that described in a previous report [23]. Briefly, rats were orally administered fructose (5 g/kg) or water (sham operation), and were used in each experiment at 12 h after administration. DPI (1 mg/kg) or sterile saline (sham operation) was intraperitoneally injected at the same time as fructose administration.

6

2.4. Uric acid excretion study

Uric acid excretion was analyzed as described in the previous report [22]. To analyze uric acid excretion, the bladder was cannulated with polyethylene tubing (SP45; Natsume Seisakusho) for urine collection, and 10-cm-long intestinal loops were made at the jejunum and ileum by ligating at both ends. Efflux buffer (saline containing 0.3 mM potassium oxonate (Wako, Osaka, Japan)) was introduced into the intestinal loops and then potassium oxonate was administered at 250 mg/kg intraperitoneally [16, 24]. Blood samples (each 0.3 mL) were collected from the jugular vein at designated time points (1, 2, 3, 6 and 8 h after administration of potassium oxonate) into tubes. Serum was prepared by allowing the blood to naturally form a blood clot and then using a centrifuge (1,000×g for 20 min at 4°C). At 8 h after administration of potassium oxonate, the efflux buffer in the intestinal loops was collected and diluted with saline. The protein contents in the intestinal loops were determined by the BCA protein assay. Urine was collected for 8 h after administration of potassium oxonate and diluted with saline. Uric acid concentration was measured by the method of Morin [25]. The area under the concentration-time curve (AUC) of uric acid was calculated using the trapezoidal rule from the concentration-time curve. The jejunal or ileal clearance (CL) of uric acid was calculated by the following equation: CL (jejunum) or CL (ileum) = D/AUC/mg protein, where D is the amount of excretion to the jejunal or ileal loop. The renal clearance of uric acid was calculated by the following equation: CL (kidney) = D/AUC, where D is the amount of excretion to urine.

2.5. Assessment of intestinal paracellular permeability

A transport experiment was carried out as described in the previous report [22]. Intestinal sheets were mounted between NaviCyte® diffusion chambers (Warner Instruments, Hamden, CT) that provided an exposed area of 0.64 cm^2. Considering a pH in the rat intestine under a physiological condition [26], Hanks' balanced salt solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 1.0 mM CaCl$_2$, 0.8 mM MgCl$_2$, 0.4 mM KH$_2$PO$_4$, 0.3 mM NaH$_2$PO$_4$ and 25 mM D-glucose) buffered with 20 mM MES (pH 6.0) or 20 mM HEPES (pH 7.4) was added to the chambers of the mucosal side (pH 6.0) and serosal side (pH 7.4). The volume of bathing solution on each side was 1.0 mL, and the solution temperature was maintained at 37°C in a water-jacketed reservoir. The buffer solution was bubbled with a 95:5 mixture of O$_2$/CO$_2$ before and during the transport experiment. The buffer solution in the chambers of the serosal side contained D-[^{3}H]-mannitol. Samples of 0.25 mL were taken from the chambers of the mucosal side after 15, 30, 45 and 60 min of incubation. A scintillation spectrometer (1600TR, Packard Instruments, Meriden, CT) was used to measure D-[^{3}H]-mannitol.

The permeation rate of D-[^{3}H]-mannitol was expressed as an apparent permeability coefficient (Papp) according to the following equation: $P_{app} = dQ/dt / SC_0$, where dQ/dt is the linear appearance rate of mass in the receiver solution, S is the exposed area (0.64 cm^2), and C_0 is the initial concentration of D-[^{3}H]-mannitol (50 nM).

2.6. Real-time PCR

Quantitative real-time PCR was performed as described in previous reports [21, 27] using

an Mx3000^{TM} Real-time PCR System (STRATAGENE, Tokyo, Japan) with a KAPA SYBR Fast

qPCR kit (KAPA Biosystems, Boston, MA) following the manufacturer's protocol. PCR was

performed using rat BCRP-specific primers and rat GAPDH-specific primers through 40 cycles of

95°C for 15 s, 50°C for 30 s and 72°C for 30 s after pre-incubation at 50°C for 2 min and 95°C for

15 min. Specific primers to rat BCRP and rat GAPDH were designed on the basis of sequences in

the GenBankTM database (accession no.: AB094089 and AF106860, respectively). The sequences

of the specific primers were as follows: the sense sequence (641-660) was 5'-GTT TGG ACT CAA

GCA CAG CA-3' and the antisense sequence (771-790) was 5'-TGA GTT TCC CAG AAG CCA

GT-3' for rat BCRP, and the sense sequence (1034-1053) was 5'-ATG GGA AGC TGG TCA TCA

AC-3' and the antisense sequence (1235-1254) was 5'-GTG GTT CAC ACC CAT CAC AA-3' for

rat GAPDH. The PCR products were normalized to amplified GAPDH, which was the internal

reference gene (housekeeping gene). Standard curves were prepared for each target and

housekeeping gene. The standard curve was established between the threshold cycles (Ct) and the

log10 (copy numbers).

2.7. Western blot analysis

Total protein extracts were prepared from the ileal mucosa of rats as previously reported

[21, 22]. Protein concentrations of the samples in clear supernatants were determined by the method

of Lowry et al. [28]. BCRP protein expression and the homodimer could be detected by sodium

dodecyl sulfate (SDS)-PAGE under reducing and non-reducing conditions, respectively [21, 22].

The reducing condition for samples was as follows. The reducing reaction mixture contained 18 μL

of a sample and 2 μL of 1 M dithiothreitol (DTT). The mixture was incubated for 2 h at 37°C and

then 4.8 μL of 1 M iodoacetamide was added. The mixture was incubated for a further 30 min at

37°C. After adding 4 μL of 200 mM NaOH, the mixture was cooled with tap water. The samples

were denatured at 100°C for 5 min in a loading buffer containing 50 mM Tris-HCl, 12.5% SDS,

20% 2-mercaptoethanol, 10% glycerol, 100 mM DTT, 5 mM sodium bisulfite, 0.002%

bromophenol blue (BPB) and 3.6 M urea. The non-reducing condition for samples was as follows.

The samples were denatured at room temperature for 5 min in a loading buffer containing 50 mM

Tris-HCl, 12.5% SDS, 20% methanol, 10% glycerol, 0.002% BPB and 3.6 M urea. Each sample

was separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred

electrophoretically onto nitrocellulose membranes at 15 V for 90 min. The membranes were

blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) and 10%

non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were

incubated overnight at room temperature with a mouse monoclonal antibody to BXP-21 (Santa

Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250 or mouse anti-actin monoclonal

antibody (Millipore, Bedford, MA) at a dilution of 1:500 and washed three times with PBS/T for 10

min each time. The membranes were subsequently incubated for 1 h at room temperature with

horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology)

at a dilution of 1:2,000 and washed three times with PBS/T for 10 min each time. The bands were

visualized by enhanced chemiluminescence according to the instructions of the manufacturer

(Amersham Biosciences Corp., Piscataway, NJ).

2.8. Measurement of Nox activity

Nox activity was quantified by lucigenin chemiluminescence as described previously [29].

The ileal mucosa of rats was homogenized in PBS. The homogenate (500 µg protein), NADPH (0.2

mM), NADH (0.2 mM) and lucigenin (20 µM) were combined to 200 µL with PBS. Lucigenin

luminescence was measured after 1 min of incubation at room temperature using the multilabel

counter Wallac 1420 ARVOse (PerkinElmer). The protein content was determined by the BCA

protein assay.

2.9. Assessment of oxidative injury

The extent of reactive oxygen species (ROS)-induced oxidative injury was assessed

indirectly by measuring the level of malondialdehyde (MDA), an intermediate product of lipid

peroxidation, as described previously [27]. The colored upper layer was placed in a glass cell for

spectrophotometer reading at 535 nm with 1, 1, 3, 3-tetraethoxypropane (TEP) as a standard. The

amount of MDA was corrected by protein content. The protein content was determined by the BCA

protein assay.

2.10. Statistical analysis

Statistical significance was evaluated using Tukey's test or Student's t-test. A value of p<0.05 was considered significant.

3. Results

3.1. Effects of fructose single administration on uric acid clearances in rats

There was no significant difference between the body weight of sham-operated rats and rats that received single administration of fructose (Table 1). Serum uric acid (Fig. 1) and $AUC_{0-8 h}$ of uric acid (Table 1) in rats that received single administration of fructose showed a tendency to be increased compared with those in sham-operated rats. Renal and jejunal uric acid clearances in rats that received single administration of fructose were the same as those in sham-operated rats (Table 1). On the other hand, ileal uric acid clearance in rats that received single administration of fructose was significantly lower than that in sham-operated rats (Table 1).

3.2. Effects of fructose single administration on paracellular and transcellular transport pathways of uric acid in the ileum of rats

There are two transport pathways that can function as uric acid excretion route in the intestine [30, 31]. One is the paracellular pathway to the lateral intercellular space via tight junctions between epithelial cells. P_{app} of mannitol, which is mainly excreted by the paracellular pathway, through the ileal membrane of rats that received single administration of fructose was the same as that in sham-operated rats (Fig. 2).

The other transport pathway is the transcellular pathway across the intestinal epithelial cell membrane. It is known that BCRP mainly contributes to intestinal excretion of uric acid via the transcellular pathway [15, 16]. As shown in Table 1, single administration of fructose decreased

uric acid clearance in the ileum, which has abundant Bcrp expression. We therefore investigated the effect of fructose on ileal Bcrp expression. Single administration of fructose did not affect Bcrp mRNA level (Fig. 3A) or protein expression (Fig. 3B) in the ileum. On the other hand, Bcrp homodimer level in the ileum was significantly decreased by the administration of fructose (Fig. 3C).

3.3. Effects of fructose single administration on Nox activity and oxidative stress in the ileum

Nox activity in the ileum of rats that received single administration of fructose was significantly higher than that in sham-operated rats (Fig. 4A). The level of MDA, which is a marker for oxidative stress, in the ileum of rats that received single administration of fructose was also significantly higher than that in sham-operated rats (Fig. 4B).

3.4. Protective effect of DPI on suppression of the function of uric acid excretion to the ileal lumen

Administration of DPI had no effect on body weight of rats that received single administration of fructose (Table 2). Nox activation (Fig. 5A) and increase in MDA level (Fig. 5B) in the ileum of rats that received single administration of fructose were diminished by administration of DPI. The decreased Bcrp homodimer level in the ileum of rats that received single administration of fructose was recovered by administration of DPI (Fig. 5E) with no effect on Bcrp mRNA level (Fig. 5C) or protein expression (Fig. 5D). The decreased ileal uric acid clearance (Table 2) in rats that received single administration of fructose was recovered by administration of

14

DPI, and the increased serum uric acid (Fig. 5F) and $AUC_{0\text{-}8\,h}$ of uric acid (Table 2) in rats that

received single administration of fructose were diminished by administration of DPI.

4. Discussion

It is well known that a high intake of fructose is associated with increasing risk for hyperuricemia/gout. The rapid effect of fructose consumption on serum uric acid concentration is attributed to hepatic fructose metabolism which leads to an increase uric acid production. Moreover, long-term fructose consumption was shown to suppress renal uric acid excretion, and increase serum uric acid level [8]. This long-term effect of fructose consumption was associated with renal dysfunction and/or abnormal expression of renal uric acid transporters [32]. It was recently reported that the change in serum uric acid following single administration of fructose was associated with SLC2A9/GLUT9 and ABCG2/BCRP genetic variants [33-35]. These findings suggest that fructose consumption rapidly affects uric acid excretion. In this study, we found that single administration of fructose suppressed the function of uric acid excretion in the ileum (Table 1). On the other hand, single administration of fructose had no effects on the function of renal and jejunal uric acid excretion function (Table 1). These results indicated that the onset of rapid fructose-induced hyperuricemia was related to suppression of the function of ileal uric acid excretion. BCRP is abundantly expressed in the ileum and transports uric acid to the intestinal lumen as an active homodimer bridged by a disulfide bond. Single administration of fructose suppressed dimerization of Bcrp in the ileum with no effect on Bcrp mRNA level or protein expression (Fig. 3). Moreover, single administration of fructose did not affect paracellular permeability in the ileum (Fig. 2). It is known that URAT1, GLUT9 and NPT1 are uric acid transporters. We investigated the effects of single administration of fructose on other uric acid transporters in the ileum. URAT1 and NPT1,

16

which mainly expresses in the kidney, did not express in the ileum (Supplementary fig. 1A). Those

expression profiles were consistent with the previous report [36]. GLUT9 expressed in the ileum

but GLUT9 mRNA level was not significantly changed by single administration of fructose

(Supplementary fig. 1B). These findings indicated that suppression of the function of ileal uric acid

excretion was caused by Bcrp dysfunction. However, there is the possibility of the contribution of

other unknown transporters on uric acid excretion in the intestine. Indeed, the meta-analysis of

genome-wide association scans from 14 studies suggests that some transporters are related to the

prevalence of hyperuricemia/gout [37]. Further studies are necessary in order to conclude that the

single administration of fructose has an influence on only BCRP.

In this study, we administered fructose at 5 g/kg to rats as the previous report [23]. This

amount could roughly estimate at 300 g in human. On the other hand, fructose was administered at

65 g containing 16 g of glucose in human clinical study for single administration of fructose [34].

We compared the changes in biochemical parameters in the reports of single administration of

fructose in human and rats. The changes in serum uric acid and glucose were almost same between

those reports [23, 34]. Therefore, there is a certain degree of validity in the rat model. We take a

large amount of fructose from daily foods. Beverages made with high-fructose corn syrup are

known as the food contains much fructose (44.63-72.31 g/L, 0.25-0.4 M) [38].Those concentrations

of fructose are higher than Km of intestinal fructose transporter GLUT5 (11-13 mM) [39-40]. The

fructose concentration (500 g/L, 2.8 M) in this study was also higher than Km of GLUT5. Those

findings suggest that fructose absorption in the intestine may be saturated state in both of

physiological condition and our study. Therefore, fructose may be able to absorb in the entire

intestinal lumen under both of physiological condition and our study, though GLUT5 expression in

the jejunum is higher than that in the duodenum or ileum [41]. Since fructose is poorly metabolized

in the rat intestine, the absorbed fructose appears in the portal blood primarily, and then

metabolized in the liver [42]. Fructose is known to be induced acceleration of purine degradation

mediated through activation of AMP deaminase. The activation of AMP-deaminase resulted in the

induction of uric acid production [7]. The induction of uric acid production was observed at 4 h

after 5 g/kg fructose administration in rats [23]. On the other hand, serum concentration of uric acid

was not changed at 12 h after the fructose administration in this study (Fig. 1 and Fig. 5F).

Moreover, xanthine oxidase activities in the jejunum, ileum and liver were not induced at 12 h after

the fructose administration (Supplementary fig. 2). These findings suggested that the effect of

fructose on uric acid production diminished at 12 h after the fructose administration. We used

uricase inhibitor for uric acid excretion study. Since uricase specifically expresses in the liver in rats

[43], uric acid, which is excreted from epithelium in ileum, might be produced in the liver in our

study.

We previously reported that BCRP dimerization was significantly suppressed by ROS

generated from xanthine oxidase [22]. Since fructose induces production of ROS, though they are

generated from Nox, we speculated that ROS generated from Nox may also affect BCRP

dimerization. In our experimental condition, single administration of fructose activated Nox and

then induced oxidative stress in the ileum (Fig. 4). Suppression of Bcrp dimerization in rats that

received single administration of fructose was recovered by a Nox inhibitor (Fig. 5), and then the

function of ileal uric acid excretion was also recovered (Table 2). In this study, we used DPI as Nox

inhibitor though DPI is also known as endothelial nitric oxide synthase (eNOS) inhibitor [44]. It is

well known that fructose consumption suppresses eNOS activities in various organs and tissues

[45-47]. Thus, the inhibitory effect of DPI on eNOS might be subtle after the administration of

fructose. These findings indicate that oxidative stress caused by Nox activation is related to

suppression of the function of uric acid excretion caused by Bcrp dysfunction.

The suppressive effect of single administration of fructose on the function of uric acid

excretion observed only in the ileum. The Nox activity in the kidney was not changed by single

administration of fructose (Supplementary fig. 3B). On the other hand, Nox activity in the jejunum

was activated by single administration of fructose as well as in the ileum (Supplementary fig. 3A).

However, the BCRP expression exhibits marked differences in different regions of the intestine.

BCRP expression in the ileum is higher than that in the duodenum, jejunum or large intestine [15,

48]. Moreover, Nox activity in the ileum is also higher than that in the jejunum or kidney (data not

shown). Taking those findings, we speculated that the function of uric acid excretion in the ileum

may be sensitive against the fructose administration compared with that in the jejunum or kidney.

Since protein disulfide is formed by an oxidative reaction, ROS may have activity for

disulfide bond formation. Yang et al. [49] reported that over-expressed mitochondrial targeting

catalase decreased protein disulfide contents in cells but that over-expressed catalase did not,

suggesting that the oxidative potential required for protein disulfide bond formation is supplied by

19

intra-mitochondrial ROS but not by cytosolic ROS. This finding suggests that ROS generated

specific intracellular compartment have activity of protein disulfide bond formation. Moreover, it is

known that protein disulfide formation is predominantly regulated by protein disulfide isomerase

(PDI). Since PDI has reactive cysteine residues, PDI is known to be one of the proteins that are

most susceptible to the effect of oxidative stress [50]. Indeed, age-associated oxidative stress

suppresses PDI function [51, 52]. These findings fortify our conclusion that ROS generated from

Nox decrease the level of BCRP homodimer, bridged by a disulfide bond, though protein disulfide

is formed by an oxidative reaction. However, the effect of ROS generated from Nox on PDI

function is still unknown. Further study is needed in order to reveal the detail mechanism of the

suppression of BCRP dimer level by ROS generated from Nox.

5. Conclusion

Single administration of fructose suppresses the function of ileal uric acid excretion. This suppression of uric acid excretion to the ileal lumen is caused by the decrease in BCRP active homodimer level. The effect of single administration of fructose is recovered by inhibition of Nox activation, indicating that ROS generated from Nox play an important role in the acute change in uric acid excretion following fructose consumption. The results of our study provide a new insight into the mechanism of fructose-induced hyperuricemia.

Acknowledgements

References

[1] S.S. Elliott, N.L. Keim, J.S. Stern, K. Teff, P.J. Havel, Fructose, weight gain, and the insulin resistance syndrome, Am. J. Clin. Nutr. 76 (2002) 911-922.

[2] M.S. Gersch, W. Mu, P. Cirillo, S. Reungjui, L. Zhang, C. Roncal, Y.Y. Sautin, R.J. Johnson, T. Nakagawa, Fructose, but not dextrose, accelerates the progression of chronic kidney disease, Am. J. Physiol. Renal. Physiol. 293 (2007) 1256-1261.

[3] R.J. Johnson, E.A. Gaucher, Y.Y. Sautin, G.N. Henderson, A.J. Angerhofer, S.A. Benner, The planetary biology of ascorbate and uric acid and their relationship with the epidemic of obesity and cardiovascular disease, Med. Hypotheses. 71 (2008) 22-31.

[4] J. Streeter, W. Thiel, K. Brieger, F.J.Jr. Miller, Opportunity nox: the future of NADPH oxidases as therapeutic targets in cardiovascular disease, Cardiovasc. Ther. 31 (2013) 125-137.

[5] K. Sharma, Obesity, oxidative stress, and fibrosis in chronic kidney disease, Kidney Int. Suppl. (2011). 4 (2014) 113-117.

[6] A. Souto Padron de Figueiredo, A.B. Salmon, F. Bruno, F. Jimenez, H.G. Martinez, G.V. Halade, S.S. Ahuja, R.A. Clark, R.A. DeFronzo, H.E. Abboud, A. El Jamali, Nox2 mediates skeletal muscle

insulin resistance induced by a high fat diet, J. Biol. Chem. 290 (2015) 13427-13439.

[7] S. Brosh, P. Boer, O. Sperling, Effects of fructose on synthesis and degradation of purine nucleotides in isolated rat hepatocytes, Biochim. Biophys. Acta. 717 (1982) 459-464.

[8] T. Nakagawa, H. Hu, S. Zharikov, K.R. Tuttle, R.A. Short, O. Glushakova, X. Ouyang, D.I. Feig, E.R. Block, J. Herrera-Acosta, J.M. Patel, R.J. Johnson, A causal role for uric acid in fructose-induced metabolic syndrome, Am. J. Physiol. Renal Physiol. 290 (2006) 625-631.

[9] A. Enomoto, H. Kimura, A. Chairoungdua, Y. Shigeta, P. Jutabha, S.H. Cha, M. Hosoyamada, M. Takeda, T. Sekine, T. Igarashi, H. Matsuo, Y. Kikuchi, T. Oda, K. Ichida, T. Hosoya, K. Shimokata, T. Niwa, Y. Kanai, H. Endou, Molecular identification of a renal urate anion exchanger that regulates blood urate levels, Nature. 417 (2002) 447-452.

[10] N. Anzai, K. Ichida, P. Jutabha, T. Kimura, E. Babu, C.J. Jin, S. Srivastava, K. Kitamura, I. Hisatome, H. Endou, H. Sakurai, Plasma urate level is directly regulated by a voltage-driven urate efflux transporter URATv1 (SLC2A9) in humans, J. Biol. Chem. 283 (2008) 26834-26838.

[11] H. Uchino, I. Tamai, K. Yamashita, Y. Minemoto, Y. Sai, H. Yabuuchi, K. Miyamoto, E. Takeda, A. Tsuji, p-aminohippuric acid transport at renal apical membrane mediated by human

inorganic phosphate transporter NPT1, Biochem. Biophys. Res. Commun. 270 (2000) 254-259.

[12] M. Iharada, T. Miyaji, T. Fujimoto, M. Hiasa, N. Anzai, H. Omote, Y. Moriyama, Type 1

sodium-dependent phosphate transporter (SLC17A1 Protein) is a Cl(-)-dependent urate exporter, J.

Biol. Chem. 285 (2010) 26107-26113.

[13] O.M. Woodward, A. Köttgen, J. Coresh, E. Boerwinkle, W.B. Guggino, M. Köttgen,

Identification of a urate transporter, ABCG2, with a common functional polymorphism causing

gout, Proc. Natl. Acad. Sci. USA. 106 (2009) 10338-10342.

[14] H. Matsuo, T. Takada, K. Ichida, T. Nakamura, A. Nakayama, Y. Ikebuchi, K. Ito, Y.

Kusanagi, T. Chiba, S. Tadokoro, Y. Takada, Y. Oikawa, H. Inoue, K. Suzuki, R. Okada, J.

Nishiyama, H. Domoto, S. Watanabe, M. Fujita, Y. Morimoto, M. Naito, K. Nishio, A. Hishida, K.

Wakai, Y. Asai, K. Niwa, K. Kamakura, S. Nonoyama, Y. Sakurai, T. Hosoya, Y. Kanai, H. Suzuki,

N. Hamajima, N. Shinomiya, Common defects of ABCG2, a high-capacity urate exporter, cause

gout: a function-based genetic analysis in a Japanese population, Sci. Transl. Med. 1 (2009) 5ra11.

[15] A. Hosomi, T. Nakanishi, T. Fujita, I. Tamai, Extra-renal elimination of uric acid via intestinal

efflux transporter BCRP/ABCG2, PLoS One. 7 (2012) e30456.

[16] K. Ichida, H. Matsuo, T. Takada, A. Nakayama, K. Murakami, T. Shimizu, Y. Yamanashi, H. Kasuga, H. Nakashima, T. Nakamura, Y. Takada, Y. Kawamura, H. Inoue, C. Okada, Y. Utsumi, Y. Ikebuchi, K. Ito, M. Nakamura, Y. Shinohara, M. Hosoyamada, Y. Sakurai, N. Shinomiya, T. Hosoya, H. Suzuki, H., Decreased extra-renal urate excretion is a common cause of hyperuricemia, Nat. Commun. 3 (2012) 764.

[17] T. Litman, U. Jensen, A. Hansen, K.M. Covitz, Z. Zhan, P. Fetsch, A. Abati, P.R. Hansen, T. Horn, T. Skovsgaard, S.E. Bates, Use of peptide antibodies to probe for the mitoxantrone resistance-associated protein MXR/BCRP/ABCP/ABCG2, Biochim. Biophys. Acta. 1565 (2002) 6-16.

[18] K. Kage, S. Tsukahara, T. Sugiyama, S. Asada, E. Ishikawa, T. Tsuruo, Y. Sugimoto, Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization, Int. J. Cancer. 97 (2002) 626-630.

[19] K. Kage, T. Fujita, Y. Sugimoto, Role of Cys-603 in dimer/oligomer formation of the breast cancer resistance protein BCRP/ABCG2, Cancer Sci. 96 (2005) 866-872.

[20] T. Takada, H. Suzuki, Y. Sugiyama, Characterization of polarized expression of point- or deletion-mutated human BCRP/ABCG2 in LLC-PK1 cells, Pharm. Res. 22 (2005) 458-464.

[21] J. Ogura, K. Kuwayama, A. Takaya, Y. Terada, T. Tsujimoto, T. Koizumi, H. Maruyama, A. Fujikawa, N. Takahashi, M. Kobayashi, S. Itagaki, T. Hirano, H. Yamaguchi, K. Iseki, Intestinal ischemia-reperfusion increases efflux for uric acid via paracellular route in the intestine, but decreases that via transcellular route mediated by BCRP, J. Pharm. Pharm. Sci. 15 (2012) 295-304.

[22] J. Ogura, K. Kuwayama, S. Sasaki, C. Kaneko, T. Koizumi, K. Yabe, T. Tsujimoto, R. Takeno, A. Takaya, M. Kobayashi, H. Yamaguchi, K. Iseki, Reactive oxygen species derived from xanthine oxidase interrupt dimerization of breast cancer resistance protein, resulting in suppression of uric acid excretion to the intestinal lumen, Biochem. Pharmacol. 97 (2015) 89-98.

[23] J.A. Moreno, E. Hong, A single oral dose of fructose induces some features of metabolic syndrome in rats: role of oxidative stress, Nutr. Metab. Cardiovasc. Dis. 23 (2013) 536-542.

[24] Y. Osada, M. Tsuchimoto, H. Fukushima, K. Takahashi, S. Kondo, M. Hasegawa, K. Komoriya, Hypouricemic effect of the novel xanthine oxidase inhibitor, TEI-6720, in rodents, Eur. J. Pharmacol. 241 (1993) 183-188.

[25] L.G. Morin, Determination of serum urate by direct acid Fe^{3+} reduction or by absorbance change (at 293 nm) on oxidation of urate with alkaline ferricyanide, Clin. Chem. 20 (1974) 51-56.

[26] E.L. McConnell, A.W. Basit, S. Murdan, Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments, J. Pharm. Pharmacol. 60 (2008) 63-70.

[27] T. Tsujimoto, J. Ogura, K. Kuwayama, T. Koizumi, S. Sasaki, Y. Terada, M. Kobayashi, H. Yamaguchi, K. Iseki, Effect of oxidative stress on expression and function of human and rat organic anion transporting polypeptides in the liver, Int. J. Pharm. 458 (2013) 262-271.

[28] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.

[29] E. Hatanaka, A. Dermargos, A.E. Hirata, M.A. Vinolo, A.R. Carpinelli, P. Newsholme, H.A. Armelin, R. Curi, Oleic, linoleic and linolenic acids increase ros production by fibroblasts via NADPH oxidase activation, PLoS One. 8, (2013) e58626.

[30] M. Hayashi, M. Tomita, Transcellular and paracellular contribution to transport processes in the colorectal route, Adv. Drug Deliv. Rev. 28 (1997) 191-204.

[31] M. Hayashi, M. Tomita, Mechanistic analysis for drug permeation through intestinal

membrane, Drug Metab. Pharmacokinet. 22 (2007) 67-77.

[32] Q.H. Hu, C. Wang, J.M. Li, D.M. Zhang, L.D. Kong, Allopurinol, rutin, and quercetin attenuate hyperuricemia and renal dysfunction in rats induced by fructose intake: renal organic ion transporter involvement, Am. J. Physiol. Renal Physiol. 297 (2009) F1080–F1091.

[33] N. Dalbeth, M.E. House, G.D. Gamble, A. Horne, B. Pool, L. Purvis, A. Stewart, M. Merriman, M. Cadzow, A. Phipps-Green, T.R. Merriman, Population-specific influence of SLC2A9 genotype on the acute hyperuricaemic response to a fructose load, Ann. Rheum. Dis. 72 (2013) 1868-1873.

[34] N. Dalbeth, M.E. House, G.D. Gamble, B. Pool, A. Horne, L. Purvis, A. Stewart, M. Merriman, M. Cadzow, A. Phipps-Green, T.R. Merriman, Influence of the ABCG2 gout risk 141 K allele on urate metabolism during a fructose challenge, Arthritis. Res. Ther. 16 (2014) R34.

[35] C. Batt, A.J. Phipps-Green, M.A. Black, M. Cadzow, M.E. Merriman, R. Topless, P. Gow, A. Harrison, J. Highton, P. Jones, L. Stamp, N. Dalbeth, T.R. Merriman, Sugar-sweetened beverage consumption: a risk factor for prevalent gout with SLC2A9 genotype-specific effects on serum urate and risk of gout, Ann. Rheum. Dis. 73 (2014) 2101-2106.

[36] X. Cheng, C.D. Klaassen, Tissue distribution, ontogeny, and hormonal regulation of xenobiotic

transporters in mouse kidneys, Drug Metab. Dispos. 37 (2009) 2178-2185.

[37] M. Kolz, T. Johnson, S. Sanna, A. Teumer, V. Vitart, M. Perola, M. Mangino, E. Albrecht, C. Wallace, M. Farrall, A. Johansson, D.R. Nyholt, Y. Aulchenko, J.S. Beckmann, S. Bergmann, M. Bochud, M. Brown, H. Campbell; EUROSPAN Consortium, J. Connell, A. Dominiczak, G. Homuth, C. Lamina, M.I. McCarthy; ENGAGE Consortium, T. Meitinger, V. Mooser, P. Munroe, M. Nauck, J. Peden, H. Prokisch, P. Salo, V. Salomaa, N.J. Samani, D. Schlessinger, M. Uda, U. Völker, G. Waeber, D. Waterworth, R. Wang-Sattler, A.F. Wright, J. Adamski, J.B. Whitfield, U. Gyllensten, J.F. Wilson, I. Rudan, P. Pramstaller, H. Watkins; PROCARDIS Consortium, A. Doering, H.E. Wichmann; KORA Study, T.D. Spector, L. Peltonen, H. Völzke, R. Nagaraja, P. Vollenweider, M. Caulfield; WTCCC, T. Illig, C. Gieger, Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations, PLoS Genet. 5 (2009) e1000504.

[38] R.W. Walker, K.A. Dumke, M.I. Goran, Fructose content in popular beverages made with and without high-fructose corn syrup, Nutrition. 30 (2014) 928-935.

[39] K. Miyamoto, S. Tatsumi, A. Morimoto, H. Minami, H. Yamamoto, K. Sone, Y. Taketani, Y. Nakabou, T. Oka, E. Takeda, Characterization of the rabbit intestinal fructose transporter (GLUT5), Biochem. J. 303 (1994) 877-883.

[40] C.P. Corpe, F.J. Bovelander, C.M. Munoz, J.H. Hoekstra, I.A. Simpson, O. Kwon, M. Levine, C.F. Burant, Cloning and functional characterization of the mouse fructose transporter, GLUT5, Biochim. Biophys. Acta. 1576 (2002) 191-197.

[41] A. Castelló, A. Gumá, L. Sevilla, M. Furriols, X. Testar, M. Palacín, A. Zorzano, Regulation of GLUT5 gene expression in rat intestinal mucosa: regional distribution, circadian rhythm, perinatal development and effect of diabetes, Biochem. J. 309 (1995) 271-277.

[42] J.Y. Kiyasu, I.L. Chaikoff, On the manner of transport of absorbed fructose, J. Biol. Chem. 224 (1957) 935-939.

[43] K. Motojima, S. Goto, Characterization of liver-specific expression of rat uricase using monoclonal antibodies and cloned cDNAs, Biochim. Biophys. Acta. 1087 (1990) 316-322.

[44] H. Peng, Y. Zhuang, Y. Chen, A.N. Rizzo, W. Chen, The Characteristics and Regulatory Mechanisms of Superoxide Generation from eNOS Reductase Domain, PLoS One. 10 (2015) e0140365.

[45] S. Kannappan, N. Palanisamy, C.V. Anuradha, Suppression of hepatic oxidative events and

regulation of eNOS expression in the liver by naringenin in fructose-administered rats, Eur. J. Pharmacol. 645 (2010) 177-184.

[46] N. Palanisamy, A.C. Venkataraman, Beneficial effect of genistein on lowering blood pressure and kidney toxicity in fructose-fed hypertensive rats, Br. J. Nutr. 109 (2013) 1806-1812.

[47] C. Lembo, F. Lopez-Aguilera, E.R. Diez, N. Renna, M. Vazquez-Prieto, R.M. Miatello, Apoptosis of endothelial progenitor cells in a metabolic syndrome experimental model, J. Cardiovasc. Dis. Res. 3 (2012) 296-304.

[48] Y. Tanak, A.L. Slitt, T.M. Leazer, J.M. Maher, C.D. Klaassen, Tissue distribution and hormonal regulation of the breast cancer resistance protein (Bcrp/Abcg2) in rats and mice, Biochem. Biophys. Res. Commun. 326 (2005) 181-187.

[49] Y. Yang, Y. Song, J. Loscalzo, Regulation of the protein disulfide proteome by mitochondria in mammalian cells, Proc. Natl. Acad. Sci. USA. 104 (2007) 10813-10817.

[50] D. van der Vlies, E.H. Pap, J.A. Post, J.E. Celis, K.W. Wirtz, Endoplasmic reticulum resident proteins of normal human dermal fibroblasts are the major targets for oxidative stress induced by hydrogen peroxide, Biochem. J. 366 (2002) 825-830.

[51] J.P. Rabek, W.H.3rd Boylston, J. Papaconstantinou, Carbonylation of ER chaperone proteins

in aged mouse liver, Biochem. Biophys. Res. Commun. 305 (2003) 566-572.

[52] J.E. Nuss, K.B. Choksi, J.H. DeFord, J. Papaconstantinou, J., Decreased enzyme activities of

chaperones PDI and BiP in aged mouse livers, Biochem. Biophys. Res. Commun. 365 (2008)

355-361.

Figure legends

Fig. 1 Time-concentration curve of serum uric acid

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each point represents the mean±S.D. of 5-6 rats.

Fig. 2 P_{app} of D-[^{3}H]-mannitol in the ileum

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each column represents the mean with S.D. of 4 rats.

Fig. 3 Bcrp mRNA level (A), Bcrp protein expression (B) and Bcrp dimer level (C) in the ileum

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each column in Fig. 3A represents the mean with S.D. of 8 rats.

Each column in Fig. 3B and 3C represents the mean with S.D. of 9-10 rats.

*: significantly different from sham-operated rats at $p < 0.05$

**Fig. 4 Nox activity (A) and MDA level (B) in the ileum**

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each column in Fig. 4A represents the mean with S.D. of 10-12 rats.

Each column in Fig. 4B represents the mean with S.D. of 9-10 rats.

*: significantly different from sham-operated rats at $p < 0.05$, **: $p < 0.01$

Fig. 5 Effects of DPI on Nox activity (A), MDA level (B), Bcrp mRNA level (C), Bcrp protein expression (D), Bcrp dimer level (E) and serum uric acid (F)

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

DPI was intraperitoneally injected at the same time as fructose administration (1 mg/kg).

Each column in Fig. 5A represents the mean with S.D. of 8-9 rats.

Each column in Fig. 5B represents the mean with S.D. of 9 rats.

Each column in Fig. 5C represents the mean with S.D. of 6-7 rats.

Each column in Fig. 5D and 5E represents the mean with S.D. of 7-9 rats.

Each point in Fig. 5F represents the mean with S.D. of 5 rats.

**: significantly different from sham-operated rats at $p < 0.01$

†; significantly different from received single oral administration of fructose at $p < 0.05$, ††; $p < 0.01$.

N.S: not significant

Supplementary fig. 1 Expression of URAT1, GLUT9 and NPT1 in the ileum (A) and effect of fructose on GLUT9 mRNA level (B)

(A) cDNAs were prepared as describe in the material and method section. RT-PCR was performed with a KAPATaq Extra PCR kit (KAPA Biosystems, Boston, MA) following the manufacturer's protocol. Kidney sample was used as positive control.

(B) Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each column in Supplementary fig. 1B represents the mean with S.D. of 9 rats.

Supplementary fig. 2 Xanthine oxidase activity in the jejunum, ileum and liver

Xanthine oxidase activity was measured by a fluorometric assay with a Xanthine Oxidase Fluorometric Assay Kit (Cayman Chemical Company, Michigan, USA) as recommended [21].

Each column in Supplementary fig. 2 represents the mean with S.D. of 3 rats.

**: significantly different from sham-operated rats at $p < 0.01$

Supplementary fig. 3 Nox activity in the jejunum and kidney

Nox activity was measured as describe in the material and method section.

Each column in Supplementary fig. 2A represents the mean with S.D. of 9-10 rats.

Each column in Supplementary fig. 2B represents the mean with S.D. of 14 rats.

*: significantly different from sham-operated rats at $p < 0.05$
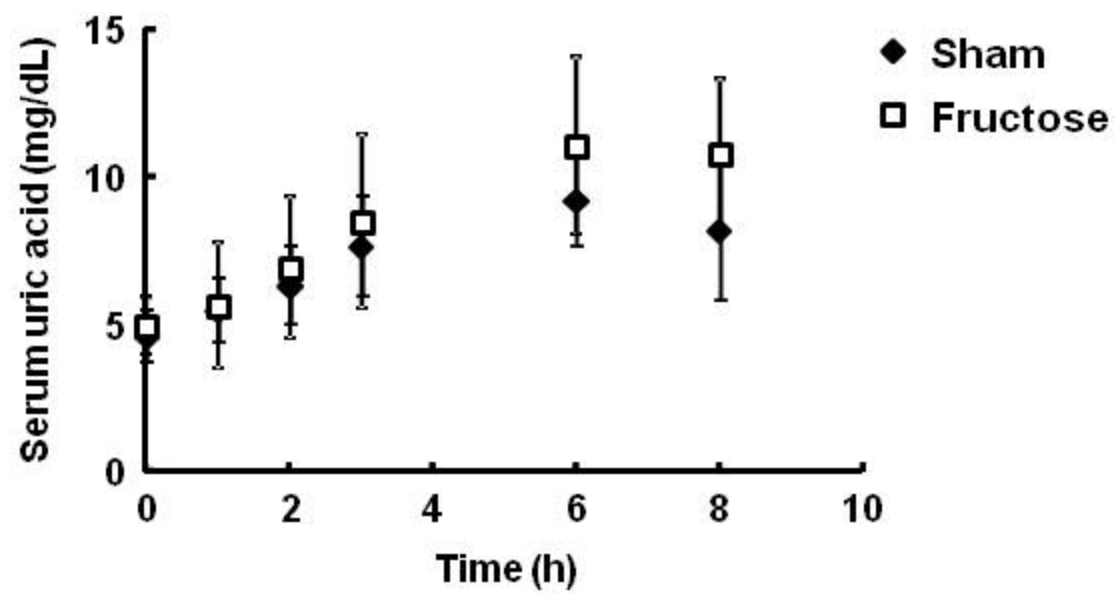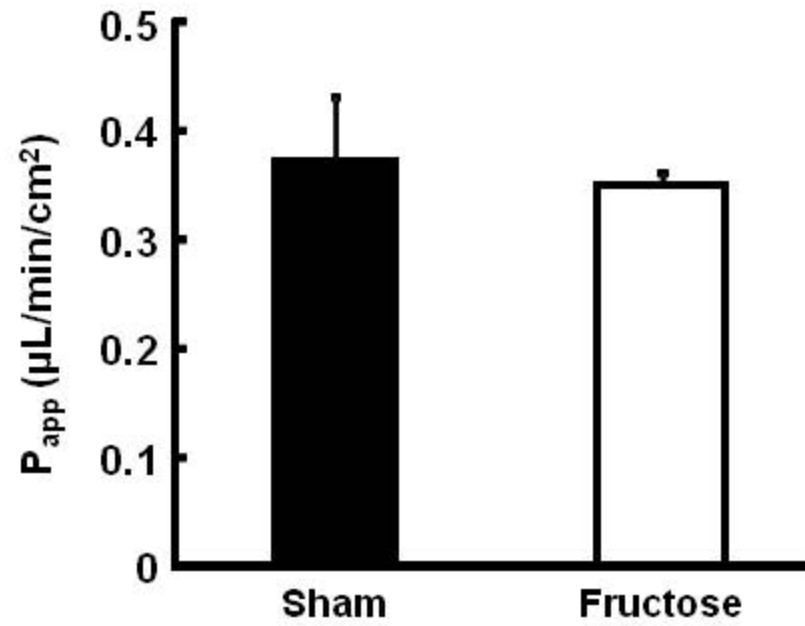
(A) Bcrp mRNA level
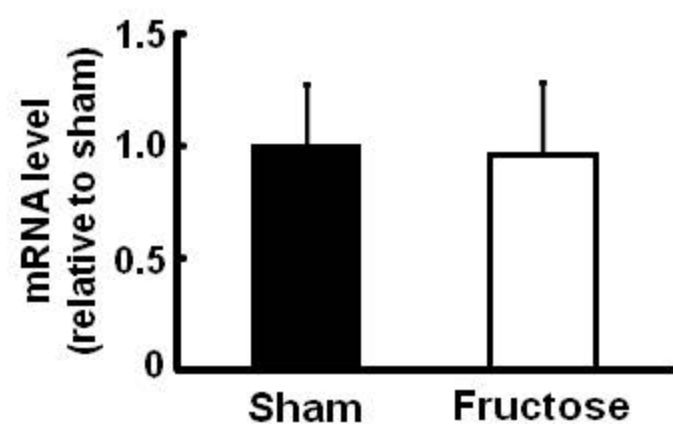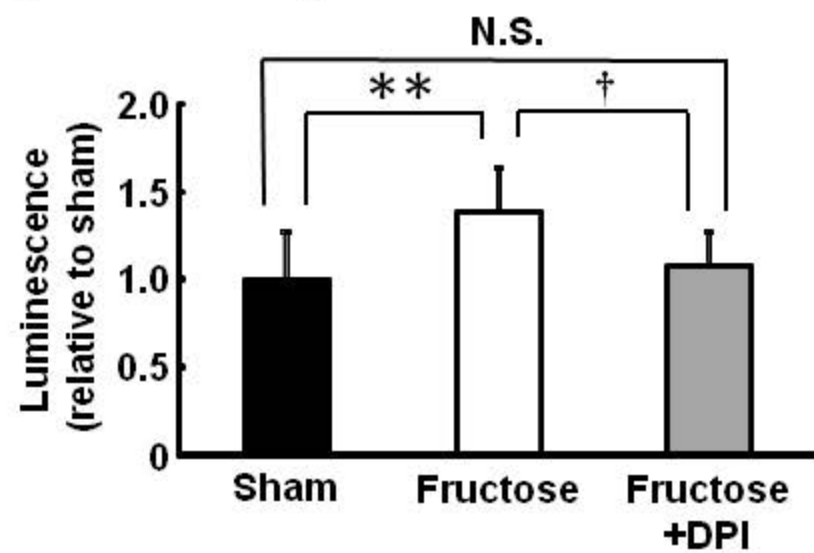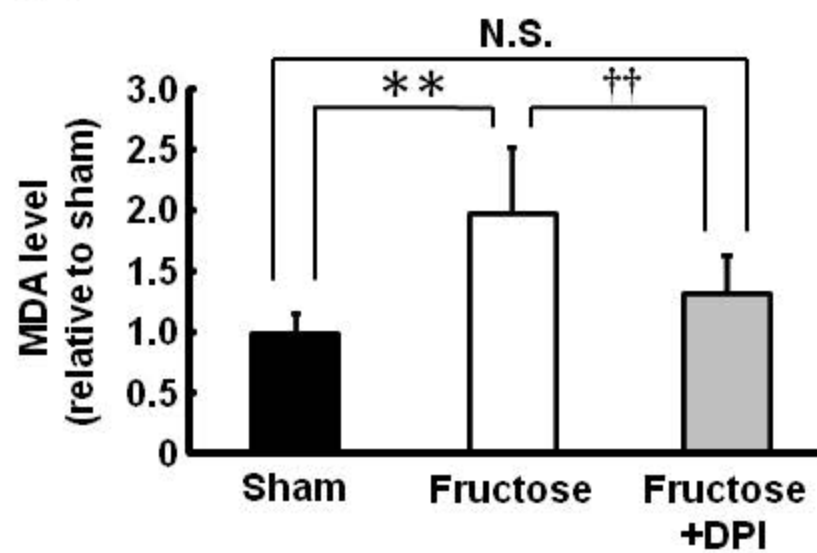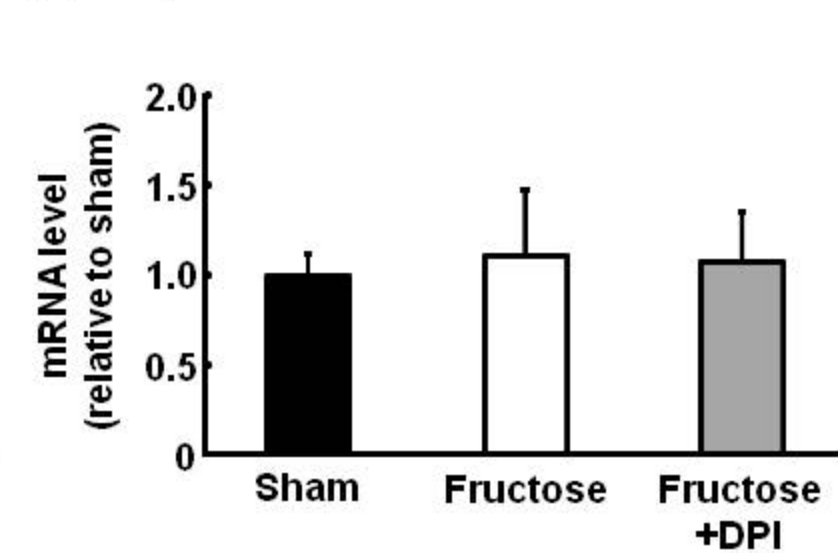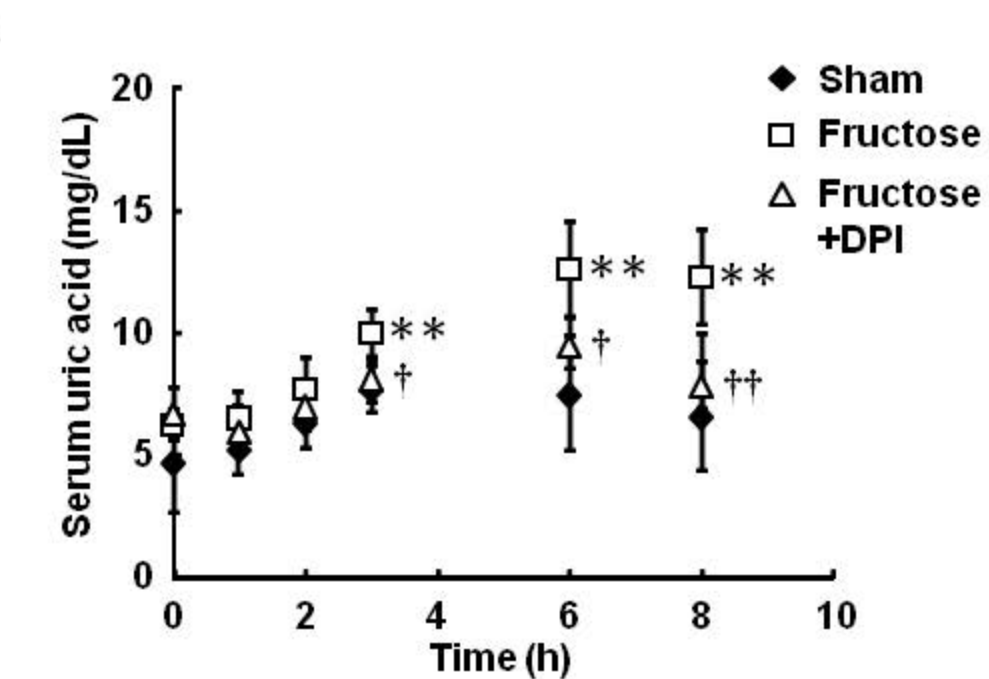
(B) Bcrp protein expression
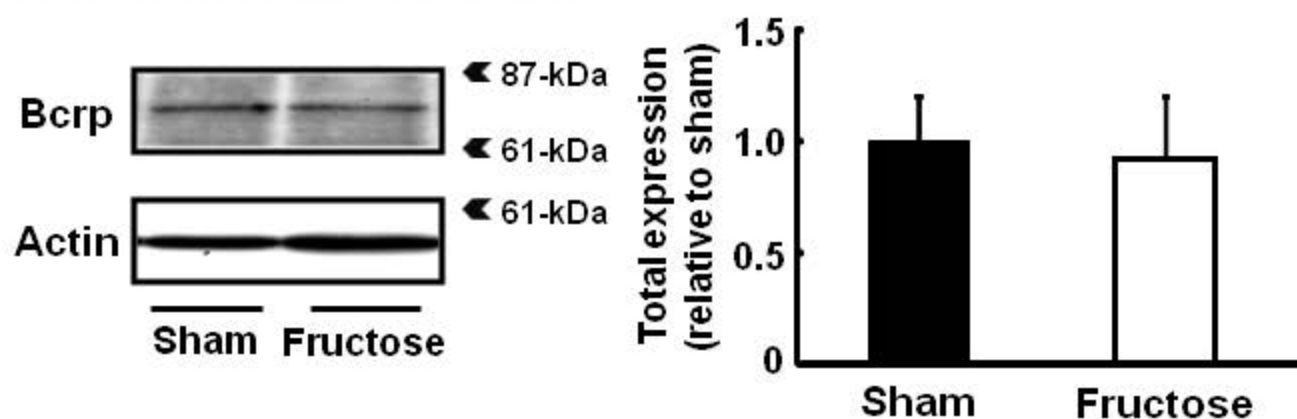
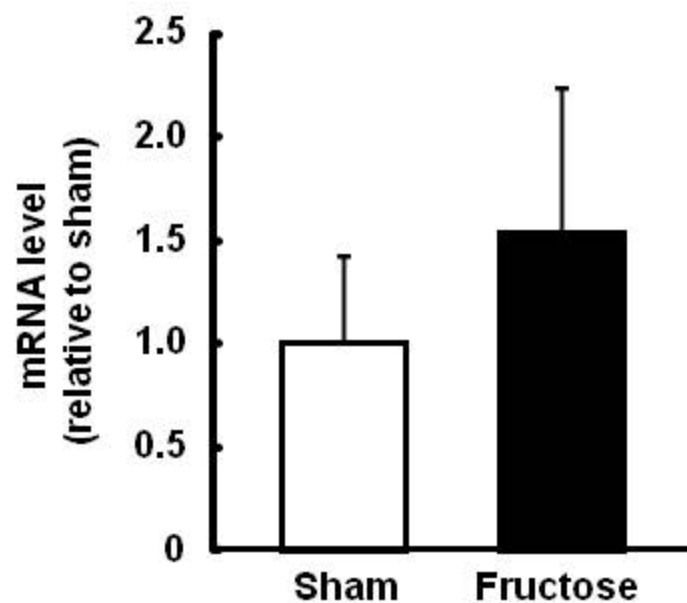**(C) Bcrp dimer level**
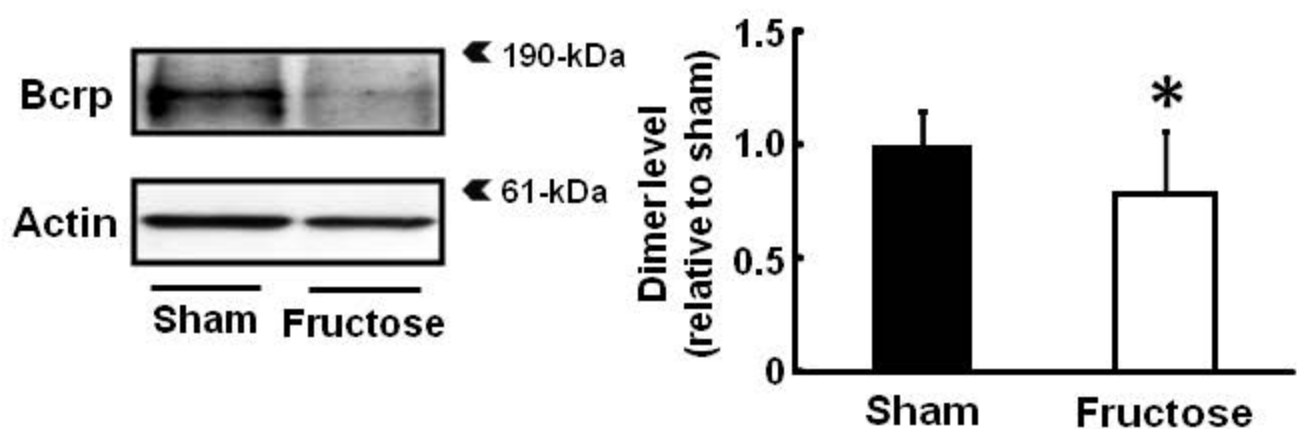
(A) Nox activity
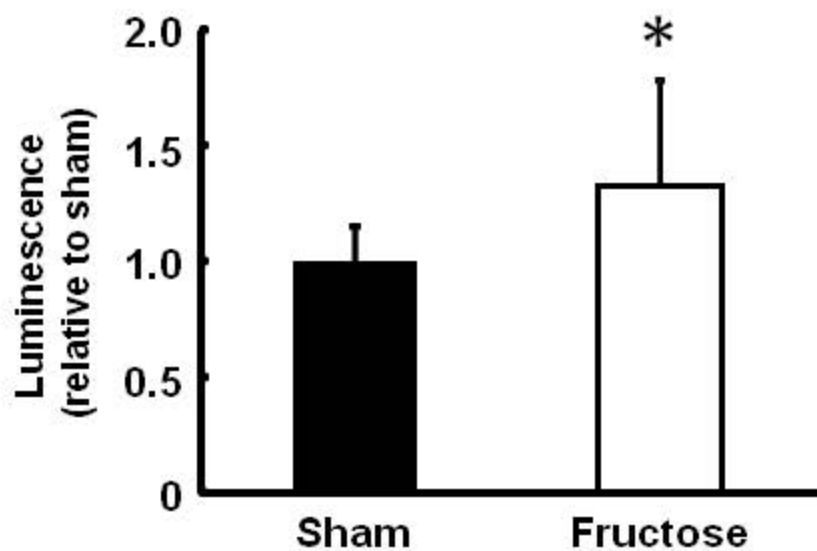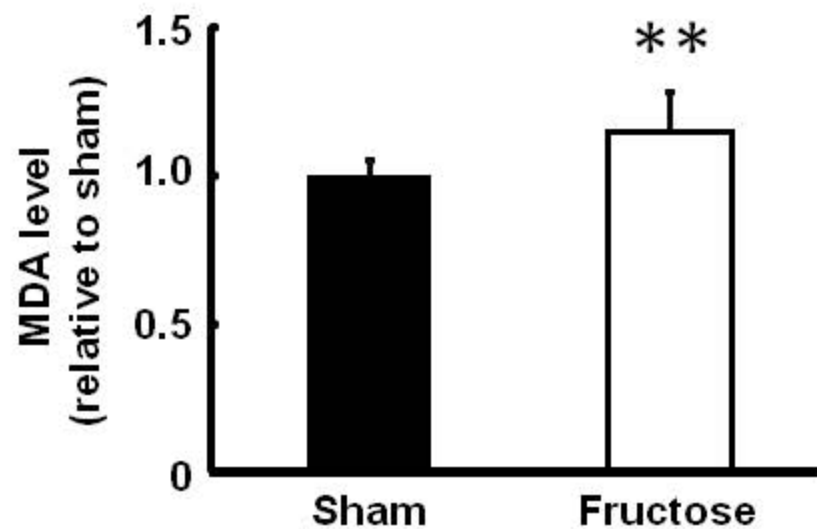
(B) MDA level

(A) Nox activity

(B) MDA level

(C) Bcrp mRNA level

(D) Bcrp protein expression

87-kDa
61-kDa
61-kDa

(E) Bcrp dimer level

190-kDa
61-kDa
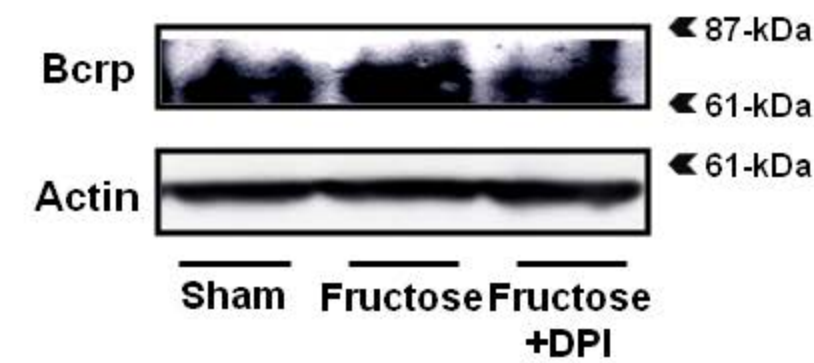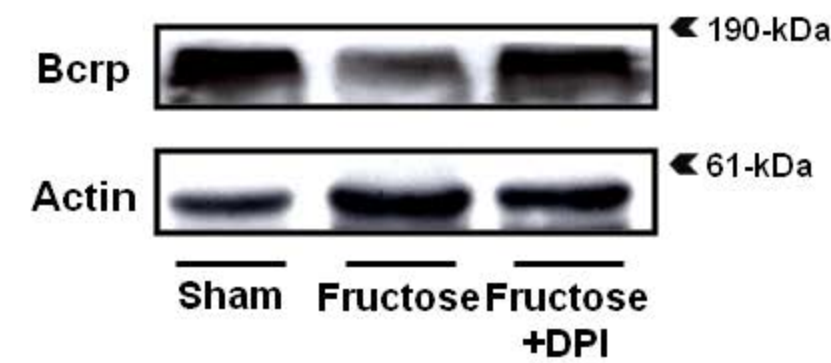
(F) Serum uric acid

(A) Uric acid transporter expression

URAT1

GLUT9

NPT1

GAPDH

Ileum   Kidney

(B) Glut9 mRNA level

mRNA level (relative to sham)

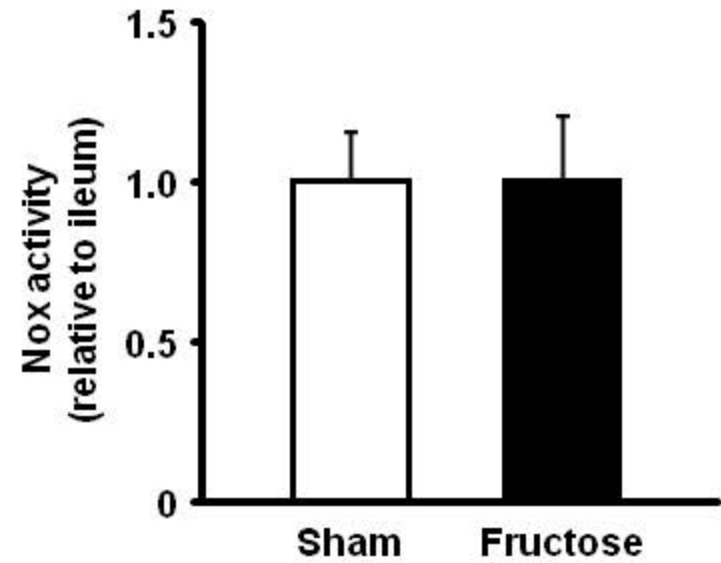Sham   Fructose
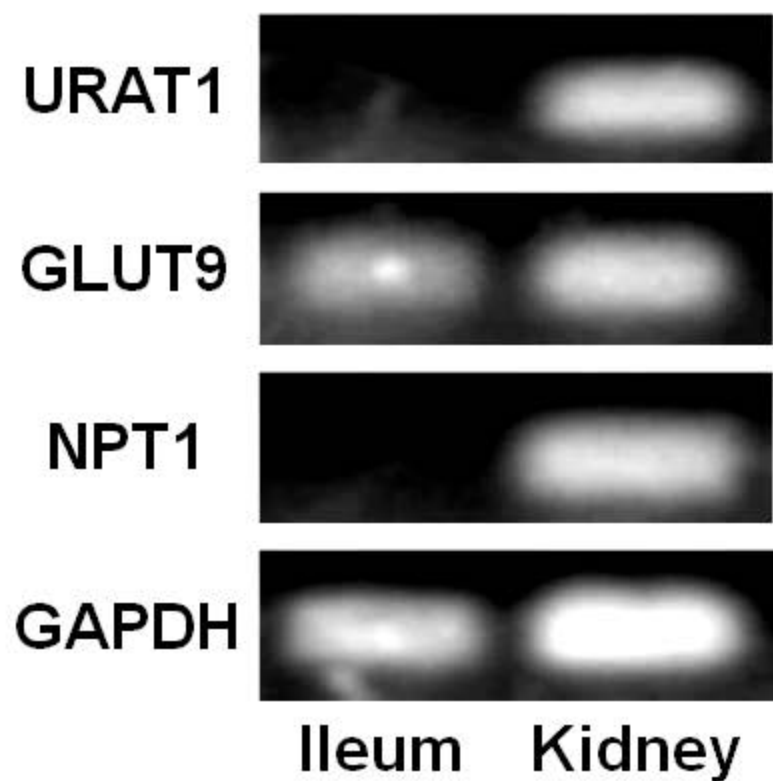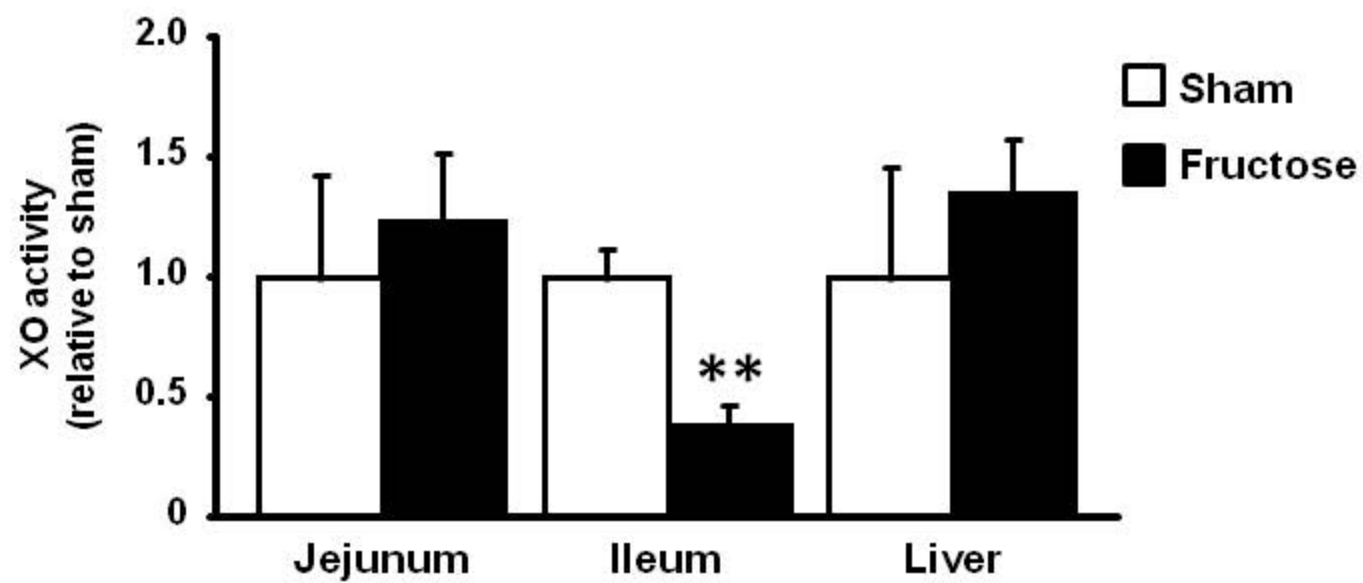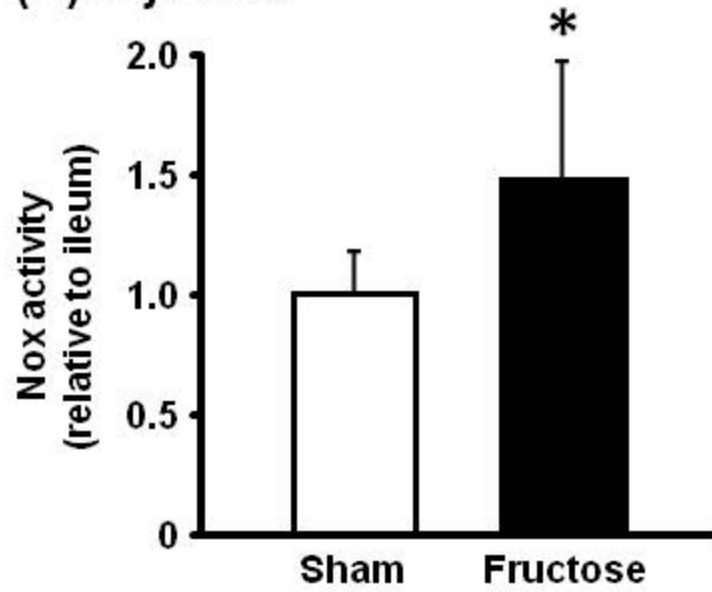
(A) Jejunum

(B) Kidney

Table 1 Body weight, $AUC_{0-8 h}$ and clearances of uric acid in the jejunum, ileum and kidney

	Before administration	12 h after administration	$AUC_{0-8 h}$ (mg/dL·h)	CL (jejunum) (μL/h/mg protein)	CL (ileum) (μL/h/mg protein)	CL (kidney) (mL/h)
Sham	221.3±21.87 g	231.5±18.56 g	60.6±10.7	5.46±2.86	11.3±1.98	5.10±1.27
Fructose	216.2±19.80 g	229.6±10.29 g	70.1±20.4	4.41±2.92	7.72±3.15 *	4.70±2.06

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each value represents the mean±S.D. of 5-6 rats.

*; significantly different from control at $p < 0.05$.

Table 2 Effect of DPI on Body weight, $AUC_{0-8\,h}$ and clearances of uric acid in the jejunum, ileum and kidney

	Before administration	12 h after administration	$AUC_{0-8\,h}$ (mg/dL·h)	CL (jejunum) (μL/h/mg protein)	CL (ileum) (μL/h/mg protein)	CL (kidney) (mL/h)
Sham	238.5±22.86 g	252.8±23.15 g	54.9±11.0	2.46±0.931	7.43±1.32	4.91±2.39
Fructose	240.9±25.11 g	256.2±27.11 g	81.2±9.46 **	1.30±1.06	3.56±1.33 **	4.04±0.750
Fructose+DPI	238.6±17.16 g	253.6±19.42 g	62.4±6.06 †	2.05±0.696	6.24±1.42 †	5.16±1.06

Rats received single oral administration of fructose (5 g/kg) and were used in the experiment at 12 h after administration.

Each value represents the mean±S.D. of 5 rats.

**; significantly different from control at $p < 0.01$.

†; significantly different from rats received single oral administration of fructose at $p < 0.05$.