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Function and expression of renal organic anion transporters in experimental diabetes in mice

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ABSTRACT: The renal clearance of organic anions (OAs) is mediated by organic anion transporters (Oats) located at the renal proximal tubules (RPTs). Decreased elimination of OAs or anionic drugs may result in severe nephrotoxicity. Up to now, no study dealt with the consequences of diabetes mellitus on renal anion transport function. This study was conducted to examine renal Oat expression and function in diabetic mice induced by streptozotocin. Transport activity was determined by measuring the uptake of fluorescein into isolated RPTs. Diabetic mice demonstrated an increase in plasma glucose, triglyceride, and growth retardation. A decrease in fluorescein accumulation in RPTs was observed in mice only after 28 and 42 days of diabetes. There was also a concomitant reduction in the level of kidney cortex membrane Oat3, but not Oat1. These results indicate that renal anion transport function is impaired after a prolonged (four weeks or longer) diabetic condition.

KEYWORDS: renal function, diabetes mellitus, nephrotoxicity, dyslipidaemia

INTRODUCTION

Diabetic nephropathy is a major cause of morbidity and mortality in patients with diabetes mellitus (DM). At least 25% of patients with Type 1 DM and 30–40% of patients with Type 2 DM eventually develop diabetic nephropathy, which may progress to end-stage renal disease[1]. In diabetic nephropathy, histopathological changes in the proximal tubules are found during the development of progressive diabetic kidney disease. In addition, the proximal tubular cells contain organic cation (OCTs) and organic anion transporters (OATs), which regulate transepithelial transport of both organic cation and anion substrates[2,3].

The OAT family represents the main renal secretory pathway for organic anions (OAs) and is also involved in the distribution of OAs in the body, drug-drug interactions, and toxicity of anionic substances such as nephrotoxic drugs and uraemic toxins. Renal secretion of OAs by proximal tubular epithelial cells occurs via a tertiary active transport[4,5]. OAT1 and OAT3 have been shown to play a major role in the basolateral uptake of OAs into the renal proximal tubular cells[6]. Their expression levels appear to be much higher than those of other OAT in the kidney cortex[7]. OATs interact with various anionic compounds such as para-aminohippurate (PAH), non-steroidal anti-inflammatory drugs, metrotrexate, loop diuretics, cephalosporins, and prostaglandin E2[8,9]. Furthermore, OAT1 and OAT3 are actively involved in the tubular uptake of various therapeutic drugs. Therefore, modulators of OAT1 and OAT3 activities would have high impact on drug-drug interaction in combination therapy and would alter the pharmacokinetics of xenobiotics, particularly those excreted into the urine via the organic anion transport process.

Oral anti-diabetic agents, such as chlorpropamide and glibenclamide, are anions at a physiological pH and have potent inhibitory effects on rOat1-mediated PAH uptake[10], indicating that they are substrates of Oat1. Pharmacokinetic changes of drugs such as acetaminophen, furosemide, and methotrexate have been reported in rat model of diabetes mellitus induced by alloxan or streptozotocin treatment[11–13]. However, no information is available regarding the function of OATs under the diabetic condition.

Morphological changes to proximal tubules in a diabetic condition consist of tubular basement membrane thickening and interstitial fibrosis[14]. There is a significant reduction in tubular cation clearance associated with a down-regulation of the expression of tubular OCTs on the basolateral surface of the proximal tubule[15,16]. Although a reduction of the renal clearance of hippurate in diabetic patients has

been reported[17], there is no report to date concerning the impact of the duration of hyperglycaemia on the function and expression of renal OATs.

In this study, we examined at various stages the effect of streptozotocin-induced Type I diabetes on the accumulation of a model organic anion, fluorescein, in epithelial cells of isolated renal proximal tubules in vitro. The expression levels of Oat1 and Oat3 in renal cortex of both control and diabetic mice were determined by western blotting.

MATERIALS AND METHODS

Animals

Male ICR mice (20–30 g) were obtained from the National Animal Centre, Nakorn Pathom, Thailand, and housed in a controlled environment with food and water ad libitum. All animal experimental protocols were approved by Animal Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (9/2007). Diabetes in mice was induced by a single intraperitoneal injection of streptozotocin (STZ, 200 mg/kg body weight) (Sigma Chemical, MO, USA) in 10 mM citrate buffer pH 4.5 during the fasting state. Control mice received an equivalent dose of citrate buffer solution. Hyperglycaemia occurred seven days after STZ injection and was verified using a glucometer (Walgreens, Co., IL, USA). Mice with blood glucose exceeding 300 mg/dl were classified as diabetic mice. Mice were divided into 4 groups: (1) control, (2) 14 days of diabetes, (3) 28 days of diabetes, and (4) 42 days of diabetes.

Measurement of serum triglyceride (TG)

Serum TG concentration was measured according to the method of Bucolo and David[18], using Triglyceride-GPO kit (Biotechnical, Bangkok). In brief, 5 µl of serum were added and mixed with 500 µl of enzyme solution provided. Then the sample was incubated at 37 °C for ten minutes and absorbance at 505 nm was measured using a spectrophotometer (UV-1700, Shimudzu Corporation, Kyoto, Japan).

Measurement of fluorescein transport into isolated renal proximal tubules

Renal proximal tubules (RPTs) were isolated as previously described[6]. In brief, animals were sacrificed under isofluorane anaesthesia and blood samples collected by cardiac puncture. Kidneys were removed, sliced and transferred to a plastic Petri dish. S2 segments of proximal tubules were dissected without the aid of enzymatic agents in buffer solution (in mM: 110 NaCl, 25 $NaHCO_3$, 5 KCl, 2 NaH_2PO_4, 1 $MgSO_4$, 1.8 $CaCl_2$, 10 Na-acetate, 8.3 D-glucose, 5 l-alanine, 0.9 glycine, 1.5 lactate, 1 malate, and 1 sodium citrate). All dissections were performed at 4 °C and aerated continuously with 95% O_2-5% CO_2 gas mixture to maintain the pH at 7.4.

RPTs were incubated in 1 µM fluorescein (FL) for 5 min at room temperature (25 °C). Subsequently, RPTs were mounted on a confocal laser-scanning microscope (Digital Eclipse C1si, Nikon Instrument Inc., NY, USA), staged and viewed through a 40× objective (numerical aperture 1.2) using 488-nm laser line (argon ion laser) (Spectra-Physics Lasers, CA, USA) and a 515-nm long-pass emission filter. Low laser intensity was used to avoid photo bleaching. The concentration of FL uptake into the epithelial cell of RPTs was analysed using the computer program EZ-C1 viewer Version 3 (Nikon Instrument Inc., NY, USA). About 10–15 fields were analysed for each tissue sample. Background fluorescence was subtracted prior to the calculation of average pixel intensity of each field. Data shown are representatives of three to four experiments for each group.

Western blot analysis

The renal cortex was homogenized and lysed in buffer containing mammalian tissue lysis buffer and protease inhibitor cocktail (Sigma Chemical, MO, USA). The lysate was centrifuged at 5000g for 15 min and the supernatant collected was designated as total protein. To obtain kidney-membrane protein, total protein was centrifuged at 100 000g for 2 h and the pellet was suspended in a small volume of buffer A. The protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, PA, USA). Total protein and membrane protein (50 µg) were separated by 12.5% SDS-polyacrylamide gel-electrophoresis, and then transferred onto a polyvinylidene difluoride membrane (Amersham Biosiences, NJ, USA) by electroblotting. After blocking with 5% skimmed milk, the membrane was incubated with rabbit anti-OAT1 (Alpha Diagnostic International, TX, USA) or anti-OAT3 (Cosmo Bio, Tokyo) polyclonal primary antibody, followed by goat anti-rabbit horseradish peroxidase-conjugated ImmunoPure secondary immunoglobulin G (IgG) (Bio-Rad Laboratory, PA, USA). Bound IgG was detected using an ECL detection kit (Amersham Biosiences, NJ, USA). The densitometric analysis was performed using SCION IMAGE for Windows.

Statistical analysis

Data are reported as mean ± SE. The level of significance for difference between means was determined

Table 1 Effects of diabetes on plasma glucose and serum triglyceride.

	Control	DM-14	DM-28	DM-42
Glucose (mg%)	105 ± 9 (n=12)	$554 \pm 24^*$ (n=8)	$598 \pm 48^*$ (n=10)	$541 \pm 60^*$ (n=4)
Triglyceride (mg%)	118 ± 19 (n=3)	$266 \pm 17^*$ (n=3)	$245 \pm 60^*$ (n=3)	$178 \pm 2^*$ (n=3)

Data are presented as mean $\pm$ SEM.
* $p < 0.001$, compared with control.

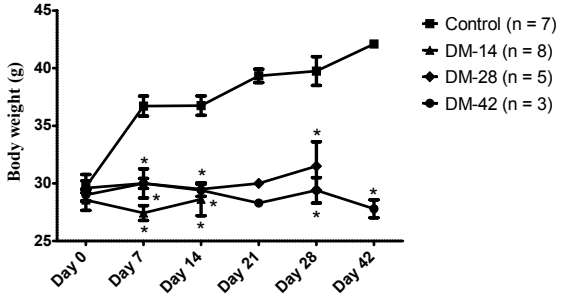

Fig. 1 Body weight of control and experimentally-induced diabetic mice. Mice were weighed on day 0, 7, 14, 21, 28, and 42 days of diabetic conditions. Values are means $\pm$ S.E.M. * Significantly different from control in each week, $P < 0.05$.

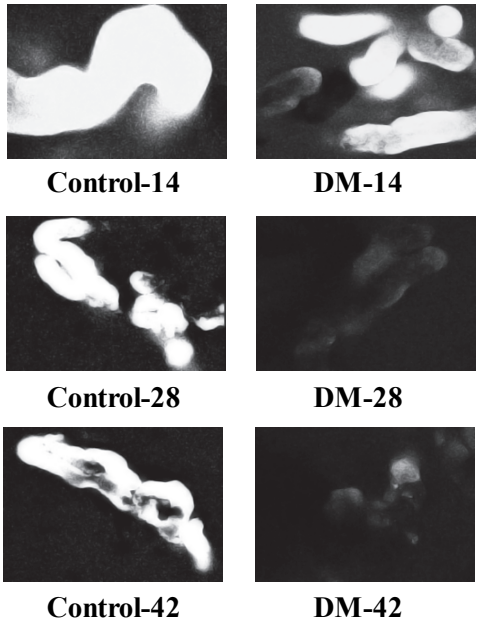

Fig. 2 Confocal image of fluorescein accumulation in isolated renal proximal tubules of control and experimentally-induced diabetic mice. FL (1 μM) uptake into RPTs was imaged using confocal microscope after 5-min incubation at room temperature (25 °C). The images chosen for display are representative of multiple replicates.

using Student's t-test for paired values, with statistical significance when $P < 0.05$.

RESULTS

Characterization of experimentally-induced diabetes condition

STZ treatment in mice led to a significant increase in fasting blood glucose in all experimental groups (Table 1). Hyperlipidaemia, a hallmark of streptozotocin-induced diabetes, was manifested by a significant increase in plasma triglyceride in all diabetic groups. In addition, diabetic mice had a marked lower body weight compared to that of control (Fig. 1).

Transport function and expression of renal Oat

FL accumulation using confocal microscopy detection method into epithelial cells of isolated mice RPT in control and diabetic conditions detection are shown in Fig. 2. After 14 days of diabetes, FL accumulation was not significantly different from that of the control. However, a significant decline in FL accumulation was observed after 28 and 42 days of diabetic conditions (Fig. 3), reflecting impairment of organic anion transport due to a decrease in membrane Oat3, but not membrane Oat1 (Fig. 4). Oat1 and Oat3 protein levels in total protein were not significantly different among all experimental diabetic and control animals.

DISCUSSION

After 28 days following the induction of experimental diabetes by streptozotocin in mice, there was a significant functional impairment of renal organic anion transport system in isolated renal proximal tubules. Measurement of anion transport was based on accumulation of FL in renal proximal tubular cells, as FL is a substrate for both Oat1 [19] and Oat3 [20]. Early stages in the development of diabetes (14 days) showed no effects in FL accumulation, indicating normal function of renal Oat1 and Oat3, although similar levels of hyperglycaemia were present. Therefore, prolonged exposure to hyperglycaemia is required to produce nephrotoxicity that affects the pharmacokinetic of drugs transported via OAT system.

The mechanism by which Oat function is reduced in experimental diabetes is by a decrease in renal proximal tubular basolateral membrane expression

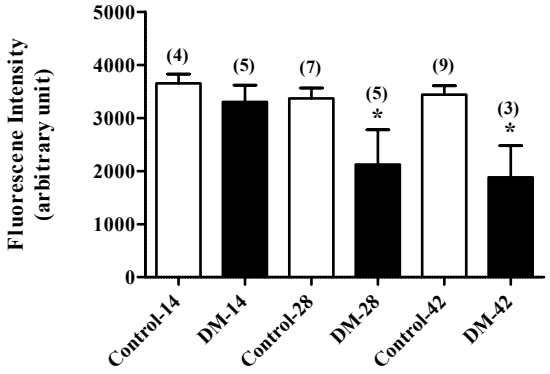

Fig. 3 Fluorescein accumulation in isolated renal proximal tubules of control and experimentally-induced diabetic mice. Values are means ± S.E.M. * Significantly different from control group, $P < 0.01$.

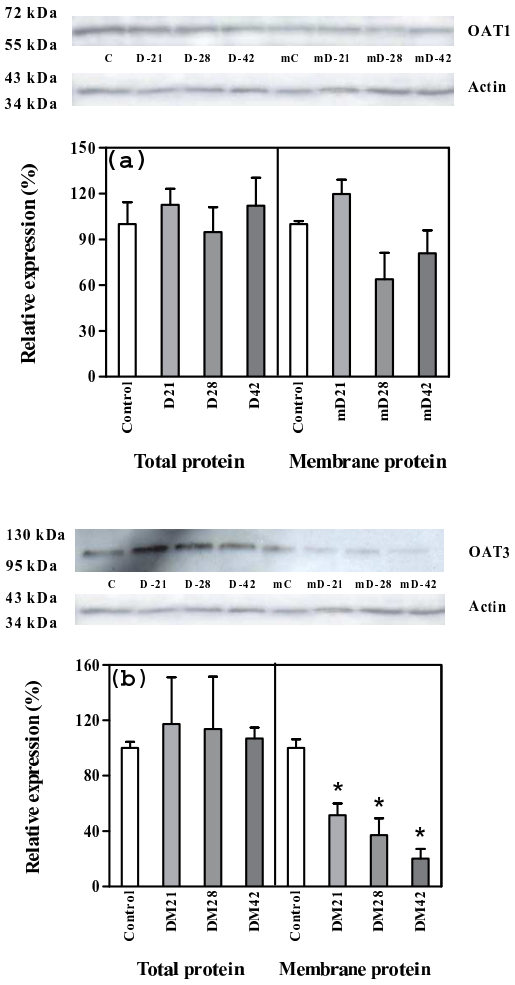

Fig. 4 Protein expression of (a) Oat1 and (b) Oat3 of control and experimentally-induced diabetic mice. Total protein and membrane proteins were subjected to a Western blot analysis as described in Materials and Methods. Data are means ± S.E.M (n=4 per group). C, total protein of control; D14, D21, D28, D42, total protein on day 14, 21, 28, and 42 of diabetic conditions, respectively; mC; membrane protein of control; mD21, mD28, mD42, membrane protein on day 21, 28, and 42 diabetic conditions. * Significantly different from control group, $P < 0.01$.

and/or in activity of Oat1 and Oat3. A decreased level of renal Oat3, but not Oat1, was observed after four weeks of diabetic condition, in agreement with earlier observations of a significant reduction in Oct expression after 4 weeks of diabetes in rats[16].

Several pathways are thought to be involved in the pathogenesis of hyperglycaemia-induced complications in diabetes. Long-term hyperglycaemia is closely associated with an increased risk of developing chronic renal failure[21]. Hyperglycaemia and dyslipidaemia which were observed in this study could give rise the oxidative stress and formation of advanced glycation and lipoxidation end products[22]. Excessive production of reactive oxygen species (ROS) has been suggested to be a common product of all pathways leading to diabetes[23]. Both Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetic patients have been described to be under enhanced oxidative stress leading to overproduction of ROS[24].

Recently, it has also been reported that mitochondria from diabetic rat kidney have an increased expression of the uncoupling protein-2 (UCP-2)[21] which is involved in mitochondria production of ROS. This phenomenon was previously reported in streptozotocin-induced diabetic rat[25,26]. Alterations in transporter function may occur via oxidation of critical amino acid residues or by alteration (formation of lipid hydroperoxides) of the membrane lipid environment of the transporter[27]. An increase in the level of lipid peroxidation by high glucose level has been shown to inhibit Na^+/glucose cotransporter activity of renal proximal tubular cells[26]. Thus oxidative stress as a consequence of hyperglycaemia may also result in dysfunction of Oats in renal proximal tubule in this study.

The level of membrane Oats is determined by membrane trafficking processes. SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) proteins, SNAP 25 (25 kDa synaptosome-associated protein) and syntaxin 4 form a complex with SNARE-associated protein, snapin,

to regulate insertion of urea transporter at inner medullary collecting duct[28]. ROS, such as H_2O_2, reacts with SNAP25 on the t-SNARE complex and induces impairment of the trafficking process[29]. A similar process may account for the reduced amount of Oat3 in the kidney cortex membrane following long term exposure to a hyperglycaemic condition. Why Oat1 is not affected still remains unclear.

In conclusion, in mice, prolonged hyperglycaemia of more than one month resulted in the impairment of renal Oat3 function due to reduced transporter expression at the renal proximal tubular basolateral membrane. Anion transport into the renal proximal tubules was conveniently tracked using a confocal microscope to measure the uptake of fluorescein. Our data indicated a significant role of renal Oat function as diabetic condition progressed.

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