Role of AMPD2 in impaired glucose tolerance induced by high fructose diet

Athanasius Wrin Hudoyo\textsuperscript{a}, Tetsuaki Hirase\textsuperscript{a}, Andreas Tandelillin\textsuperscript{a}, Masahiko Honda\textsuperscript{a}, Manabu Shiraia\textsuperscript{b}, Jidong Cheng\textsuperscript{b}, Hiroko Morisakia\textsuperscript{b}, Takayuki Morisakib\textsuperscript{b,c,*}

\textsuperscript{a} Department of Bioscience and Genetics, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan

\textsuperscript{b} Tokyo University of Technology School of Health Sciences, Ota, Tokyo, Japan

\textbf{ABSTRACT}

A high intake of products containing fructose is known to mediate insulin resistance. In the liver, AMPD2, an isoform of AMPD, has important glucose metabolic homeostasis functions including maintenance of AMP-activated protein kinase (AMPK). We speculated that AMPD2 induces impaired glucose tolerance in individuals who consume a high-fructose diet. We gave either a normal-chow (NCD) or high-fructose (HFrD) diet for 40 days to 8-week-old male wild-type (WT) and \textit{Ampd2}\textsuperscript{−/−} homozygote (A2\textsubscript{−/−}) C57BL/6 mice. A glucose tolerance test (GTT) and pyruvate tolerance test (PTT) were used to evaluate glucose metabolism. In addition, gluconeogenesis and glycolysis enzymes, and AMPK phosphorylation in the liver were investigated. With consumption of the HFrD, A2\textsubscript{−/−} mice showed enhanced glucose tolerance in GTT and PTT results as compared to the WT mice, which were independent of changes in body weight. Also, the levels of phosphoenolpyruvate carboxy kinase and glucose-6-phosphatase (hepatic gluconeogenic enzymes) were significantly reduced in A2\textsubscript{−/−} as compared to WT mice. The hepatic glycolytic enzymes glucokinase, phosphofructokinase, and pyruvate kinase were also examined, though there were no significant differences between genotypes in regard to both mRNA expression and protein expression under HFrD. Surprisingly, hepatic AMPK phosphorylation resulted in no changes in the A2\textsubscript{−/−} as compared to WT mice under these conditions. Our results indicated that \textit{Ampd2}\textsuperscript{−/−} mice are protected from high fructose diet-induced glycemic dysregulation, mainly because of gluconeogenesis inhibition, and indicate a novel therapeutic target for type 2 diabetes mellitus.

1. Introduction

Increasing fructose intake in recent decades because of increases in consumption of soft drinks and other sweetened beverages containing high fructose corn syrup is associated with numerous metabolic disorders, such as hyperlipidemia, non-alcoholic fatty liver disease (NAFLD), insulin resistance, and obesity [1–4]. Fructose is mainly metabolized in the liver via its specific-transporter GLUT5 [4,5]. Through the activity of phosphofructokinase, insulin level and energy status maintain glucose metabolism, whereas fructose is metabolized by an insulin-independent mechanism and excessive consumption leads to excessive hepatic fructose absorption [6]. In the liver, fructokinase converts fructose to fructose-1-phosphate in an ATP-dependent manner, resulting in an increased adenine monophosphate (AMP) level. Elevation of AMP stimulates AMP deaminase (AMPD) activity and triggers conversion of AMP into inosine monophosphate (IMP), resulting in generation of uric acid as a waste product [7,8]. Furthermore, AMP accumulation also induces AMP-activated protein kinase (AMPK), which plays a central role in glucose and lipid metabolism. However, it has been reported that AMPD has an effect to hinder AMPK activities in human hepatocytes [9] and its activity is also shown to diminish the favorable impact of AMPK to improve insulin sensitivity in metabolic disorders, presumably through inhibition of uric acid [10]. Hence, abrogation of AMPD activity might enhance the positive effect of AMPK for controlling glucose metabolism.

AMPD2 is one of three different AMPD isoforms and exclusively distributed from the liver, though has also been detected in other organs such as the kidneys and brain [11]. In humans, a mutation of the \textit{Ampd2} gene is related to pontocerebellar hypoplasia [12], while it is associated with nephrotic syndrome in mice [13]. In regard to insulin resistance and glucose metabolism, silencing of \textit{Ampd2} in hepatocytes successfully augmented AMPK activation, resulting in elevated fatty acid oxidation [9]. Also, recent studies have shown that deletion of AMPD1, another form of AMPD expressed mainly in skeletal muscle [14], attenuates glucose intolerance and improves glucose clearance via up-regulation of the AMPK/Akt/mTORC1/p70s6 kinase axis in mice fed a high fat diet [15,16].

In the present study, we investigated the effects of a high fructose
diet (HFrD) on glucose metabolism in C57BL6 wild-type (WT) and Ampd2−/− (A2−/−) mice in order to elucidate the role of AMPD2 in HFrD-induced impaired glucose tolerance. We speculated that Ampd2 knockout mice would display suppression of glucose dysregulation induced by excessive fructose intake. Additionally, due to the specific attribute of fructose to induce gluconeogenesis, we examined two key enzymes that play a crucial part in that process, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). In addition, the glycolytic enzymes glucokinase/hexokinase IV (GCK), phosphofructokinase (PFKL), and pyruvate kinase (PKLR) were analyzed to obtain mechanistic insight into the process by which Ampd2 deficiency affects whole body glucose regulation. Finally, AMPK phosphorylation was also investigated to understand its role in Ampd2 deficiency associated with a high fructose diet.

2. Materials and methods

2.1. Mice

Generation of C57BL/6 WT (control) and Ampd2-deficient homozygous (Ampd2−/−; A2−/−) mice was performed as previously described [11]. Animals were housed in an SPF environment with a 12-h light-dark cycle and constant temperature (25 °C). Male 8-week-old mice with a body weight ≥20 g were selected, and given either a normal chow diet (NCD) (CE-2; CLEA Japan, Tokyo, Japan) or high fructose diet (HFrD) (TD 89247; Harlan Laboratories, Indiana, USA), the latter containing 60% Kcal from fructose, and had free access to food and water. All animal experiments were approved by the Committee on Animal Research of the National Cerebral and Cardiovascular Center and performed according to the Guidelines for protection of experimental animals of the National Cerebral and Cardiovascular Center. The body weight of each mouse in both groups was measured twice per week throughout the study.

2.2. Intraperitoneal glucose and pyruvate tolerance test

An intraperitoneal glucose tolerance test (IpGTT) was performed on day 29 after a 16-h overnight fast. Blood samples were taken from the tail vein at various times. After measuring fasting blood glucose, a glucose (2 g/kg of body weight) was administered by intraperitoneal injection and blood glucose was determined again at 15, 30, 60, 90, 120, and 180 min following the injection. After completing the IpGTT, food was resumed and the mice were allowed to recover for 11 days.

Next, an intraperitoneal pyruvate tolerance test (IpPITT) was performed on day 40. After 4 h of fasting in the morning (7:00–11:00), blood glucose was measured, then pyruvate (2 g/kg of body weight prepared in saline) was injected in an intraperitoneal manner. Blood glucose was determined again at 15, 30, 60, 90, 120, and 180 min following that injection.

2.3. Blood and tissue collection

Two hours after finishing the IpPITT, the mice were euthanized with intraperitoneal sodium phenobarbital anesthesia. Blood was collected via a cardiac puncture and centrifuged at 6000 rpm (4 °C) for 15 min, then serum specimens were stored at −80 °C. In addition, tissues were isolated, and quickly frozen in liquid nitrogen and stored at −80 °C.

2.4. Western blot analysis

Approximately 20–30 mg of liver tissue was homogenized in RIPA buffer containing 10 mM Tris (pH 7.4), 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, eComplete ULTRA protease inhibitor cocktail (Roche Diagnosis, Mannheim, Germany), and PhosSTOP phosphate inhibitor cocktail (Roche Diagnosis). Each sample was centrifuged at 15,000 rpm (4 °C) for 15 min, then the supernatant homogenate was collected and protein determination was performed using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Samples at the same volume were loaded onto SDS-PAGE gels and transferred in an electrophoretic manner to PVDF membranes using a Trans Blot Turbo System (Bio Rad Laboratories Inc., Hercules, LA, USA).

The membranes were then probed with the following primary antibodies: anti-PEPCK (Pck1) 1:2000 (Protein Tech, code 16754-1-P), anti-GCK 1:2000 (Santa Cruz Biotechnology, code sc-25840), anti-PFKL 1:2000 (Santa Cruz Biotechnology, code sc-7908), anti-PFKL 1:1000 (Cell Signaling Technology, #8175), anti-PKLR 1:1000 (Protein Tech, code 22456-1-AP), anti-phospho Ampk-α (Thr172) 1:1000 (Cell Signaling Technology, #2535), anti-AMPKα antibody 1:1000 (Cell Signaling Technology, #2532), and anti-α tubulin 1:1000 (Cell Signaling Technology, #2125). An anti-rabbit IgG HRP-Linked Antibody (Cell Signalling Technology, #7074) was used as the secondary antibody.

Bands were detected by chemiluminescence using an ECL Prime Kit (GE Healthcare, Piscataway, NJ, USA) and images were obtained with a GE Amersham Imager 600 (GE Healthcare Life Sciences). Band Intensity was quantified using Image Quant TL 8.1 (GE Healthcare Life Sciences).

2.5. RNA extraction and quantitative PCR

Total RNA was extracted from liver samples with Isogen II, following the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed using Superscript III (Invitrogen, Carlsband, CA, USA). An ABI Prism 7900 Sequence Detection System was used to perform real-time PCR with Power SYBR Green Master Mix (Life Technologies, California, USA). Expression data for each gene of interest were normalized with β-actin mRNA level as the control. The specific primer sets for the target genes were as follows: mPCK1 (PEPCK), forward primer GAT GAC ATT GCC TGG ATG AA, reverse primer CAC GTT GGA TAT GTT GT; mG6Pc (G6Pase), forward primer CCT GAG GAA CGC CTT CTA TG, reverse primer TGT CCA GCA GGC ACC AAT AC; GCK (GCK), forward primer AGT ACG ACC GGA TGG TGG ATG, reverse primer TTG AGC AGC ACA GTG CTT ACC AGC AGA; mPKL (PFKL), forward primer CAGT CCG GTC ACA ACA GAA CAA GGA CCA GCC ACC ACC ACC ACC AC; mPKL (PFKL), forward primer CAGT CCG GTC ACA ACA GAA CAA GGA CCA GCC ACC ACC ACC ACC AA.

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2.6. Statistical analysis

Values are presented as the mean ± SEM. Intergroup comparisons were analyzed using Mann-Whitney's test. A p level < 0.05 in comparisons between groups was considered to indicate statistical significance.

3. Results

To assess body weight changes with the normal chow and high fructose diets, we regularly measured the body weights of the WT and A2−/− mice. After 40 days of the NCD, that for the A2−/− mice tended to be lighter, while similar findings were noted for mice that received the HFrD (Fig. 1). These results suggested that Ampd2 had a major role in body weight regulation even under the HFrD condition during the experimental period.

We used IpGTT results to determine the effect of Ampd2 deficiency on glucose metabolism. After 29 days of the diet protocols, we subjected all mice in both groups to an IpGTT after an overnight fast. For mice who received the NCD, fasting blood glucose levels were similar between the WT and A2−/− groups (Fig. 2A). However, following a bolus glucose injection, the A2−/− mice displayed a significantly lower level as compared to the WT mice at 15, 30, 60, and 90 min after the injection, then the level returned to near the baseline towards the end of the observation period (Fig. 2A). Under the HFrD condition as well, we observed similar fasting blood glucose levels in WT as...
compared to A2+/+ mice (Fig. 2B). Thereafter, the A2−/− mice demonstrated significantly decreased blood glucose concentrations as compared to WT mice at 60, 90, and 120 min after bolus glucose injection and then sustained a higher glucose concentration until the end of the experiment (180 min) (Fig. 2B). As expected, the area under the curve (AUC) for blood glucose level in IpGTT findings was significantly increased in WT mice as compared to those fed the NCD, while the AUC of A2−/− mice was significantly decreased as compared to that of the WT mice under both the NCD and HFrD conditions (Fig. 2C). These results indicate that Ampd2 deficiency contributes to attenuation of impaired glucose tolerance.

An IpPTT was also performed after a 4-h fast at 40 days after beginning the feeding period to investigate the effect of Ampd2 deletion on gluconeogenesis. Blood glucose concentration in the A2−/− mice was not significantly different as compared to that in the WT mice under the NCD (Fig. 3A). In contrast, with the HFrD, fasting blood glucose level in the A2−/− mice was slightly reduced (Fig. 3B). Furthermore, blood glucose in the A2−/− mice was significantly lower as compared to the WT mice at 15 min after the pyruvate injection and different levels were continuously observed at all time points until the end of the experiment (180 min) (Fig. 3B). As shown by the IpGTT findings, the AUC for blood glucose using IpPTT results was calculated (Fig. 3C). Based on the IpPTT results, the AUC of blood glucose in the WT mice was not significantly different from that in the A2−/− mice under the NCD condition, whereas that under the HFrD condition was significantly increased as compared to the NCD condition in the WT mice. Also, the AUC of blood glucose in the A2−/− mice was significantly decreased as compared to that in the WT mice under the HFrD condition. Together, these results suggest that Ampd2 deficiency plays a role in inhibition of gluconeogenesis stimulated by HFrD.

Results obtained from the IpPTT indicate that Ampd2 deficiency may alter gluconeogenesis and/or glycolysis under a high fructose condition. To examine that, we studied the expressions of genes related to the gluconeogenic pathway in the liver by performing quantitative RT-PCR assays, including PEPCK and G6Pase, which are involved in regulation of gluconeogenesis. The mRNA expression of PEPCK was significantly decreased in the A2−/− mice as compared to the WT mice under both the NCD and HFrD conditions (Fig. 4A). On the other hand, G6Pase mRNA expression was not significantly different between the mouse groups regardless of feeding condition, though G6Pase mRNA expression in WT mice was different between the NCD and HFrD conditions. As for protein expression, that of PEPCK was not statistically significantly different between the mouse groups, though was...
Fig. 3. Intraperitoneal pyruvate tolerance test (IpPTT) results showed that Ampd2 deficiency resulted in reduced blood glucose after consumption of high-fructose diet (HFrD).

Blood glucose shown by IpPTT results in mice fed the normal chow diet (NCD) (n = 5). B. Blood glucose shown by IpPTT results in mice fed the HFrD (n = 5). C. Quantification of area under curve (AUC) of blood glucose levels in IpPTT results. Values shown represent the mean ± SEM. *p < 0.05, WT vs. A2 −/−. #p < 0.05, WT NCD vs. WT HFrD. WT: wild-type, A2 −/−: Ampd2 −/− homozygotes.

significantly different in WT mice between feeding conditions (Fig. 4B). As for G6Pase protein expression, there was a statistically significant decrease in the A2 −/− as compared to the WT mice under the HFrD condition (Fig. 4B).

To elucidate glycolytic regulation, we examined the expressions of GCK, PFKL and PKLR, which play important roles in glycogenic pathways (Fig. 5A). Under the NCD condition, the mRNA expression of GCK was not different between the WT and A2 −/− mice. However, that in A2 −/− mice under the HFrD condition showed an increasing tendency as compared with the WT mice, though the differences were not statistically significant. PFKL and PKLR mRNA expressions were at similar levels in the WT and A2 −/− mice. We also investigated the protein expression of these glycolytic enzymes (Fig. 5A). There were no statistically significant differences regarding GCK protein expression between the WT and A2 −/− mice under either of the feeding conditions. As for the PFKL protein, its expression was not different under the HFrD condition between the mouse groups, while it was significantly decreased in the A2 −/− as compared to the WT mice under the NCD condition. There were no statistically significant differences regarding PKLR protein expression between the mouse groups under either feeding condition.

AMPD is known to regulate intracellular adenine nucleotides, especially AMP [26]. Also, intracellular AMP levels in the liver were reported to be increased in Ampd2-deficient mice [9]. Therefore, changes in expression of gluconeogenic enzyme genes might be due to changes in the phosphorylated condition of AMP-activated protein kinase, as those are regulated by intracellular AMP levels as well as ATP/AMP ratio [26]. To examine whether the improvement in glucose tolerance in Ampd2-deficient mice that received a high fructose diet is associated with enhanced insulin signaling, we determined phosphorylated AMPK in liver tissues using western blot analysis.

Surprisingly, there were no statistically significant differences between the WT and A2 −/− mice regarding the ratio of phosphorylated AMPK protein expression (p-AMPK/AMPK) observed in liver tissues (Fig. 6).

4. Discussion

In the present study, the body weights of A2 −/− and WT mice were not significantly different after receiving the NCD or HFrD, suggesting that Ampd2 had a limited impact on body weight changes under our experimental settings. On the other hand, the adverse effects of high fructose intake were modulated in a manner independent of weight gain. These results are consistent with those reported by Janevski, et al., who found no alteration of body weight in rats fed a high fructose diet for 28 days, while several factors related to hepatic lipidogenesis were upregulated [17]. Schultz, et al., also noted that insulin resistance occurred in rats without increased body weight after 16 weeks of fructose feeding [18]. In humans, metabolic syndrome was shown to develop with a fructose diet despite no change in body weight [6].

A previous animal study documented that chronic consumption of fructose leads to insulin resistance [19]. In the present investigation, after 40 days consuming the HFrD, fasting blood glucose levels in the A2 −/− mice seemed to be reduced as compared to the WT mice, indicating a role of Ampd2 deficiency to enhance insulin sensitivity under a basal condition. Another study of rats reported fasting hyperglycemia and hyper-insulinemia after 30 days of consuming fructose [20], while a human study found that hepatic insulin resistance was largely influenced by fasting from glucose [21]. Hence, augmentation of hepatic insulin signaling is a critical point to lower the degree of insulin resistance. Our findings of a higher AUC in WT mice fed the HFrD as compared to those fed the NCD showed the deleterious effect of fructose.
on glucose metabolism. Furthermore, the significant difference in blood glucose between WT and A2−/− mice under the HFrD condition shown by IpGTT and IpPTT results indicated the major effect of Ampd2 deficiency on improvement of glucose tolerance, especially when gluconeogenesis is limited. Based on our results, Ampd2 dysfunction may interfere hepatic glucose production, presumably through reduction in the amount of the gluconeogenic precursor, up-regulation of glycolysis, or suppression of gluconeogenic gene expression.

Since Ampd2 showed the highest level of expression in liver tissues among Ampd genes and in consideration of the central importance of the liver in fructose metabolism, we speculated that that organ likely has a predominant role in glucose metabolism changes throughout the body. We found that expression of genes encoding the gluconeogenic enzymes PEPCK and G6Pase had differential effects towards Ampd2 deletion, though glucose production was inhibited. Ampd2 deficiency suppressed the gene expression of PEPCK under both the NCD and HFrD conditions, as compared to the control. In addition, we observed that G6Pase protein expression with Ampd2 deficiency was decreased with the HFrD. Although we found differences between mRNA and protein expressions, the changes were consistent with decrease in the gluconeogenic pathway. Our results are also similar to those of other studies that showed indispensable functions of both enzymes, especially G6Pase, for regulating hepatic gluconeogenesis [22–25]. Gluconeogenesis is a complex process, which may be partially attributed to decreased expression of those enzymes.

We also examined three irreversible steps of the glycolytic pathway, namely, the expressions of GCK, PFKL, and PKLR. There were no significant differences regarding the expression of any of those genes or proteins between the WT and A2−/− mice under the HFrD condition, though we could not exclude the influence of change of glycolytic pathway caused by Ampd2 deficiency. However, Wu et al., reported that overexpression of glucokinase in an obese mice model promoted hepatic glycolysis up-regulation, resulting in decreased hepatic glucose production and increased whole-body glucose disposal [26].

AMP-activated protein kinase (AMPK), an enzyme widely known as a key regulator of metabolic homeostasis [27], is stimulated by changes in intracellular AMP/ATP ratio, and mediates numerous favorable regulations that control glucose production and lipid synthesis [28].
Physiologically, AMPD generates increases in uric acid as a result of increased AMP driven by fructose exposure, which inhibits the activity of AMPK [9]. Therefore, counteracting AMPD might be a good strategy to enhance AMPK activation. Surprisingly, our findings showed that phosphorylated AMP-activated protein kinase in the liver, considered to be one of the main changes affected by Ampd2 deficiency, was unaltered in the A2−/− mice as compared to the WT mice under the HFrD. In line with this result, Foretz et al. showed that AMPK was not necessary for gluconeogenesis inhibition stimulated by metformin [24]. Also, Woods et al. recently reported that Ampk activation did not improve glucose tolerance in D361A-transgenic mice fed a high fructose diet for 12 weeks [29].

Baena et al. reported that fructose, but not glucose, exhibited adverse effects towards insulin signaling in white adipose tissues and skeletal muscle, which were also seen in the liver [30]. Taken together with our findings, it is suggested that insulin signaling in other

Ampd2 deficiency resulted in no changes in mRNA or protein expression of glycolytic enzymes in mice fed the high-fructose diet (HFrD).

A. The mRNA expression of the examined glycolytic enzyme genes was quantified by real-time PCR using RNA extracted from liver tissues of mice fed the normal chow diet (NCD) or HFrD. mRNA expression was normalized to that of β-actin. GCK: glucokinase, PFKL: phosphofructokinase L-type gene, PKLR: pyruvate kinase liver/red blood cell type gene. Values shown represent the mean ± SEM (n = 6).

B. Immunoblotting of glycolytic enzymes. Protein extracted from livers of mice fed the NCD or HFrD were used to analyze the phosphorylation of GCK, PFKL, and PKLR. The band intensity of the phosphorylated form was quantified and adjusted with that of α-tubulin. Values for the fold change shown represent the mean ± SEM (n = 6).

*p < 0.05. WT: wild-type, A2−/−: Ampd2−/− homozygotes.

Physiologically, AMPD generates increases in uric acid as a result of increased AMP driven by fructose exposure, which inhibits the activity of AMPK [9]. Therefore, counteracting AMPD might be a good strategy to enhance AMPK activation. Surprisingly, our findings showed that phosphorylated AMP-activated protein kinase in the liver, considered to be one of the main changes affected by Ampd2 deficiency, was unaltered in the A2−/− mice as compared to the WT mice under the HFrD. In line with this result, Foretz et al. showed that AMPK was not necessary for gluconeogenesis inhibition stimulated by metformin [24]. Also, Woods et al. recently reported that Ampk activation did not improve glucose tolerance in D361A-transgenic mice fed a high fructose diet for 12 weeks [29].

Baena et al. reported that fructose, but not glucose, exhibited adverse effects towards insulin signaling in white adipose tissues and skeletal muscle, which were also seen in the liver [30]. Taken together with our findings, it is suggested that insulin signaling in other
pathways, including the role of other insulin-sensitive tissues such as skeletal muscles and adipose tissue, may play a role in glucose metabolism, especially in the setting of a high fructose diet. On the other hand, an alternative mechanism in the kidneys could explain these metabolic changes. AMPD2 is known to be expressed in the kidneys, which lose AMPD2 protein immunoreactivity in the presence of Amp2d deficiency [11]. In addition, GLUT5, a specific transporter of fructose, has been detected in kidney as well as liver tissues [5]. The kidneys have a significant role in glucose metabolism, especially under conditions associated with deep fasting [31] or diabetes [32], since they are functionally in the liver in mice did not lead to hypoglycemia in amp2d-deficient mice. Our results indicate a potential model for the fold change shown represent the mean ± SEM (n = 6). In conclusion, we found a protective effect via high fructose diet-induced glycosmic dysregulation induced by suppression of gluconeogenesis in Amp2d-deficient mice. Our results indicate a potential model for attenuation of abnormal metabolism including insulin resistance, especially in connection with changes in gluconeogenesis as well as glycolysis.

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