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ORIGINAL ARTICLE

Investigation of the Expression of CYP3A4 in Diabetic Rats in Xenobiotic Metabolism

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ABSTRACT

Objectives: This study investigated the impact of a high-fat diet streptozotocin (STZ)-induced diabetes and dapagliflozin treatment on hepatic protein expression of CYP3A4.

Materials and Methods: In our study, 34 male Sprague-Dawley rats were randomly divided into four groups: Control, high-fat diet and STZ-induced diabetes, dapagliflozin-treated control, and dapagliflozin-treated diabetes. In the microsomes obtained from the livers of these rats, the protein expression levels of CYP3A4 were determined by Western blotting.

Results: Hepatic CYP3A4 protein expression levels in the control group treated with dapagliflozin were significantly decreased compared with those in the control group. In addition, hepatic CYP3A4 protein expression levels were decreased in dapagliflozin-treated diabetic Sprague-Dawley rats compared with those in both control and diabetic group rats, but the difference between the groups was not statistically significant.

Conclusion: According to these two results, the use of dapagliflozin inhibited hepatic CYP3A4 protein expression.

Keywords: CYP3A4, dapagliflozin, diabetes mellitus, microsome, protein expression

INTRODUCTION

Cytochrome P450 monooxygenases (CYP450), a superfamily of heme-containing proteins, are responsible for the biotransformation of a vast majority of both endobiotics and xenobiotics by converting these lipophilic compounds into their hidrophilic forms.[1,2] More than 95% of available pharmaceuticals used clinically are metabolized by CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. Different CYP450s, which have varying degrees of abundance in the smooth endoplasmic reticulum of the human hepatocyte, have been determined to be 13% CYP1A2, 4% CYP2A6, 1% CYP2B6, 20% CYP2C, 2% CYP2D6, 7% CYP2E1, and 30% CYP3A4.[3] CYP3A4 is a major CYP450 enzyme that particularly mediates biotransformation of approximately 50% of marketed drugs, including benzodiazepines (alprazolam, diazepam, and midazolam), calcium channel blockers (amlodipine, diltiazem, and verapamil), immunosuppressives (cyclosporine, tacrolimus, and sirolimus), macrolide antibiotics (clarithromycin, erythromycin), and statins (atorvastatin, simvastatin). Along with many medications, CYP3A4 also catalyzes the metabolism of several endogenous molecules such as steroids (estradiol, progesterone, testosterone) and vitamin D.[4,5] CYP3A4 is also responsible for inactivating aflatoxin B1, which is an environmental carcinogen.[6] A high level of CYP3A4 gene expression is found in the liver of humans. In addition, extrahepatic tissues expressing CYP3A4 include the prostate, breast, intestine, colon, small intestine, and brain.[7]

Diabetes mellitus (DM) is a heterogeneous group of diseases characterized by hyperglycemia due to an absolute or relative deficiency of insulin secretion and/or action. Hyperglycemia associated with DM, which is a chronic disease, damages the heart, blood vessels, eyes, kidneys, and nerves.[8] There are two

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main types of DM: type 1 and type 2. Type 1 DM (T1DM), an autoimmune disease, results from a lack of insulin production caused by damage to pancreatic beta cells. Type 2 DM (T2DM) has three characteristic features: insulin resistance, beta cell secretory dysfunction, and increased production of hepatic glucose.[9,10] The consequences of DM, which is a treatable disease, can be prevented or delayed by diet, physical activity, medications, regular screening, and treatment for complications.[11] Dapagliflozin, chosen as an antidiabetic agent in our study, is a medicine used in the management of T2DM by selectively inhibiting sodium-glucose co-transporter 2 (SGLT2), thus preventing the reabsorption of glucose from the urine. In January 2014, the United States Food and Drug Administration approved its use in combination with diet and exercise to treat adult T2DM patients by providing glycemic control. According to the results of some *in vitro* studies examining the relationship between dapagliflozin metabolism and CYP450 enzymes, dapagliflozin metabolism can be catalyzed by CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2D6, and CYP3A4.[12,13] Sprague-Dawley rats with T2DM induced by a combination of a high-fat diet and STZ were used in our study. High-fat diet leads to insulin resistance in rats. In addition, treatment with STZ contributes to beta cell dysfunction. Namely, co-administration of a high-fat diet and STZ creates the metabolic profile observed in humans who suffer from T2DM.[14]

Various components influence the expression of each CYP450, including genetic polymorphisms, xenobiotics, cytokines, hormones, disease states, sex, age, and others.[15] Factors that induce CYP3A4 include a wide variety of medicines such as antiandrogens, antibiotics, antiemetics, antiepileptics, antineoplastic agents, antipyretic analgesics antiretroviral barbiturates, cystic fibrosis medications, glucocorticoid, retinoid receptor modulators, steroidogenesis inhibitors, kinase inhibitors, and different types of herbal compounds such as ginkgolide A and B, hyperforin, and quercetin. Moreover, dichlorodiphenyltrichloroethane and endrin, which are organochlorine pesticides, as well as ethanol have been associated with CYP3A4 induction.[16] The induction of CYP3A4 by different exogenous substances, which upregulate gene transcription by binding to the pregnan X receptor or constitutive androstane receptor, is an important factor regulating its expression.[17] The activity and expression level of the CYP3A4 enzyme has also been associated with environmental factors such as diet and xenobiotic exposure. Because CYP3A4 is widely distributed throughout the intestinal mucosa, CYP3A4 enzyme levels are affected by fasting symptoms and are increased during starvation. There is a gender-specific difference in CYP3A4 induction in humans. Studies have shown that women are more likely to have a higher CYP3A4 metabolism rate.[18]

Among the factors mentioned above, DM can modulate CYP450 enzyme levels, drug metabolism, and drug response. Moreover, patients with DM often require pharmaceutical therapy more frequently than healthy individuals. For these two reasons, it is important to understand how DM affects the biotransformation of endogenous and exogenous compounds.[19,20] The effects of both types of diabetes on CYP450 enzyme expression and activity have been shown in different human samples, experimental animal models, and cell lines. A number of xenobiotic-metabolizing enzymes are affected by DM, including CYP1A1, CYP1B1, CYP2B1, CYP2B4, CYP2C6, CYP2C11, CYP2C23, CYP2E1, CYP3A1, CYP3A4, CYP3A11, CYP3A5, CYP7A1.[21-28] However, the number of studies examining the effects of DM on CYP3A4 protein expression is limited. Researchers found that the levels of CYP3A4 proteins and catalytic activity were significantly reduced by DM.[28] T2DM is acknowledged as a chronic condition characterized by low inflammation. In patients with T2DM, certain cytokines are associated with changes in CYP450 enzyme expression levels and/or activity. There is a correlation between T2DM and increased levels of inflammatory markers, especially interleukin-6 and tumor necrosis factor-alpha. Many drug-metabolizing enzymes, particularly CYP450 enzymes in the CYP3A subfamily, are downregulated in response to higher levels of interleukin-6 and tumor necrosis factor-alpha.[29] In contrast to these findings, another study showed that both T1DM and T2DM significantly increased hepatic CYP3A expression.[30] CYP3A4 enzyme inhibition and/or induction increases the risk of undesirable drug-drug interactions and subsequent drug toxicity. Because CYP3A4 is the main and most important enzyme involved in the metabolism of more than half of the drugs prescribed and administered, it is thought that this may be the principal cause for clinical failures and withdrawal of marketed drugs.[31]

Our study aimed to understand the alteration of CYP3A4 expression under diabetic conditions. For this purpose, CYP3A4 hepatic expression was investigated in liver microsomes obtained from control, high-fat diet, STZ-induced diabetes, dapagliflozin-treated control, and dapagliflozin-treated diabetes Sprague-Dawley rats using Western blot.

MATERIALS AND METHODS

Animals and study design

Four to five-week-old male Sprague-Dawley rats (100-150 g) were obtained from Bilkent University, Genetics and Biotechnology Research Center (Ankara, Türkiye). The rats were housed with 2 or 3 rats in each cage and maintained on a 12 hours light/dark cycle at constant room temperature (22 ± 1 °C) with tap water and standard rat chow (Purina) *ad libitum*. One week after quarantine, rats were given either standard chow or a high-fat diet (35% fat; Arden Research & Experiment) during the rest of the experiment. Rats in the control group received only citrate buffer (pH: 4.5) intraperitoneally. After another 4-5 weeks, diabetes was induced, followed by a single-dose injection of STZ (25 mg/kg; *i.p.*) dissolved in citrate buffer (0.1 N; pH 4.5) in rats fed a high-fat diet. After 72 hours of STZ injection, blood glucose levels were evaluated for each rat from the tail. A second or third STZ injection was administered to animals whose blood glucose levels were <200 and <140 mg/dL. Rats were accepted as diabetic when their blood glucose level was higher than 140 mg/dL. After diabetes had been established, half of the control and diabetic group rats

were orally administered 1 mg/kg/day of dapagliflozin for 12 weeks. Dapagliflozin suspension was prepared by pulverizing Forziga® tablets (10 mg as 12.3 mg dapagliflozin propanediol monohydrate) and then dissolving them in distilled water. Among the pharmaceutically inactive ingredients in the tablets were microcrystalline cellulose, anhydrous lactose, crospovidone, silicon dioxide, and magnesium stearate. They are coated with polyvinyl alcohol, titanium dioxide, polyethylene glycol, talc, and yellow iron oxide. Thus, the animals were divided into four groups: Control (n= 10), diabetic rats (n= 6), control rats treated with dapagliflozin (n= 10), and diabetic rats treated with dapagliflozin (n= 8). All animal procedures were performed in accordance with the guidelines of the Ankara University Animal Care and Use Committee (14.03.2018/2018-6-45).

Preparation of microsomes

Twelve weeks after treatment, rats were sacrificed under anesthesia. Liver tissue was rapidly excised, weighed, and preserved at -80 ˚C until use. Liver tissues (approximately 1.5 g) were homogenized in a Potter Elvehjem homogenizer using 1.15% potassium chloride (*w/v*) (Sigma-Aldrich) at 3000 rpm in an ice-cold bath. The homogenate was then centrifuged at 11,000 g for 25 min. The supernatant fractions were centrifuged again at 108,000 g for 60 min. After ultracentrifugation, microsomal pellets were resuspended in 20% glycerol (Sigma-Aldrich), and microsomal fractions were stored at -80 ˚C.

Western blotting

Protein levels of CYP3A4 were assessed by western blotting. First, the total protein content was measured using the BCA Protein Assay Kit (Pierce). Samples were heated with sample buffer (Sigma-Aldrich) at 70 ˚C for 5 minutes to denature the protein. 30 µg of samples were loaded onto a 10% SDS-PAGE gel. Proteins were separated using electrophoresis conducted at 100 V for approximately 2 hours. In the following gel electrophoresis, separated proteins were transferred onto nitrocellulose membranes (Biorad) *via* the wet transfer method at 100 V for 2 hours. After transfer, the membranes were blocked with 5% BSA (Sigma-Aldrich) in Tris-buffered saline containing Tween 20 (Sigma-Aldrich) at room temperature for 1 hour. The membranes were then incubated with primary antibody (Abcam; 1:5000 dilution) at 4 ˚C overnight. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Advansta; 1:5000 dilution) was used as the secondary antibody. Detection of specific bands was performed by chemiluminescence using an ECL reagent (Advansta). Imaging was conducted using the Li-Cor Odyssey imaging system. Beta-actin (Biolegend; 1/2000 dilution) was used as a loading control to normalize the density of each band.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 25). The Shapiro-Wilk test was used to check the normal distribution of all variables. Statistically significant differences between groups were analyzed using a one-way analysis of variance, followed by a *post*-hoc least significant difference test. The data are expressed as mean, standard error, and standard deviation. A $p < 0.05$ was considered statistically significant.

RESULTS

Blood glucose levels and body weight of the animals

Diabetic rats exhibited statistically significantly higher blood glucose levels, as compared with rats of control and treatment groups ($p < 0.05$). The body weight of the dapagliflozin-treated control group was found to be statistically significantly lower than that of the control group ($p < 0.05$). Body weights of the dapagliflozin-treated control group were lower than those of the diabetes and dapagliflozin-treated diabetes groups. However, no statistically significant difference in body weight was found between the dapagliflozin-treated control group and each of the diabetes and dapagliflozin-treated diabetes groups ($p > 0.05$). Data on blood glucose levels and body weights at the time of death are indicated in Table 1.

CYP3A4 protein levels in the livers of animals

The protein expression level of CYP3A4 in dapagliflozin-treated rats was statistically significantly decreased compared with the control rats ($p < 0.05$). In addition, the hepatic CYP3A4 protein expression level in the high-fat diet and STZ-induced diabetes group was lower than that in the control group. Still, the difference between these two groups was not statistically significant ($p > 0.05$). It was also reported that CYP3A4 protein expression levels of rats placed in dapagliflozin-treated diabetes were lower than those of the control and diabetes groups, but higher than those of the dapagliflozin-treated control group. Still, the difference between groups was not statistically significant ($p > 0.05$). Protein expression bar graphs and representative protein bands for each group are given in Figures 1 and 2.

DISCUSSION

DM is a metabolic disease characterized by insufficient benefit of organisms from carbohydrates, lipids, and proteins and hyperglycemia caused by defects in insulin secretion, insulin effect, or both. DM has been demonstrated to regulate protein expression of the CYP450 enzyme. Alterations in the expression of CYP450 enzymes are associated with changes in metabolism caused by diabetes (increased ketone bodies,

Table 1. Blood glucose levels and body weights at the time of death for each group of animals

	Control	Diabetes	Dapagliflozin-treated control	Dapagliflozin treated diabetes
Body weight (g)	430.2 ± 7.374	410.67 ± 18.204	303.3 ± 55.059*	418.75 ± 7.343
Blood glucose levels (mg/dL)	100.2 ± 2.149	233.67 ± 54.769$^{\Delta}$	98.7 ± 2.135	129 ± 12.186

Significant changes are expressed as *($p < 0.05$; One-Way ANOVA, *post*-hoc-LSD) compared with the control group, $^{\Delta}$($p < 0.05$; One-Way ANOVA, *post*-hoc-LSD) compared with the control and treatment groups, LSD: Least significant difference test

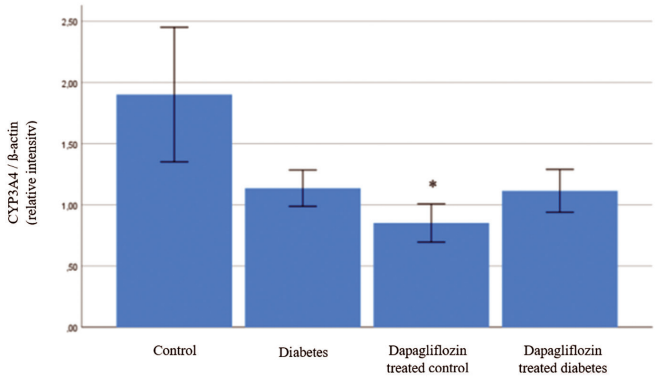

Figure 1. Hepatic CYP3A4 protein expression levels for each animal group. Significant changes are expressed as *($p < 0.05$; One-Way ANOVA, post-hoc-LSD) compared with the control group, LSD: Least significant difference test

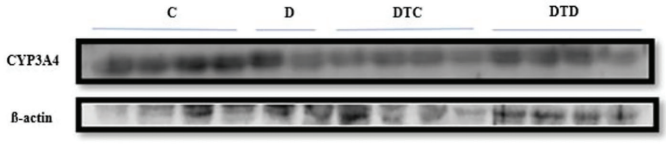

Figure 2. Western blot images of protein/loading control for each group
C: Control; D: Diabetes, DTC: Dapagliflozin-treated control, DTD: Dapagliflozin-treated diabetes

lipids, and carbohydrates) and regulation of some hormones such as insulin, and glucagon. Research has reported that the protein expression of CYP3A4, which is one of the most important enzymes in the processes of biotransformation, is affected by DM. Considering this information, our study has several implications. First, it shows that diabetes downregulates CYP3A4 protein expression in the rat liver microsome. This result was consistent with a previous study, which revealed that the CYP3A4 hepatic expression level was significantly lower in diabetic human liver microsomes. The same study also showed that there was no significant difference in CYP3A5 protein levels between diabetic and non-diabetic individuals.[28] However, little is known about how CYP3A5 protein expression is affected in rat liver microsomes under diabetic conditions. Changes caused by DM in metabolic processes, such as elevations in ketone bodies, lipids, and carbohydrates, as well as hormonal regulation, such as insulin, glucagon, leptin, and growth hormone, may influence hepatic CYP450 expression.[32] Different opinions have been suggested regarding the mechanism of decreased CYP3A4 hepatic expression in individuals with DM. These mechanisms include the effects of pro-inflammatory cytokines, non-cytokinin components, oxidative stress, and obesity. It has been reported that elevated levels of cytokines (interleukin-1ß, interleukin-6, and tumor necrosis factor alpha) contribute to a decrease in CYP3A4 enzyme expression in individuals with DM.[28] In addition, another study suggested that the protein level and activity of CYP3A4 were reduced in liver microsomes of DM and non-alcoholic fatty liver disease patients. Contrary to these results, it was demonstrated that the induction activity and upregulated protein level of CYP3A4 were found in HepG2 cells incubated with serum from rats developing diabetes with STZ. Based on the results of the study, AMP-activated protein kinase, protein kinase C (PKC), and nuclear factor kappa B pathways were most likely involved in oleic acid-induced CYP3A4 activity, whereas PKC might be involved in palmitic acid-induced activity.[33] Similar to the results of the previous study, markedly increased hepatic CYP3A protein levels were determined in both STZ-induced T1DM and db/db T2DM mice. Although there are differences in their pathophysiology, these two diseases seem to have the same modulating effect on CYP3A expression.[30] When all these results are considered together, it seems that CYP3A4 protein expression and activity are modulated differently by DM in human samples, diabetic animal models, and cell cultures. A disruption of CYP3A4 protein expression and activity associated with DM may alter the xenobiotic elimination half-life and bioavailability, efficacy, and safety of CYP3A4 substrates. A second important implication of our study is that the hepatic CYP3A4 protein expression level of dapagliflozin-treated control rats was found to be significantly lower than that of the control group. Based on this result, the use of dapagliflozin is most likely to inhibit hepatic CYP3A4 protein expression. Therefore, drugs that are CYP3A4 substrates, such as acetaminophen, lovastatin, diltiazem, and vardenafil, should be used with caution in patients with DM using dapagliflozin.[34] According to rat, dog, monkey, and human liver microsomal studies, dapagliflozin undergoes oxidative metabolism. Various human CYP450 enzymes metabolized dapagliflozin *in vitro*, and the highest metabolism was attributed to the enzymes CYP2D6, CYP1A2, CYP3A4, CYP2C9, CYP1A2, CYP3A5, and CYP2E1 in the order of highest to lowest.[35] According to this information and the results obtained from our study, inhibition of the hepatic expression of CYP3A4, which is involved in the metabolism of dapagliflozin, by dapagliflozin may also cause a decrease in the biotransformation of the aforementioned drug. In a study in which STZ-induced T1DM rats, it was found that combining dapagliflozin with a low dose of insulin stabilizes CYP1A, CYP2D, CYP2E, and CYP3A activities.[36] In addition, blood glucose levels and body weights at the time of death were measured for each group of animals. Blood glucose levels in diabetic rats were significantly higher than those in control and dapagliflozin-treated control rats. Accordingly, it was demonstrated that the STZ-induced DM model was confirmed. Body weights were found to be statistically significantly lower in the dapagliflozin-treated control group than in the control group. The mechanism of action of dapagliflozin is glucose excretion, suggesting that it may have decreased body weight in the treated groups.

Our study reported the effect of DM on CYP3A4 expression in rat liver, but the impact of DM on CYP3A4 enzymatic activity and/or mRNA level is still needed to be studied. In addition, the mechanism behind this decrease in CYP3A4 expression has yet to be clarified in our study. Further research is required to shed light on these issues. To the best of our knowledge, this study is the first to investigate the effect of dapagliflozin on CYP3A4 expression. Therefore, it is essential to support our study by designing studies in which the number of animals

is increased and the relationship between dapagliflozin and CYP3A4 expression in different species of experimental animals is evaluated. Our study was performed exclusively on male rats. However, the impact of the gender factor should also be assessed in other studies involving female rats. Moreover, the effect of STZ administration on CYP3A4 expression in early and late applications can be evaluated. When the studies are examined, it is seen that the antidiabetic agent whose effect on CYP450 expression and activity is investigated is generally insulin. Therefore, it is important to design studies that examine the effect of antidiabetic agents other than insulin on CYP450 enzyme expression. Research on the expression of other CYP450 enzymes involved in dapagliflozin metabolism in addition to CYP3A4 in diabetic conditions will also be complementary to our study.

CONCLUSION

The findings of our study showed that hepatic CYP3A4 protein expression levels in the control group treated with dapagliflozin were significantly decreased compared with those in the control group. Besides, we reported that hepatic CYP3A4 protein expression levels were decreased in dapagliflozin-treated diabetic Sprague-Dawley rats compared with those in both control and diabetic group rats. But, the difference between groups was not statistically significant. According to these two results, the use of dapagliflozin inhibited hepatic CYP3A4 protein expression. This result was consistent with the data of two previous studies. Another important conclusion of our study was regarding the physical and biochemical characteristics of rats. Blood glucose levels of both control and dapagliflozin-treated control groups were lower than those of the diabetes and dapagliglozin-treated diabetes groups, but this difference was not statistically significant. On the other hand, blood glucose levels of diabetic rats were found to be significantly higher than those of the control and administration groups. Accordingly, the control of the DM model induced by STZ and a high-fat diet was provided. Body weights of the dapagliflozin-treated control group at the time of death were lower than those of the diabetes and dapagliglozin-treated diabetes groups, but this difference was not statistically significant. Moreover, body weights at the time of death were found to be statistically significantly lower in rats of the dapagliflozin-treated control group than in rats of the control group. According to these data, a significant decrease in body weight of dapagliflozin-treated control rats was associated with the mechanism of action of dapagliflozin, leading to the excretion of urinary glucose. Finally, we showed for the first time the impact of dapagliflozin treatment on hepatic CYP3A4 protein expression levels. CYP3A4 metabolically processes many clinically used drugs, including dapagliflozin and endogenous substances. Diabetic conditions, complications related to diabetes, and antidiabetic agents are among the factors that play a role in regulating CYP3A4 protein expression. Therefore, it is necessary to design studies that examine the relationship between these factors and CYP3A4 protein expression.

Ethics

Ethics Committee Approval: All procedures used in this study were approved by the Ankara University Local Ethics Committee for Animal Experiments (2018-6-45).

Informed Consent: Not required.

Authorship Contributions

Surgical and Medical Practices: E.A.I., I.K., Concept: N.M.G., B.C.E., Design: N.M.G., B.C.E., Data Collection or Processing: N.M.G., B.C.E., Analysis or Interpretation: N.M.G., B.C.E., Literature Search: N.M.G., B.C.E., Writing: N.M.G., B.C.E.

Conflict of Interest: No conflict of interest was declared by the authors.

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