様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　３ 年　 ６ 月 １６ 日現在

機関番号：８４４０４
研究種目：若手研究
研究期間：2019～2020
課題番号：１９Ｋ１７７５９

研究課題名（和文）A new therapeutic approach to diabetic nephropathy: targeting renal hypoxia by inhibition of sodium-glucose co-transport in the proximal tubule

研究課題名（英文）A new therapeutic approach to diabetic nephropathy: targeting renal hypoxia by inhibition of sodium-glucose co-transport in the proximal tubule

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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：2型糖尿病ラットの腎皮質は糖尿病初期において低酸素状態にあったが、組織低酸素状態は高血糖の重症度に応じて悪化はしなかった。ダパグリフロジン投与は腎皮質組織酸素分圧を87 ± 18%（9週齢）および42 ± 8%（24週齢）と増加させたことから、糖尿病初期の治療が効果的であることを示唆している。組織低酸素の軽減は腎酸素消費量の減少に依存し、主に糖再取り込みの減少に起因していた。SGLT2阻害薬の慢性投与によりHIF-1a発現レベルが有意に低下したことから、腎低酸素状態の改善が維持されていたと考えられた。また、慢性投与群ではEDHsによる血管拡張作用が改善し、腎血管内皮機能障害の改善が示唆された。

研究成果の学術的意義や社会的意義
We showed that treatment with SGLT2 inhibitors may prevent the glucotoxicity on the kidney as excessive glucose in the circulation is excreted into the urine. In doing so, it relieves the kidney from hypoxia, an effect commonly described to contribute to the severity of diabetic nephropathy.

研究成果の概要（英文）：The renal cortex of rats with type 2 diabetes is hypoxic in early diabetes but does not worsen with severity of hyperglycemia. Following acute dapagliflozin treatment, cortical PO2 increased by 87.1 ± 17.5% in the 9 wk old rats and 42.4 ± 8.4% in the 24 wk old rats. This may indicate that the effects of preventing hypoxia maybe greater when treated in early diabetes. The alleviation of tissue hypoxia is due to the significantly greater reductions in renal oxygen consumption, presumably due to the reduction in glucose reabsorption, than alterations in oxygen delivered to the kidney. The alleviation of cortical hypoxia appeared to be sustained with long term treatment of SGLT2 inhibitors as levels of HIF-1a was significantly less than non-treated rats. Further, chronic treatment improved endothelial dysfunction, evident by the observations of well-perfused vessels during vasoconstrictor challenges. Vasodilatory effects of EDHs on the vasculature was restored following treatment.

研究分野：Renal physiology

キーワード：Diabetic nephropathy　SGLT2 inhibitor　renal hypoxia

１．研究開始当初の背景

Chronic kidney disease (CKD) is an ever-growing concern and CKD associated with diabetes accounted for ~ 35% of new cases of end-stage renal failure. Furthermore, diabetic nephropathy is predicted to increase in proportion with the prevalence of diabetes and will continue to plague developing and developed countries alike. Thus, there is an urgent need for development of drugs that reduce the mortality, morbidity and disability associated with diabetic nephropathy. Currently, clinical trials with a new class of drugs, insulin-independent sodium-glucose co-transporter 2 (SGLT2) inhibitors not only showed significant improvements in hyperglycemia and thus renal damage, but also reduced the risk of adverse cardiovascular events. Despite the promising results from clinical trials, pre-clinical studies into the mechanisms by which SGLT2 inhibitors prevent/alleviate diabetic nephropathy are lacking. It was suggested that tissue hypoxia, due in part to excessive and inefficient utilization of oxygen, is a major contributing factor in the pathogenesis of diabetic nephropathy and that SGLT2 inhibitors combat diabetic nephropathy by preventing hypoxia-induced damage to the kidney.

２．研究の目的

SGLT2 inhibitors, the newest class of anti-diabetic drug, currently being tested in clinical trials for patients with type 2 diabetes in many countries, inhibit the reabsorption of glucose and sodium in the proximal tubules of the kidney. Thus, SGLT2 inhibitors exert potent anti-diabetic effects by increasing urinary excretion of glucose. Diabetic nephropathy is characterized by glomerular hyperfiltration, and thus glomerular hypertrophy. Under euglycemic conditions, SGLT2 activity accounts for ~97% of glucose reabsorbed in the kidney and back into the bloodstream. The inhibition of reabsorption of glucose and sodium reabsorption at SGLT2 co-transporters in the proximal tubule not only increases urinary excretion of glucose, but also decreases reabsorption of sodium at the NHE and the Na/K-ATPase pump. These dual consequences of SGLT2 inhibition may abolish glomerular hyperfiltration and thus prevention of the subsequent development of CKD. Therefore, our aims and hypothesis are as below:

Study 1: [Hypothesis]: Chronic treatment with dapagliflozin alleviates hyperglycemia and blunts glomerular hyperfiltration, and thus prevents glucose-induced renal damage in diabetic rats. [Aim]: To characterize the effects of dapagliflozin on glucose control in rats with type 2 diabetes.

Study 2:　Microvessels supply local tissue oxygen demands and are important controllers of regional perfusion. Endothelial dysfunction has been posited to be an important factor driving the pathogenesis of diabetic nephropathy, largely through impaired eNOS activity and thus reduced nitric oxide bioavailability. We assessed the state of endothelial function and the ability of microvessels to maintain perfusion in the presence of vasoconstriction induced by inhibition of nitric oxide synthase and prostaglandin blockade. [Hypothesis]: Diabetic rats chronically treated with dapagliflozin improves renal endothelial function and the microvessels are better able to maintain adequate perfusion in response to the inhibition of nitric oxide synthase and prostaglandin blockade. [Aim]: To determine the impact of dapagliflozin on vasodilator function in the renal vasculature

Study 3: The ‘chronic hypoxia hypothesis’ proposes that kidney disease is partly driven by a vicious cycle of tissue hypoxia, tubular atrophy, hypertrophy of remaining nephrons, fibrosis and vascular rarefaction, leading to sustained renal tissue injury. Cellular hypoxia can initiate downstream signaling events, through stabilization of hypoxia-inducible factors (HIFs), leading to apoptosis, epithelial to mesenchymal transition and mitochondrial production of superoxide. [Hypothesis]: Cellular hypoxia is predominant in the renal cortex of type 2 diabetics and chronic dapagliflozin treatment improves renal damage by alleviating cortical hypoxia. [Aim]: To generate preliminary data regarding the effects of dapagliflozin on renal oxygenation in rats with type 2 diabetes.

Study 4: There is growing evidence that tissue hypoxia is a major driver of diabetic nephropathy. Renal tissue hypoxia and impaired vasodilatation has been observed in many forms of experimental diabetes using various methods. Further to that, in the absence of confounding effects of other injury, tissue hypoxia *per se* leads to nephropathy. Thus, it appears that tissue hypoxia is a critical mediator of CKD but is often overlooked. I propose that SGLT2 inhibition is an attractive therapeutic target for CKD because amelioration of hypoxia, either by preventing

the inefficient utilization of oxygen by the kidney or increasing the oxygen delivered to tissues, will abolish hypoxia-induced damage and thus retard the progress of CKD. [Hypothesis]: Dapagliflozin ameliorates hypoxia in early diabetes by blunting hyperfiltration, and in more advanced stages, by enhancing the metabolic efficiency of sodium reabsorption. [Aim]: To quantify the impact of dapagliflozin on the determinants of renal oxygenation (oxygen delivery and oxygen consumption) and the factors that govern them (renal blood flow, blood hemoglobin content, GFR, sodium reabsorption, and the metabolic efficiency of sodium reabsorption) in early and advanced diabetes.

３．研究の方法

Study 1-3: Zucker fatty diabetic (ZFDM), a rodent model of type 2 diabetes, and control rats were studied at 8 weeks of age. They were given daily oral gavage of 1 mg/kg dapagliflozin or its vehicle for up to 22 weeks of age. Renal excretory function was assessed before the commencement of treatment protocol and after every 4 weeks up until 22 weeks of age. Rats were placed in metabolic cages with free access to food and water for 24 h. The next day, a 0.2 ml blood sample was taken from the tail vein, under brief anesthesia so that indices of renal injury (e.g. serum creatinine, albuminuria and proteinuria) can be determined. Glomerular filtration rate (GFR) in these rats was assessed via transcutaneous clearance of FITC-sinistrin while they were awake and freely moving. At 22 weeks of age, rats were fasted for 24 h so that fasting blood glucose and HbA1c (hemoglobin A1c) levels can be determined. Rats were then anesthetized, and vasodilator function of the renal microvasculature was assessed via X-ray microangiography. An equilibration period of 30 min ensued once surgical preparation for renal microangiography was completed. A bolus (~ 250 µl) of an iodinated contrast agent delivered into the kidney through the renal artery allowed for the acquisition of an X-ray image of the renal microvessels. We then conducted successive imaging during infusion of acetylcholine (ACh) and sodium nitroprusside (SNP) through the jugular vein which facilitated the assessment of endothelium dependent and independent dilation respectively. After which, rats were given successive boluses of L-NAME, a nitric oxide synthase inhibitor and indomethacin, a cyclooxygenase inhibitor, to assess the vasodilatory function of the microvasculature independent of nitric oxide and prostaglandins respectively. These inhibitors were given under the conditions of SNP clamp which allowed for us to titrate the blood pressure of the rats to baseline levels. Lastly, we assessed the ability of endothelium hyperpolarizing factors to maintain microvascular perfusion when SNP infusion ceased and an infusion of ACh commenced while still under the effects of L-NAME and indomethacin.

Study 4: ZFDM (n=10) and control rats (n=10) will be studied at 8 or 22 weeks of age. This will allow me to determine the acute effects of SGLT2 inhibition on alleviating hypoxia in early and advanced diabetic nephropathy. We will use methods well established in our laboratory [Ow *et al*, *AJP Regul Integr Comp Physiol* 2014, 307: R1207-R1215, Ow *et al*, *AJP Renal* 2018, 315: F1358-1369]. Briefly, each rat will be anesthetized with thiobutabarbital sodium and artificially ventilated. Catheters will be placed in the carotid artery (for measurement of arterial pressure and collection of arterial blood) and left renal vein (for collection of renal venous blood and thus the determination of renal oxygen consumption). The left kidney will be placed in a stable cup and a transit-time ultrasound flowprobe will be placed around the renal artery for measurement of total renal blood flow. Laser Doppler flow probes will be placed in the medulla and cortex for measurement of regional kidney perfusion. To allow for the measurement of GFR and sodium reabsorption by clearance methods, [^{3}H]-inulin will be infused at a constant rate and urine will be collected from a bladder catheter. A Clarke electrode (10-50 µm tip) will be advanced, from the surface of the cortex, at 1 mm intervals, to map the profile of tissue PO_2 from the cortex to inner medulla. Arterial and renal venous blood samples will then be collected for oximetry and to allow measurement of GFR and sodium reabsorption. These measurements will be obtained during a 60 min control period, and then again during the period 30-90 min after acute administration of dapagliflozin (1 mg/kg, i.v.)

４．研究成果

Study 1: As expected, blood glucose was consistently less in rats fed dapagliflozin than in vehicle-treated diabetic rats across the 12 week treatment period. Consequently, hemoglobin A1c (HbA1c) and fasting blood glucose at 22 wks of age were significantly lesser in treated diabetic rats. (Fig 1).

[Renal function & indices of renal damage]: Time taken for the clearance of FITC-sinistrin increased across the study period in vehicle treated diabetic rats while it was

relatively well-maintained for diabetic rats fed dapagliflozin. There was a tendency for dapagliflozin to ameliorate the decline of GFR in diabetic rats, although this apparent effect was not statistically significant. Dapagliflozin did not appear to improve blood urea nitrogen, urinary creatinine levels nor albuminuria but tended to improve urinary albumin to creatinine ratio. Treatment with dapagliflozin alleviated polyuria in diabetic rats. Dapagliflozin did not appear to have an impact on urine osmolarity or sodium excretion in diabetic rats, but significantly increased sodium excretion in control rats fed dapagliflozin.

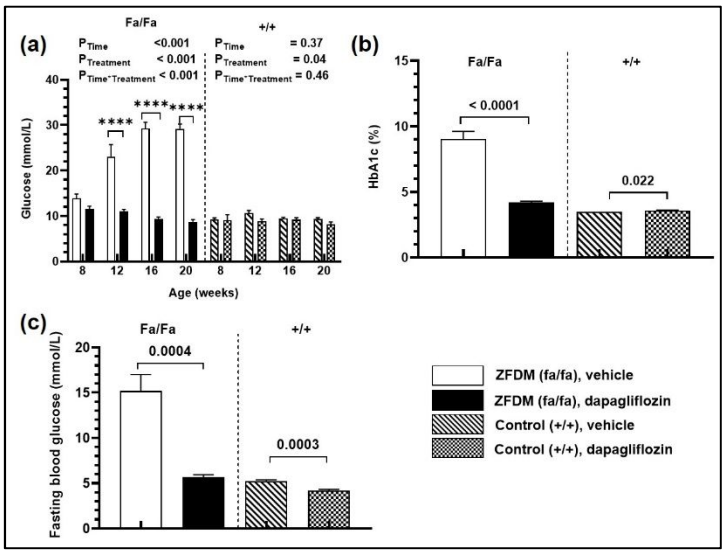

Fig 1: Blood glucose levels (a) across the 12 week treatment period. At the end of the treatment protocol, rats were fasted for 12 h and the blood glucose (b) and HbA1c (c) levels were determined prior to renal angiography. Data are presented as mean ± SEM. P_{time}, $P_{treatment}$ and $P_{time*treatment}$ are the outcomes of 2-way ANOVA and dichotomous comparisons were made by 2-tailed unpaired students' t-test. $P \leq 0.05$ was considered statistically significant.

Study 2: After 12 weeks of daily oral gavage with dapagliflozin, the responses (vessel diameter) of renal microvessels to vasodilators, ACh and SNP, after 12 weeks treatment were greater than in vehicle fed diabetic rats (Fig 2). The renal microvessels of diabetic rats appeared to undergo relative constriction in response to nitric oxide synthase and cyclooxygenase blockade even under the effects of SNP clamp. In contrast, diabetic rats treated with dapagliflozin appeared to be relatively well-perfused after NOS and COX blockade. This suggests that dapagliflozin may improve endothelial dysfunction commonly associated with diabetic nephropathy. Following NOS and COX blockade, ACh was infused in rats to determine the status of vasodilatory function mediated by endothelium dependent hyperpolarizing factors (EDHF). The renal microvessels in diabetic rats did not appear to be dilated after infusion of ACh, suggesting that vasodilatory effects of EDHF on the vasculature is diminished in diabetic rats. Treatment of dapagliflozin improved this effect in that the renal microvessels were dilated even when NOS and COX production was blocked/inhibited (Fig 3).

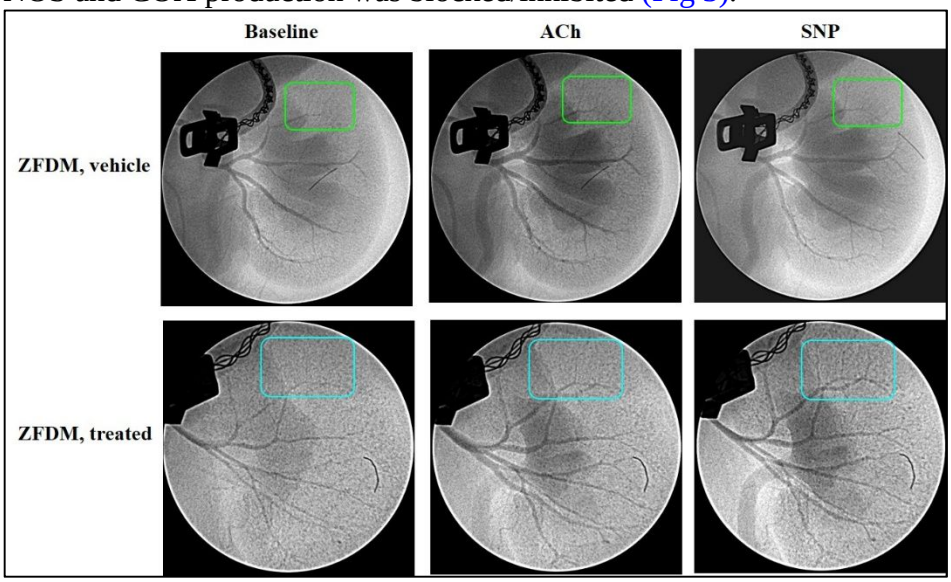

Fig 2: Renal microangiography. X-ray images of the rat microvasculature was taken during the baseline period and after successive intravenous infusions of acetylcholine (ACh) and sodium nitroprusside (SNP). Note the relative improved response to both vasodilators in dapagaliflozin fed rats.

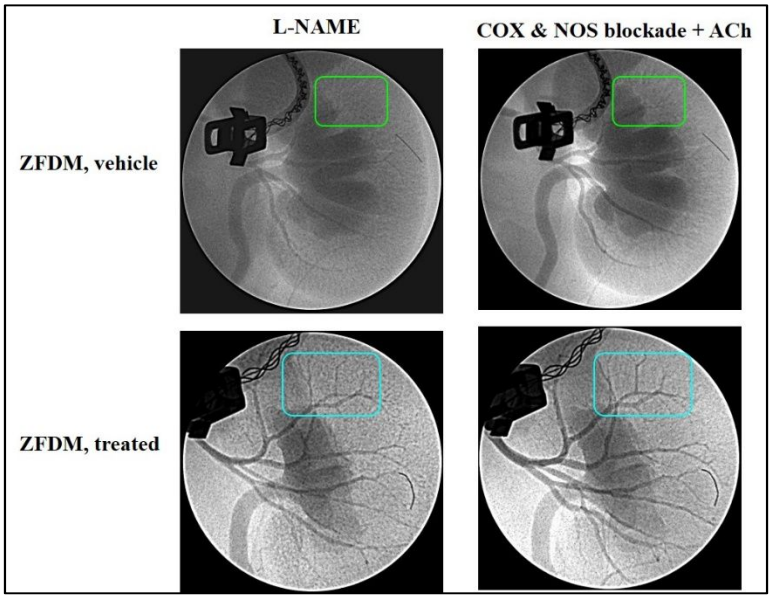

Fig 3: Renal microangiography. X-ray images of the rat microvasculature taken after a bolus adminsitration of L-NAME and during an intravenous infusion of acetylcholine (ACh) while under the effects of cyclogenase (COX) and nitric oxide synthase (NOS) blockade so as to determine the contributions of endothelium dependent hyperpolarizing factors in maintaining renal perfusion. Note the relative dilation of the vasculature after infusion of acetylcholine in dapagliflozin treated rats compared to vehicle treated rats.

Study 3: Western blot analysis showed that the vehicle treated diabetic rats had significantly elevated renal cortical HIF-1α compared to control rats, suggesting that the cortex is hypoxic in diabetic rats. HIF-1α levels were significantly less in diabetic rats fed dapagliflozin than in vehicle fed diabetic rats, suggesting that dapagliflozin alleviates cortical hypoxia. There was significantly more SGLT1 protein in the renal cortex of diabetic rats than in control rats. Treatment with dapagliflozin resulted in lesser expression of SGLT1 in the cortex of diabetic rats. The expression of both SGLT1 and HIF-1α proteins was apparently unaffected by both diabetic nephropathy and dapagliflozin treatment.

Study 4: The cortical tissue PO_2 of 9 week old rats was 27.1 ± 3.2 mmHg and that of 24 week old rats was 24.0 ± 2.1 mmHg. These values are relatively lower than the average, ~50-60 mmHg, of the cortex in a healthy rat. Following administration of dapagliflozin, cortical tissue PO_2 significantly increased by 87.1 ± 17.5% in the 9 week old rats and 42.4 ± 8.4% in the 24 week old rats. In contrast, the increase in medullary tissue PO_2 was relatively modest in the 9 and 24 week old rats respectively. Renal oxygen delivery tended to be lower in 24 week old rats prior to treatment but renal oxygen consumption was not significantly different between groups at baseline. Following dapagliflozin treatment, renal oxygen delivery was not significantly altered but renal oxygen consumption was significantly decreased in both groups of rats. Acute administration of dapagliflozin significantly decreased mean arterial pressure and heart rate by 14.6 ± 1.5 and 5.5 ± 0.8% in 9 week and by 8.2 ± 2.0 and 6.4 ± 0.7% in 24 week old rats. Renal vascular resistance decreased by 31.0 ± 5.3% following treatment in 9 week old rats but this effect was considerably lesser in 24 week old rats. Severity of hyperglycemia increases with age but the magnitude of decrease in plasma glucose in response to dapagliflozin was statistically indistinguishable between both groups suggestive of a ceiling effect. The severity of polyuria worsened with age and in response to treatment, urine flow significantly increased by 261.9 ± 75.4 and 53.4 ± 19.2% in 9 week and 24 week old rats respectively. Urinary glucose excretion tended to be greater at baseline in 24 week old rats. Following dapagliflozin treatment, urinary glucose excretion significantly increased although this effect was considerably greater in 9 than in 24 week old rats. Urinary sodium excretion and fractional excretion of sodium, on the other hand, was not significantly changed in response to dapagliflozin.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Ow Connie P. C.、Ullah Md Mahbub、Ngo Jennifer P.、Sayakkarage Adheeshee、Evans Roger G.	４．巻 317
２．論文標題 Detection of cellular hypoxia by pimonidazole adduct immunohistochemistry in kidney disease: methodological pitfalls and their solution	５．発行年 2019年
３．雑誌名 American Journal of Physiology-Renal Physiology	６．最初と最後の頁 F322～F332
掲載論文のDOI（デジタルオブジェクト識別子） 10.1152/ajprenal.00219.2019	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Ullah Md Mahbub、Ow Connie P.C.、Evans Roger G.、Hilliard Krause Lucinda M.	４．巻 2020
２．論文標題 Impact of choice of kinetic model for the determination of transcutaneous FITC‐sinistrin clearance in rats with streptozotocin‐induced type 1 diabetes	５．発行年 2020年
３．雑誌名 Clinical and Experimental Pharmacology and Physiology	６．最初と最後の頁 2020
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/1440-1681.13301	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 Connie P. C. Ow, Vijayakumar Sukumaran, Akihiro Fujiwara, Hirotsugu Tsuchimochi, Hiroshi Hosoda and James T. Pearson
２．発表標題 Characterization of renal vascular responses in a rat model of cisplatin-induced kidney injury
３．学会等名 BCVR 2019
４．発表年 2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関