รายงานวิจัยฉบับสมบูรณ์

โครงการ Effects of sodium-glucose cotransporter 2 (SGLT2) inhibitor on the improvement of insulin resistance, renal glucose transporters and renal function in obese-insulin resistant rats

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และมหาวิทยาลัยเชียงใหม่

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และมหาวิทยาลัยเชียงใหม่ ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

งานวิจัยนี้ สนับสนุนโดยทุนอุดหนุนการวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัย ผู้วิจัยขอขอบคุณสำหรับทุนสนับสนุนการวิจัยทั้งหมดตลอดโครงการ และขอขอบคุณคณะแพทยศาสตร์ และมหาวิทยาลัยเชียงใหม่ที่ให้ทุนสนับสนุนบางส่วนในการวิจัยครั้งนี้ นอกจากนี้ ผู้วิจัยขอขอบคุณภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ สำหรับการเอื้อเฟื้อสถานที่ทำการวิจัย

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ผศ. ดร. อนุสรณ์ ลังกาพินธ์

Introduction

An increasing prevalence of obesity is emerging as a serious world health problem/ situation [1]. A high-fat diet (HFD) linked with obesity has been recognized as an independent risk factor for the development and progression of renal disease with or without developing diabetes [2,3]. The link between an increase in glomerular filtration rate (GFR) with microalbuminuria, an indicative early feature of renal injury, has been reported [4,5]. In obese Zucker rats, renal dysfunction was accompanied by albuminuria, mesangial expansion, and glomerulosclerosis [6]. However, the molecular mechanisms underlying the obesity-induced renal injury are not yet understood.

Evidence from human and animal studies found that HFD consumption and obesity-related renal disease was closely correlated with systemic oxidative stress [7,8]. Studies in HFD-induced insulin resistant rats demonstrated that an increased malondialdehyde (MDA) level and a decreased superoxide dismutase (SOD) activity in the kidney were associated with massive proteinuria and severe glomerular filtration barrier injury [9]. It has been reported that the up-regulation of NAD(P)H oxidase 4 (NOX4) has an important role in causing renal oxidative stress and kidney injury in animal models of chronic kidney disease (CKD) and diabetic nephropathy (DN)[10,11]. The inhibition of NOX by an antisense NOX cDNA could lower oxidative stress and prevent the development of DN as reflected by the reduction in kidney and glomerular hypertrophy and the attenuation of fibrosis marker expression in the renal cortex [8,10]. Oxidative stress also plays an important role in podocyte injury and the down-regulation of glomerular filtration barrier proteins, nephrin and podocin, and the damage of podocytes results in proteinuria and eventually renal failure [12-14]. Therefore, pointing targeting of the NOX complex may be an effective strategy against reactive oxygen species (ROS)-induced renal injury in cases where there are pathological complications.

It has been well known that obesity induced by HFD feeding is associated with insulin resistance [15]. Cross-sectional studies, either case-control or prospective cohort in design, have demonstrated the correlations of the microalbuminuria and the associated hyperinsulinemia and hypertension [16,17]. In cases of metabolic syndrome, hyperinsulinemia and oxidative stressinduced an increase in microvascular density which accounted for the elevation of renal blood flow (RBF) and GFR [18,19]. Increased levels of ROS are caused not only by enhanced oxidant activity, but also upregulation of angiotensin II type I receptor

(AT_1R) via nuclear factor kappa B (NF-κB)activation and these could play a role in the development of hypertension [20]. Ang II, acting through the AT_1R, increased the generation of ROS in the vasculature and renal tubules, primarily through the activation of the membrane-bound NADPH oxidase enzyme complex [21,22]. Chronic exposure of proximal tubular epithelial cells to Ang II caused up-regulation of NOX4-dependent ROS production, indicating the potential NOX4-dependent mechanism to instigate progressive renal injury [23]. Studies investigating gentamicin-induced nephrotoxicity have demonstrated that nuclear factor E2-related factor2 (Nrf2) protected rat kidneys against oxidative stress through the activation of antioxidant enzymes such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1) and glutamate-cysteine ligase (GCLC) [24,25]. However, the role of the action of Nrf2 against renal oxidative stress in the obese insulin-resistant condition has not been investigated.

Giving weight to the concept that obesity is recognized as a chronic low-grade systemic inflammatory disease with accumulation of pro-inflammatory cytokines, during the onset and progress of obesity and insulin resistance, inflammation and oxidative stress play important roles in the pathogenesis of renal injury [26-28]. Interestingly, a recent study has shown the role of inflammation in kidney disease in the development of kidney lipid accumulation in obese mice in turn triggers inflammation and the release of multiple inflammatory cytokines causing oxidative injury, and structural changes which further aggravate renal injury [29-31]. In addition, lipids or their breakdown products in non-adipose tissue also produce the key profibrotic factor, transforming growth factor beta 1(TGF-β1), which causes a fibrotic response subsequently leading to organ dysfunction [32]. Moreover, data from animals with metabolic syndrome have showed a correlation between hyperinsulinemia and progressive tubulointersitial fibrosis, involving proliferation of the extracellular matrix and collagen deposition which are characteristic of renal injury in diabetes [33-36]. Thus, the control of inflammation has great therapeutic potential as regards the inhibition of progressive renal fibrosis.

Emerging data evidence from animal studies have demonstrated that abnormal tissue lipid metabolism in conditions of obesity or insulin resistance leads to an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen which consequently activate an unfolded protein response (UPR), and subsequently triggering ER stress [37-39].

To restore ER homeostasis, ER luminal domains with the protein chaperone, immunoglobulin heavy-chain binding protein BiP, (also known as GRP78) is displaced to interact with unfolded/misfolded proteins [40]. However, prolonged or massive ER stress could induce activation of apoptosis. This is mediated by the transcriptional CHOP protein and classical route of Calpain 2 and Caspase-12 through up-regulated pro-apoptotic protein Bax and down-regulated anti-apoptotic protein Bcl-2, leading to activation of apoptotic caspases cascades [41-43]. Recently, the increase in renal lipid deposition has showed to be associated with increased ER stress-mediated apoptosis, and renal dysfunction [44,45]. Hence, regulation of ER stress and its related pathways would be a good strategy for the prevention of renal injury in obesity.

Organic anion transporter 3 (Oat3) plays a major role in the elimination of organic anion substances which is one of the major routes for body drug clearance/detoxification [46,47]. Oats are expressed in many tissues, including kidney, liver, choroid plexus, olfactory mucosa, brain, retina, and placenta [48]. The previous reports from metabolomics studies revealed that Oats may function in remote interorgan communication by regulating levels of signaling molecules and key metabolites in tissues and body fluids [48]. The expression of Oat3 at the basolateral membrane of the proximal tubular cells is regulated by protein kinase C (PKC) [49]. Previous studies clearly shown that the down-regulations of renal Oat3 function and expression in type 1 diabetes and gentamicin-induced nephrotoxicity were associated with oxidative stress-induced PKCα activation [25,50].However, the alteration of renal Oat3 function and expression in obese-insulin resistant model has not been determined.

Pharmacological inhibition of the renal sodium glucose cotransporter 2 (SGLT2) is a new approach which inhibits the renal reabsorption of filtered glucose, thereby lowering blood glucose levels without increasing body weight [51-53]. Several clinical trials with dapagliflozin, a potent and selective SGLT2 inhibitor, showed that it reduced hyperglycemia and improved glycemic control in patients with type 2 diabetes (T2DM) [43,54,55]. In addition to its anti-diabetic effect, dapagliflozin has been found to prevent the development of nephropathy, probably not only by instigating decreases in body weight, lipid metabolism, oxidative stress, inflammation, and fibrosis but also by improving insulin resistance in experimental models. [56-59]. A previous study demonstrated that the SGLT2 inhibitor empagliflozin facilitated better glycemic control and reduction of renal inflammation than metformin treatment in *db/db* mice [60]. These findings suggested that an SGLT2 inhibitor might exert greater benefit than metformin in the improvement of glycemic control in the diabetic condition. Moreover, the SGLT2 inhibitor luseogliflozin had higher efficacy than the ACE inhibitor lisinopril, which is the standard therapy used to reduce renal injury in diabetic patients, in the preservation of renal function as shown by the lowered degree of glomerular and tubular injury [58]. These findings suggested that SGLT2 inhibitor had greater renoprotective effects indicating by slowing the rate of progression of renal injury in diabetic rats.

Although several studies in animal models suggest that administration of SGLT2 inhibitors improve insulin sensitivity and slow the progression of DN, the influence roles of SGLT2 inhibition on the renal function in the obese insulin-resistance have never been investigated. Therefore, to address these issues, the purpose of this study was to explore the renal function and molecular mechanisms that inhibition of SGLT2 by dapagliflozin in rat model of obese-insulin resistant condition. Our hypothesis was that dapagliflozin reversed renal dysfunction and restored renal Oat3 function underlying: oxidative stress, ER stress, inflammation/fibrosis, apoptosis as well as modulation of renal insulin signaling in the obese-insulin resistance.

Literature review

1. Epidemiology of obesity

Obesity is the major risk factor for the metabolic syndrome, characterized by insulin resistance, dyslipidemia, and hypertension [61]. It is defined by the World Health Organization as a body mass index (BMI) $\geq$ 30 kg/m^2[62]. The rising of obesity epidemic in almost every industrialized country continues to be a growing problem worldwide, with increasing rates in adults and children[63]. In 2014, 600 million adults (13%) and 42 million children under the age of five were obese.Obesity is predicted to become a leading cause of morbidity and mortality, driven by an increase in related life-threatening disorders, including dyslipidemia, hypertension, cancer, and T2DM [64].Furthermore, obesity is also a major and independent risk factor for the development of kidney disease [63-65]. Therefore, it is one of the most pervasive, chronic diseases in need of new strategies for medical treatment and prevention [65].

2. Obesity-induced insulin resistance

Insulin is the principal hormone of glucose homeostasis via stimulating glucose influx into muscle, glycogen synthesis in the liver and muscle, and fat deposition in adipocytes [66]. Moreover, insulin also enhances protein synthesis, cell survival and growth, prevents protein catabolism, and has anti-inflammatory effects [67,68]. Insulin acts via binding to the α-subunit of the insulin receptor molecule which induces rapid autophosphorylation of the β-subunit, and subsequently turns on its tyrosine kinase activity [66]. This gives insulin receptor ' ability to phosphorylate insulin receptor substrates-1 and-2 (IRS-1, IRS-2). The tyrosine phosphorylation of IRS proteins leads to the second intracellular step of insulin action, the association of phosphorylated IRS-1 or IRS-2 with the enzyme, phosphoinositide-3-kinase (PI3K)[69]. The IRS-activated PI3K in turn affects several downstream signaling pathways through the generation of a lipid second messenger, phosphatidyl-inositol-3, 4, 5-triphosphosphate (PI3K). A critical target for PI3K is Akt/PKB (protein kinase B) [70]. A serine/threonine kinase, Akt/PKB is the major effector of the insulin receptor-IRS-1-PI3K pathway and is activated by the phosphorylation of its threonine 308 and serine 473 residues. Akt/PKB drives the metabolic actions of insulin including glucose transport, glycogen synthesis, fat deposition, and protein synthesis and trafficking [50,67].

In the prediabetic or early stages of type 2 diabetes, insulin resistance leads to increase of pancreatic insulin production to maintain euglycemia and normal biological responses. In obesity, data obtained from various studies has shown that hyperinsulinemia is derived from either over production or decreased clearance of insulin [71,72]. The balance of insulin production rate and insulin clearance rate determines plasma insulin level. The β-cells in the pancreatic islets are the only source of insulin. In obesity, β-cells function is enhanced and the cell number is increased in the pancreatic islets during the weight gain [72].

The increase of size and number of adipose tissue accounts for an excess adipose tissue in obesity. A recent and striking discovery is that obesity is associated with a low-grade inflammation process in adipose tissues, the pathophysiological mechanisms of which remained poorly understood, underlining the relationship between fat cells and the immune system [73]. A link between obesity and inflammation was initially demonstrated that the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) was expressed in adipose tissue of obese mice and linked to insulin resistance[74]. In addition, adipose tissue hypoxia secondary to reduced blood flow may directly or indirectly induce expression of proinflammatory cytokines, which subsequently release to circulation [75]. The macrophage chemo attractant protein-1 (MCP-1) released from adipocytes plays a role in the recruitment of macrophages [76]. This was supported by cell death-associated infiltration of macrophages [77]. These adipogenic cytokines and chemokines activate intracellular pathways that promote the development of insulin resistance. Also, the concomitant generation of ROS exaggerates or plays a causal role in cytokine-related insulin resistance [78].

The intracellular mediators of inflammatory response include NF-κB, c-Jun amino-terminal kinase/stress activated protein kinase (JNK/SAPK), and induction of the suppressor-of-cytokine-signaling-3 (SOCS3) [79]. The associated activation of the JNK/SAPK pathway also promotes the development of hepatic inflammation leading to hepatic steatosis, lipid peroxidation, and hepatic apoptosis, all of which are seen with obesity-induced diabetes [80]. Moreover, inflammation has been shown to inhibit the insulin signaling in adipocytes and hepatocytes through several mechanisms. It has been suggested that these inflammatory mediators also lead to serine phosphorylation of IRS-1 [81].Serine phosphorylation, as opposed to tyrosine phosphorylation, inhibits insulin signaling [82]. The second is inhibition of peroxisome proliferator-activated receptor gamma(PPAR γ) function [83,84]. PPAR γ is a nuclear receptor that drives lipid synthesis and fat storage in cells. Its activity is dependent on ligands, which include long chain fatty acids and thiazolidinedione (TZD). It induces expression of enzymes or proteins in lipogenesis or storage through transcriptional activation. Reduction of PPAR γ activity contributes to insulin resistance. The third is to increase plasma free fatty acid (FFA) through stimulation of lipolysis and blocking triglyceride synthesis [72]. Previous reports have shown that insulin resistance can increase lipolysis from adipocytes. FFA can adversely affect insulin signaling and cause insulin resistance within adipocytes, skeletal muscle and other tissues [85]. It was recently shown that short chain fatty acid (SFAs) can be converted to diacylglycerides (DAG) and ceramides-induced insulin resistance [86]. DAG stimulate protein kinase C (PKC) which causes inhibits IRS phosphorylation in serine residues. Ceramide directly activates protein phosphatase 2A (PP2A) that in turn deactivates Akt/PKB [87]. Accordingly, the implication of inflammation pathways is also suggested by the protective effect of some anti-inflammatory compounds against obesity-associated insulin resistance [73].

Many of the human studies demonstrated the link between hyperglycemia-induced generation of ROS and insulin resistance in diabetic patients [88]. The increase in ROS in the pre-diabetic stage is more likely due to obesity-related elevations of FFA that cause oxidative stress due to increased mitochondrial uncoupling and β-oxidation, leading to the increased production of ROS [89]. In healthy subjects, infusion of FFA causes increased oxidative stress and insulin resistance that is reversed by infusion with antioxidants such as glutathione [90]. Recent studies have addressed the molecular mechanisms by which oxidative stress might lead to insulin resistance. In vitro study, ROS and oxidative stress lead to the activation of multiple serine/threonine kinase signaling cascades [91,92]. These activated kinases can act on a number of potential targets in the insulin signaling pathway, including the insulin receptor and the family of IRS proteins. For IRS-1 and IRS-2, an increase in serine phosphorylation decreases the extent of the activating tyrosine phosphorylation [92,93]. Petersen et al. (2003) found that severe insulin resistance was associated with significantly higher levels of triglycerides in both muscle and liver in the elderly [94]. These changes were accompanied by the reduction in mitochondrial function as shown by the decreases in both mitochondrial oxidative activity and mitochondrial ATP synthesis. In the insulin-resistant subjects, a decrease in the number of muscle mitochondria a decrease in the expression of nuclear-encoded genes that regulate mitochondrial biogenesis, such as PPAR γ coactivator α (PGC-1α) leads to intramyocellular fat accumulation [95]. Since functional mitochondria is source of FFA-induced ROS generation, it is possible that an increase in ROS due to FFA oxidation occurs early during the development of insulin resistance and prior to mitochondrial dysfunction. At a later stage, ROS might lead to a decrease in mitochondrial function that then leads to the accumulation of fat in the muscle and liver, exacerbating the insulin resistance phenotype via the mechanisms mentioned above.

3. Obesity and kidney injury

In each decade of the last two, the number of people with end-stage kidney disease were doubled, and in 2010, there were 600,000 people had required dialysis treatment [4]. There has been report of microalbuminuria and variable degree of proteinuria in obese patient which significantly increases risk factors for chronic kidney disease (CKD). It has been estimated that 20 million people in the United States have either persistent proteinuria or substantial kidney damage [4]. Clinically, an early histological feature found in massive obese patients with proteinuria is glomerular hypertrophy [96]. Obesity is mechanistically tied to renal disease associated with hypertension and type 2 diabetes, the 2 most common etiologies of end stage renal disease (ESRD) [97].In high fat induced obese mouse model, renal pathophysiology consists of glomerulomegaly such as increased extracellular matrix, irregular thickening glomerular basement membrane, and foot process effacements in some part of the glomeruli along with lipid accumulation, macrophage infiltration, impaired sodium handling [98,99], and vacuolization in proximal tubules [100]. Renal lipid accumulation associated with generation of oxidative stress, inflammation and fibrosis further contributes to progressive renal injury [101].

3.1Effects of hyperinsulinemia in obesity on renal injury

Insulin plays a homeostatic role in normal kidney function and has direct dose-dependent vasodilator effects on the renal microvasculature. However, in hyperinsulinemic states, such as those found in the obese Zucker rats, insulin-induced dilation of the renal microvessels was impaired [102]. Renal microvessels from obese Zucker rats also displayed impaired endothelial nitric oxide (eNOS) production, which could account for the reduction in insulin-dependent vasodilatation [102]. These microvascular alterations affects renal morphological changes. In obese *db/db* mouse, glomerular hypertrophy and increased mesangial cell density were evident [103]. There were also an increase in glomerular capillary length and surface area in addition to glomerular basement membrane thickening, as a result of the exposure of the renal vasculature to a hyperglycemic environment [103]. Hyperinsulinemia can also cause cell proliferation and renal injury by way of promoting the expression of other growth factors such as transforming growth factor beta (TGF-β) and the downregulation of matrix metalloproteinase (MMP)-2, an enzyme responsible for matrix degradation [35]. TGF-β can promote renal fibrosis, and insulin has been shown to act directly to induce an increased production of TGF-β in proximal tubular cells, which could be involved in extracellular matrix proliferation that is a characteristic of diabetic nephropathy [34]. Insulin has also angiogenic effects on the glomerulus via the MAP kinase pathway [104].Hyperinsulinemia has been shown to stimulate the synthesis of growth factors such as

insulin-like growth factor (IGF)-1 and receptor, which may promote glomerular hypertrophy [105].IGF and IGF receptor levels were found to increase in animal models of diabetes and in the presence of high glucose in cell culture [106]. The increased glomerular IGF-1 led to mesangial cell proliferation, extracellular matrix expansion, and podocyte apoptosis. These were supported by the increased IGF receptor mRNA in the kidney of the obese Zucker rat compared with the lean Zucker rat [107]. Moreover, there were correlation of circulating levels of IGF with the markers of proliferative kidney disease in diabetic patients and animal models of diabetic disease [108].

Obesity is associated with a state of chronic, low-grade inflammation, which may cause obesity-induced insulin resistance [82,109,110]. In animal models of obesity, there has been reported macrophage infiltrations into white adipose tissue and the kidney which give rise to obesity-induced dysregulation of adipokine production and may also participate in tissue fibrosis [111]. Moreover, during the course of obesity, proinflammatory cytokines that release from adipose tissue macrophages contribute to chronic inflammatory responses [82,110]. In addition, the increased oxidized low density lipoprotein (Ox-LDL) levels in obesity has been shown to stimulate monocyte adhesion to the glomerular endothelial cells which subsequently increase glomerular injury [112]. Furthermore, the ectopic lipid in the kidney is predominantly found in the proximal tubules and to a lesser degree in glomeruli [113]. Ectopic lipid in the kidney or a ‘fatty kidney’ may constitute a quintessential biomarker of obesity-related kidney disease [114].

Emerging data evidence from animal studies have demonstrated that abnormal tissue lipid metabolism in conditions of obesity or insulin resistance leads to an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen which consequently activate an unfolded protein response (UPR), subsequently triggering ER stress [37-39]. To restore ER homeostasis, ER luminal domains with the protein chaperone, immunoglobulin heavy-chain binding protein BiP, (also known as GRP78) is displaced to interact with unfolded/misfolded proteins [40]. However, prolonged or massive ER stress could induce activation of apoptosis. This is mediated by the transcriptional CHOP protein and classical route of calpain 2 and caspase-12 through up-regulated pro-apoptotic protein Bax and down-regulated anti-apoptotic protein Bcl-2, leading to activation of apoptotic caspases cascades [41-43]. Recently, the increase in renal lipid deposition has showed to be associated with increased ER stress-mediated apoptosis, and renal dysfunction [44,45]. Study in obese porcine demonstrated that the increased renal triglyceride accumulation was associated with microvascular proliferation and increased glomerular filtration rate (GFR), expression of pro-angiogenic and inflammatory factors along with albumin leakage [115].

3.2 Renal hemodynamic changes associated with excess weight gain and insulin resistance

Alterations in glomerular hemodynamics in obesity and insulin resistance are characterized by the increases in GFRand effective renal plasma flow, accompanied with elevations of filtration fraction and albumin excretion [116-118].An increase in GFR in the leptin receptor-deficient obese *db/db* mouse [119] was consistent with the report that obese individuals exhibited an increase in GFR of about 50% above lean subjects [120]. Also, the increased renal plasma flow (RPF) accompanied with increased renal perfusion and and enhanced (single-nephron) glomerular filtration rate was found in severe obesity (BMI $>$ 38 kg/m^2), even in the presence of normal blood pressure[120]. One plausible explanation for augmenting effective renal plasma flow may be associated with the increased blood volume and cardiac output secondary to the high metabolic demand in obesity [121]. Although the mechanisms remainincompletely understood, the novel pathogenesis of renal hyperfiltration has been attributed to tubular and glomerular hemodynamics factors [122].

The classic concept is the primary hemodynamic hypothesis, in which the primary event is vasodilation of the afferent arteriole, resulting in glomerular hyperfiltration [123]. Glomerular hyerfiltration also induces an increase in protein concentration in the postglomerular circulation, resulting in a higher oncotic pressure in the plasma entering the peritubular capillary (PTC) than in the systemic circulation. Since hydrostatic pressure drops along the efferent arterioles [26–28], in the normal kidney, the high PTC oncotic pressure generates a transcapillary pressure gradient favoring net uptake of the reabsorbate from the intercellular space into the PTC [124-126]. In the obese subject, a raised postglomerular oncotic pressure is responsible in part for the increased proximal tubular sodium reabsorption.

Hyperinsulinaemia secondary to insulin resistance probably also has a role in obesity-associated renal dysfunction. Under the physiological condition, insulin may regulate GFR through local renal vasodilation, which can be blocked by indomethacin (prostaglandin inhibitor) and augmented by activation of endothelial nitric oxide (eNOS) synthase [127,128]. In metabolic syndromes subjects with insulin resistance, the loss of renal vasodilation effect of insulin is associated withendothelial dysfunction due to downregulated expression of eNOS and increased endothelin-1(ET-1) levels [129,130]. In metabolic syndromes, the increase in microvascular density was associated with the upregulated expression of vascular endothelial growth factor (VEGF), possibly secondary to oxidative stress and the direct effect of hyperinsulinemia on VEGF production [18,19]. These proliferated small microvessels may contribute to maintain increased renal perfusion, and may initially account for elevated renal blood flow and GFR that characterize the early stage of metabolic syndromes [131]. As mentioned earlier, hyperinsulinemia may induce glomerular hyperfiltration, endothelial dysfunction, and increased vascular permeability, leading to albuminuria. In nondiabetic subjects, even a short-term insulin infusion increases urinary albumin excretion [132]. In turn, leakage of albumin in the tubular lumen may results in a cycle of chronic tubular injury, inflammation and dysfunction through activation of the Toll-like recptor 4 (TLR4) pathway [132,133].

Furthermore, the elevated insulin levels have been found to stimulate insulin-like growthfactor (IGF-1) production, which maypromote connective tissue growth factor, causing renal fibrosis [134]. Study in Zucker obese rats fed a high-protein diet demonstrated that insulin sensitizing compounds, such as thiazolidinediones (TZD), indeed abrogate interstitial fibrosis [135]. These findings suggest that the interaction of insulin with its receptor bears direct ramification for renal structural and functional impairment in metabolic syndromes. Yet, obesity-related glomerulopathy (ORG) may not be mediated solely by hemodynamic factors. As in individuals with ORG, it has been found that each podocyte must undergo mechanical stretch to cover a larger surface area to accommodate the increased glomerular volume, resulting in decreased podocyte density and increased foot process width [136,137]. Over time, when podocyte enlargement is no longer proportional to glomerular hypertrophy, podocytes fail and detach causing localized denudation of the glomerular basement membrane, subsequent adhesions to the bowman capsule and parietal cell coverage, forming a nidus for development of segmental sclerosis, and resulting in proteinuria [138,139]. In the Fischer rat model, proteinuria and glomerulosclerosis were linearly related to increasing body weight and could be accelerated by unilateral nephrectomy, which promotes further compensatory filtration demand [139].

The tubular hypothesis proposes that hyperfiltration is initiated by increased sodium reabsorption in the proximal tubule which involves stimulation of sodium-glucose cotransporter 2 (SGLT2)[140]. These results in reduced delivery of sodium to the macula densa, which senses the decrease in volume depletion or renal perfusion. The physiological response to the increased GFR, via tubuloglomerular feedback (TGF)activation, is to reduce adenosine generation in the juxtaglomerular apparatus, leading to enhanced afferent arteriolar vasodilatation, and subsequently to increased renal perfusion and a normalization in GFR [51]. Thus, the increased GFR under this conditions of effective circulating volume contraction, a physiological response would be to reduce adenosine generation in the juxtaglomerular apparatus, leading to enhanced afferent arteriolar vasodilatation, an subsequent to increase renal perfusion and a normalization in GFR via TGF mechanism [141-143]. Consequently, the increased proximal reabsorption is associated with a supranormal rise in GFR into the hyperfiltration range. Data from experimental studies revealed the marked increment of proximal sodium reabsorption in the setting of obesity resulting from the increased activation of sodium transporters along the nephron [144,145]. In addition, accumulation of visceral fat in obese subjects almost completely encapsulates the kidneys and penetrates into medullary sinuses. These cause compression and increase of intrarenal pressure [146]. Obesity also causes marked changes in renal histology that could compress the renal medulla, which slows the flow in tubular fluid, and subsequently increases sodium reabsorbtion, retention and hypertension [146]. Because the kidney is surrounded by a capsule with low compliance, the increased renal interstitial pressure and solid tissue pressure secondary to elevated extracellular matrix would cause compression of the thin loops of Henle, reduce vasa recta blood flow, and also increase tubular reabsorption by slow tubular flow. These were supported by the markedly increased renal interstitial fluid pressure found in obese dogs [146]. The study in high-fat diet induced obese dogs for 6th weeks demonstrated the promptly increased glomerular hyperfiltration, sodium retention and extracellular fluid along with the increased sodium reabsorption [116].Moreover, in a swine model,the increased GFR in early metabolic syndrome was associated with enhanced renal adiposity,microvascular proliferation and tubular injury as indicated by proximal tubular vacuolization. These might preface significant activation of oxidative stress and inflammation [131].

Furthermore, systemic hypertensionwhich is highly prevalent in individuals who are overweight or obese [147] also contributes to the pathogenesis of hyperfiltration. Due to renal vasodilation and increased transcapillary hydraulic pressure difference noted in obese patients, an abnormal transmission of increased arterial pressure to the glomerular capillaries through the dilated afferent arteriole is expected to augment transcapillary hydraulic pressure difference and thus increases GFR [120]. In prolonged metabolic syndromes, renal microvascular endothelial dysfunction also increases glomerular capillary wall permeability and albuminuria, which may also promote glomerular capillary loss and progression of renal injury [148].

3.3 Hormonal and neuronal activation in obesity

Several findings indicate that insulin interferes with the systemic renin angiotensin aldosterone system (RAAS) and the intrarenal RAAS. In experiments performed in dogs, hyperinsulinemia was shown to significantly increase both the aldosterone and pressor responses to intravenous infusion of angiotensin II (Ang II) [149]. It has been reported that adipocytes are rich sources of the precursor protein of Ang II and angiotensinogen as well as aldosterone synthase [150]. Previous studies demonstrated that oxidative stress in the pathogenesis of obesity also caused the upregulation of angiotensin II type I receptor (AT_1R) via nuclear factor kappa B (NF-ΚB) activation that could play a role in the development of hypertension [20,151]. In the obese condition, the RAAS is overactivated and the levels of RAAS components are increased in the circulation and in renal tissue [152]. RAAS overactivation might be involved in the pathogenesis of hyperfiltration. Firstly, Ang II and aldosterone vasoconstrict glomerular arterioles and have a greater effect on the efferent than the afferent arterioles, which lead to increases of transcapillary hydraulic pressure and GFR [153,154]. Secondly, Ang II increases sodium reabsorption proximally by stimulation of the luminal Na^+-H^+exchanger and the basolateral Na^+-K^+-ATPase, and distally by activation of the epithelial Na^+ channel (ENaC) [155]. Ang II might also directly activate the mineralocorticoid receptor, resulting in increased sodium reabsorption and a positive sodium balance. RAAS overactivation might therefore lead to excessive sodium reabsorption and resulting in systemic hypertension and hyperfiltration [156]. In addition, hyperinsulinemia might increase the tubular reabsorption of sodium by stimulating the activity of ENaC in the late distal tubule, and less prominently in the proximal tubule and the loop of Henle [157]. Taken together, these effects contribute to glomerular injury and nephron loss associated with obesity because Ang II formation constricts the efferent arterioles and exacerbates the rise in glomerular hydrostatic pressure secondary to systemic arterial hypertension. A significant role of Ang II in stimulating sodium reabsorption, impairing renal-pressure natriuresis, and causing hypertension in obesity, is supported by the finding that treatment of obese dogs with an Ang II antagonist or ACE inhibitor blunts sodium retention and volume expansion, as well as increasing arterial pressure [105]. Likeise, clinical treatment with ACE inhibitors and Ang II receptor antagonists clearly showed the slow progression of renal disease in overweight patients with type II diabetes [158].

Evidence from in vitro study has shown that insulin is necessary for the Ang II-induced contraction of mesangial cells, providing another link between insulinand Ang II mediated renal injury [159]. In the above-mentioned study performed by Anderson et al. [160], Ang II alone had a slight effect on TGF-β1 and collagen protein production from cultured mesangial cells, but this effect was multiplied by the addition of insulin. Moreover, in cultured vascular smooth muscle cells, insulin increased both the mRNA levels and the protein expression of AT_1R in a dose-dependent fashion [161]. A similar stimulating effect of insulin on the AT_1R mRNA expression was demonstrated in renal mesangial cells [162], providing a possible explanation for the enhancement of Ang II actions from insulin.

A major source of ROS in metabolic syndromes is the NADPH oxidase (NOX) family of enzymes. Accumulating evidences have shown that NOX, particularly NOX1, 2, and 4 which are highly expressed in the kidney, plays vital roles in intrarenal oxidative stress [163]. In metabolic syndromes, the augmented Ang II, increases mRNA expression of NOX1 and NOX4 several fold, suggesting that NOX activity is Ang II-dependent [164]. Furthermore, additional mechanisms in injured kidneys may exacerbate oxidative stress, resulting in a vicious circle. In addition, NOX4-derived ROS not only decreases mitochondrial function in endothelial cells via disruption of the electron transport chain complex I but also causes extracellular matrix protein accumulation in mesangial cells [165,166]. Interestingly, these changes are associated with AMPK inactivation and its activation reduces renal fibrogenesis [166]. Therefore, kidney dysfunction in obesity might involve renal oxidative stress.

3.4 Mechanism of kidney injury development in obesity

The well-known that central (visceral or abdominal) obesity is associated with an increased risk for atherosclerosis, stroke, and coronary heart disease [105]. Framingham study and supported by others [167] have reported that BMI is one of the predictors of chronic kidney disease (CKD). It predicts not only CKD, but also end-stage renal disease (ESRD) [168,169]. Accumulation of fat in and around the kidneys observed in visceral obesity may have additional lipotoxic effects on the kidneys. Lipid accumulation in key organs (ectopic fat storage) accompanying with increased oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress lead to impairment of organ function [170]. These observations establish metabolic syndrome as a trigger for renal injury which magnifies the adverse impact of other insults. Several new injurious pathways that metabolic syndrome activates in the kidney have been identified (Figure 1) [171]. However, the pathways activated by obesity to induce kidney remains to be clarified.

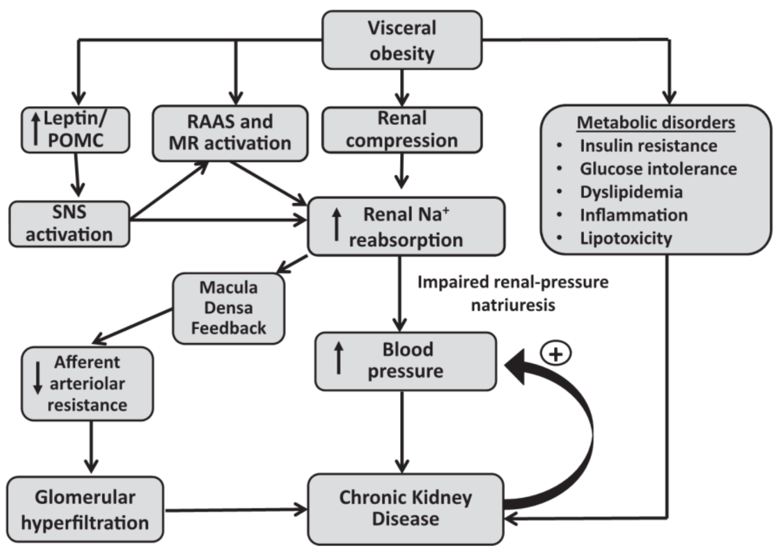


Figure 1 Summary of mechanisms by which obesity initiates development of renal injury. MR; mineralocorticoid receptor, POMC; proopiomelanocortin, RAAS; renin angiotensin aldosterone system, and SNS; sympathetic nervous system [172].

It has been reported the decreased activity of antioxidant enzymes in prolonged obesity. Previous study demonstrated the significantly lowered activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in individuals with obesity as compared with healthy persons [7]. Nuclear factor E2-related factor-2 (Nrf2) and nuclear factor-ΚB (NF-ΚB) are two stress-sensing transcription factors that regulate antioxidant and anti-inflammatory pathways (Figure 2) [173]. NF-ΚB can activate gene expression encoding proinflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1β), interleukin-6 (IL-6), cycloooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Antioxidant enzymes such as catalase and superoxide dismutase (SOD) can inhibit ROS generation and reduce the activation of NF-κB, resulting in the decreased production of proinflammatory cytokines. Nrf2 is a key transcription factor in regulating intracellular redox balance, and is a sensor of oxidative and electrophilic stress [174]. Oxidant and electrophiles are known to stimulate Nrf2. Oxidative stress and electrophiles also activate the disruption of Nrf2/Keap1 complex (Keap1 is the regulatory protein of Nrf2). Then, Nrf2 translocates into the nucleus called Nrf2-ARE (antioxidant response element) pathway that plays a role in promoting the antioxidant defense system or protecting against oxidative stress. The previous *in vitro* and *in vivo* studies have shown that Nrf2 is essential for ARE-mediated induction of genes including phase II detoxifying enzymes such as heme oxygenase 1 (HO-1), superoxide dismutase (SOD) and glutamate-cysteine ligase (GCLC) [175]. In addition, Nrf2 also has an important role in protecting cell and suppressing TNF-α against oxidative stress [175,176]. Previous study in metabolic syndrome-induced kidney dysfunction showed he promoted Nrf2 activation through ERK1/2 pathway to increase cyto-protective activity [177]. Recent research has found that Nrf2 inhibits lipid accumulation and oxidative stress in mouse liver after feeding a HFD, suggesting that Nrf2 inhibits oxidative stress induced by free fatty acid accumulation associated with induction of genes involved in lipogenic and cholesterologenic pathways [178]. The Nrf2/Keap1 pathway has been involved in the control of NF-κB through reduction of IkBα phosphorylation, thereby favouring NF-κB degradation (Figure 3) [179]. In addition, *Nrf2* knockout mice showed enhanced NF-κB activity and expression of inflammatory genes in response to different insults than the corresponding wild-type mice [180]. A recent review by Wakabayashi et al. [179] has pointed out the complexity of the interactions between downstream targets of Nrf2 and NF-κB that may lead to modulation of transcription factor activity. For example, induction of Nrf2-dependent antioxidative proteins such as HO-1 which was able to limit NF-κB activity by inhibiting IkBα degradation, while inhibition of HO-1 which increased NF-κB-p65 activity. HO-1 exhibits in fact anti-inflammatory effects that complement the antioxidant properties. An NF-κB target such as COX-2 was found to cause a reduction of transcription of Nrf2 and antioxidant genes [179].

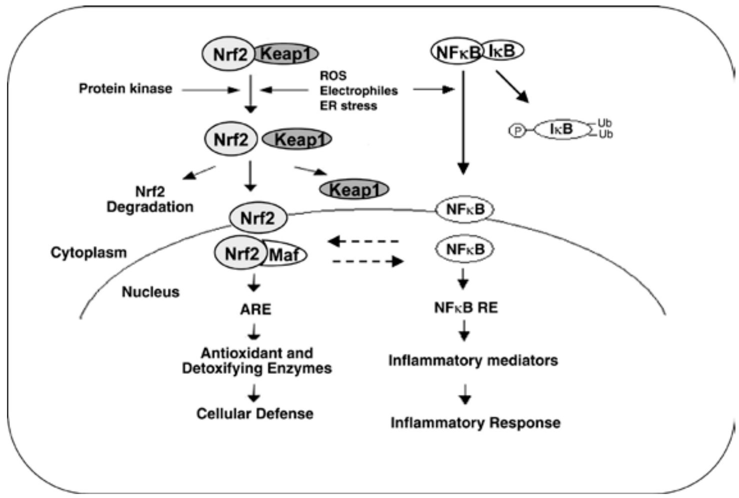


Figure 2 Pathway of Nrf2 and NF-ΚB activation by oxidative stress and inflammation [181].

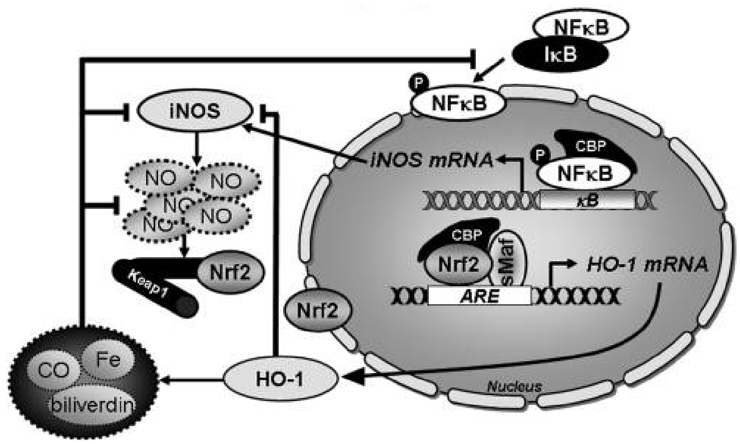


Figure 3 The interactions of the Nrf2 and NF-ΚB signaling pathways

4. Organic anion transporter

Kidney plays an important role in the elimination and detoxification of harmful substance of several compounds, including drugs, toxins, and endogenous substances. Thus, the changes in expressions and/or functions of renal transporters could also affect their substrate concentrations and pharmacokinetics [182]. Recently, obese-insulin resistance condition related glomerular and proximal tubular epithelial damage which indicated by increased glomerular and tubular urinary space volume [126]. The epithelial cells in renal proximal tubule have the expression of transporters to eliminate toxic substances and much of substance elimination will occur in the renal proximal tubule [46]. The two major super families of transport proteins have been identified including the ATP binding cassette transporters and the solute carrier transporters (SLC). The ATP binding cassette transporters transport substance across cell membrane by directly binding and hydrolyzing ATP as a driving force for unidirectional transport of substrates. The solute carrier transporters (SLC) transport substances by indirectly coupling to cellular energy, and using the membrane potential difference and/or concentration gradient as driving force [183].

The solute carrier transporter 22 (SLC22) family contains 13 functionally characterized human plasma membrane proteins, consisting of organic cation transporters (Octs), organic anion transporters (Oats) and organic zwitterions/cation transporters (OCTNs). Oats play a major role in the elimination of organic anion substances such as many drugs, including anti-cancer agents, antiviral, antibiotic, antihypertensive and diuretic drugs. Moreover, Oats can transport endogenous substances (metabolic intermediates/by products and hormones) and environmental toxins. The previous reports from metabolomics studies revealed that Oats may play a role in remote interorgan communication by controlling levels of signaling molecules and key metabolites in tissues and body fluids called “Remote Sensing and Signaling Hypothesis” [48]. Several families of multispecific organic anion transporters have been cloned and identified. All members of Oats family are expressed in the kidneys and some Oats are also expressed in the liver, brain, and placenta [184-186].

Several renal organic anion transporters have been found including Oat1, Oat2, Oat3, Oat4, Oat5, Oat8, Oat9 and Oat10 [47]. Among these, only Oat1 and Oat3 have been shown to play a major role in the cellular uptake of organic anions across the basolateral membrane of renal proximal tubules [47]. Oat3 protein consists of 536-542 amino acids with 12 transmembrane domains and has a large intracellular loop between transmembrane domains 1, 2 and 6, 7 including potential phosphorylation sites that regulated by protein kinase C (PKC) (Figure 4) [49]. In humans and rodents, Oat3 is highly expressed on the basolateral membrane of renal proximal tubules and at the apical membrane of choroid plexus, and has the role in secretion of exogenous and endogenous anions [187,188]. Recently, Oat3 has been cloned from human, monkey, pig, rabbit, rat and mouse[189]. The rOat3 mRNA is expressed in the liver, kidneys and brain, and weakly in the eye while hOAT3 is expressed at the basolateral membrane of human kidney, with less expression in brain and skeletal muscle [190].

Oat3 mediates the high-affinity transport of endogenous substrates such as the second messenger cAMP, cGMP, cortisol, prostaglandin E2 as well as prostaglandin F2α, estrone sulfate (ES), dehydroepiandrosterone sulfate (DHEAS), estradiol glucuronide ($E_2 17\beta G$), bile salt taurocholate, and purine metabolite urate. Exogenous substrates which are transported by Oat3 are antibiotics, antivirals, anti-epileptics, anti-neoplastics, non-steroidal, anti-inflammatory drugs, ochratoxinA, and para–aminohippuric (PAH) [190].

The common test substrate for OAT3/Oat3 is estrone-3-sulfate (ES) because the uptake is easily detectable, and the affinity of OAT3/Oat3 to ES is high. Therefore, ES is the specific substance that has been usually used to assess Oat3 activity[190]. In addition, the ES uptake, an index of Oat3 activity, can be inhibited by other anions such as sulfobromophthalein, furosemide, and benzylpenicillin, but not by cationic substance such as tetraethylammonium (TEA).

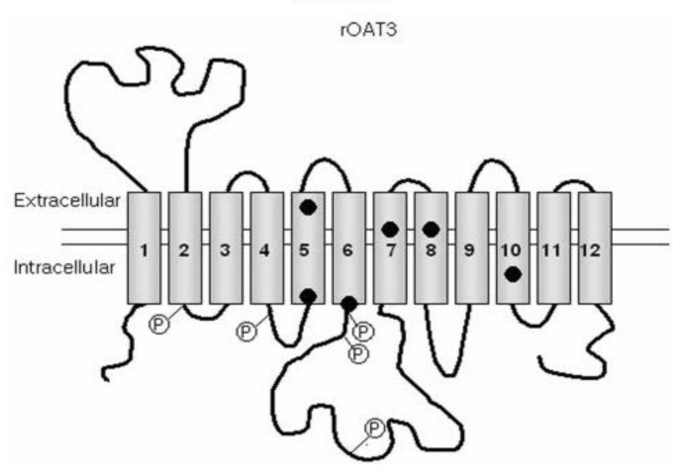


Figure 4 The structure of organic anion transporter 3 (Oat 3). Twelve transmembrane domains are numbered from 1-12. Potential phosphorylation sites are labeled as “P” [191].

4.1 Mechanism of the basolateral organic anion transport system in renal tubular cell

Oat1 and Oat3, locating in the basolateral membrane of the renal proximal tubule, make a major contribution to actively eliminate endogenous substances or organic anion compounds and their metabolites from the body. The organic anion compounds are transported from the blood across the basolateral membrane into the tubule cells against their electrochemical gradients via a tertiary transport system. The terminal step involves moving out of the cells down its electrochemical gradient. This tertiary active transport process is the rate-limiting step in tubular secretion of these compounds. The outwardly directed gradient of alpha ketoglutarate (αKG) is maintained by the combination of intracellular metabolic generation of αKG and the active uptake of αKG into the cells across both luminal and basolateral membranes via Na-dicarboxylate co-transporter (NaDC1 and NaDC3). The inwardly directed gradient for sodium is in turn established by Na/K-ATPase, the primary energy requiring step in the tertiary process (Figure 5) [192].

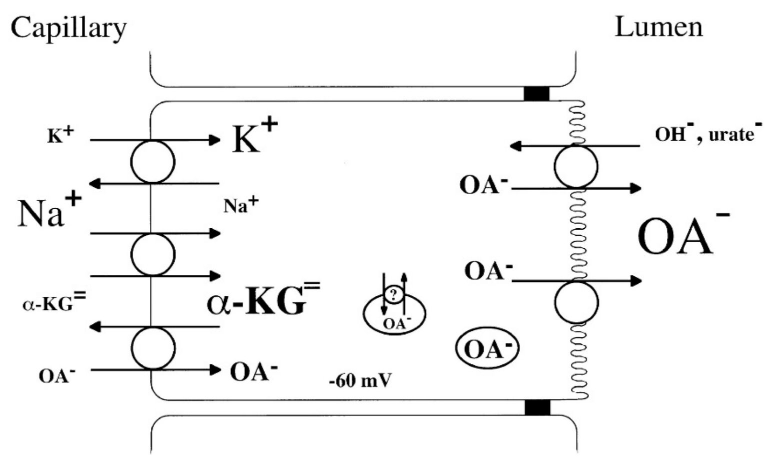


Figure 5 The classic model of the mechanism of basolateral organic anion transport system [193].

4.2 The regulation of organic anion transporter 3 (Oat3)

The regulations of Oat3 functions have been studied extensively in the last decade. Recently, it has been reported that Oat3 activity is regulated by epithelium growth factor (EGF) that leads to activation of mitogen-activated protein kinase (MAPK) pathway and protein kinase A (PKA) respectively. EGF stimulates OAT3 activity via MAPK, mitogen-activated protein kinase, and extracellular signal-regulated kinases1/2 (ERK1/2), which activates phospholipases A2 (PLA2), leading to the release of arachidonic acid (AA). Arachidonic acid is then metabolized to prostaglandins (PGE2), which activates PKA via adenylate cyclase and finally stimulates OAT3 activity [194]. Several investigations have shown that tyrosine kinase and phospho-inositide-3-kinase (PI3K) are the significant mediators of EGF signaling in cell growth and differentiation of renal cells [195-197]. Binding of EGF to its receptor leads to the activation of PI3K and some of tyrosine kinases. A growing evidence has been demonstrated that metabolic disorder could alter Oat3 function and affect several organs function [50,198,199] by regulating or modulating the key metabolites and signaling molecules through the gut-liver-kidney axis [200]. On the other side, Oat3 activity was inhibited by activation of PKCα that leads to internalization of Oat3 from plasma membrane [201]. Our results similarly showed that down-regulation of Oat3 by the increased ROS production via PKCα pathway in obese insulin-resistance and type I diabetic condition [27,202].

Moreover, Oat3-mediated organic anion transport was also down-regulated by the impairment of renal insulin signaling in diabetic rats [50]. ROS generate in diabetic condition can stimulate PKCα. The activation of PKCα can cause the internalization of renal Oat3 into cytoplasm leading to the decrease of renal Oat3 expression at basolateral membrane and finally down-regulates transporter function in diabetic condition (Figure 6). More recently, kidney biopsy specimens from patients proved DN have revealed a marked reduction of Oat3 mRNA expressions in parallel with a significant decrease of urinary organic anion metabolite [203]. This indicated that renal Oat3 in proximal tubules were influenced by certain pathological status and strongly correlated with the progression of diabetic kidney disease [203].

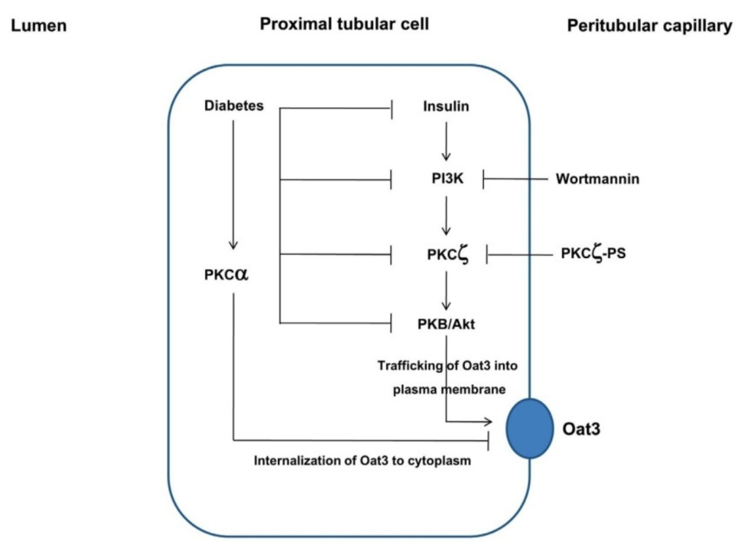


Figure 6 The regulation of renal Oat3 in diabetic condition [50].

5. Renal glucose filtration and reabsorption

The kidney can also influence glucose homeostasis not only by release of synthesized glucose via gluconeogenesis into the circulation and its utilization of glucose but also by returning glucose to the circulation via the reabsorption of glucose from glomerular filtrate [204]. Approximate 160 to 180 grams of glucose per day are filtered through kidneys. Reabsorption of glucose occurs mainly in the proximal tubule and almost filtrated glucoses are completely reabsorbed under normal conditions [205]. There are two different energy transporter proteins, sodium glucose co-transporters (SGLTs) (Figure 7) :- SGLT1, which is found in the straight portion of the proximal tubule (S2 and S3 segment), and SGLT2, which is located in the convoluted portion of the proximal tubule (S1 segment)[206]. The most of 90% of filtered glucose is reabsorbed through by SGLT2, and the energy for SGLT-mediated active transport of glucose across the cell membrane is derived from the electrochemical potential gradient of sodium which is maintained by the transport of intracellular sodium ions into the blood circulation via Na^+-K^+ ATPase pump, which is located in the basolateral membrane [206]. In the tubular cell, glucose binds to glucose transporters (GLUTs), and is passively transported across the cell membrane from the intracellular compartment into the blood circulation (Figure 7) [207].

An elevated renal tubular transport maximum for glucose (T_mG) reported in individuals with diabetes, also contributes to the worsening of hyperglycemia [208]. The increase in renal threshold and T_mG most likely represents an evolutionary adaptation to prevent glucose loss in response to hyperglycemia and to conserve energy during condition of starvation [204]. Furthermore, the increases in renal SGLTs and GLUTs expressions or activities might be the possible mechanisms for increasing glucose reabsorption. Clinical studies in proximal tubular cells isolated from the urine showed that in a hyperglycemic culture environment, both SGLT2 and GLUT2 mRNA levels and glucose transport were significantly higher in the T2DM group versus controls [209]. In rodent models of diabetes, similar results demonstrated that the expressions of renal SGLT1, SGLT2, and GLUT2 were significantly increased compared with normal controls [210,211]. However, the impact of obese-insulin resistance on renal tubular SGLTs levels is unknown.

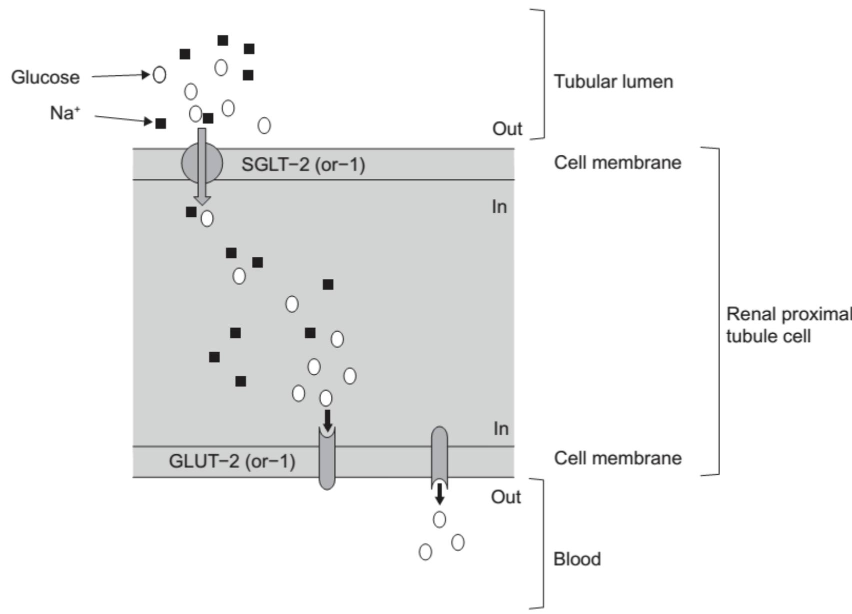


Figure 7 Renal glucose transportation in proximal tubular epithelial cell [212].

6. Sodium glucose co-transporter type 2 (SGLT2) inhibitors

The most of 90% of filtered glucose is reabsorbed through by SGLT2 which is almost located exclusively in the convoluted portion of the proximal tubule (S1 segment)[206]. Therefore, selective inhibition of this protein affects glucose reabsorption in the kidney but not in other tissues. Pharmacological inhibition of SGLT2 promotes renal glucose excretion, thereby lowering plasma glucose levels without affecting hypoglycemia (Figure 8) [205]. At present, the sodium glucose co-transporter 2 inhibitors (SGLT2 inhibitors) which have been clinically approved for the treatment of T2DM include canagliflozin, dapagliflozin, and empagliflozin [183].

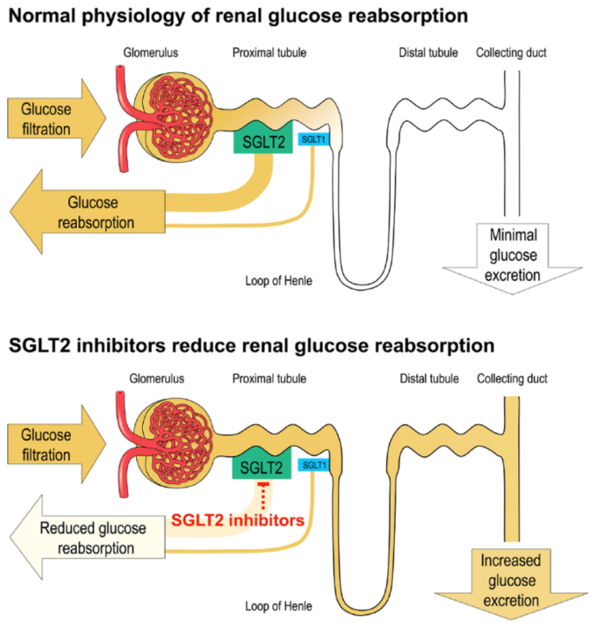


Figure 8 Mechanism of action of SGLT2 inhibitors [213].

7. The renal effects of SGLT2 inhibitors in diabetes

SGLT2 inhibitor targets the kidney to promote urinary glucose excretion (UGE), reduce hyperglycemia, and thereby improving glycemic control independent of insulin. Clinically, SGLT2 inhibitors have been used in combination with any of the existing classes of glucose-lowering agents, including insulin [214]. However, the continued treatment with SGLT2 inhibition has been associated with modest, transient decrease in eGFR ranging from roughly 3% to 10% and volume loss due to the osmotic diuresis [215]. Other adverse events seen with SGLT2 inhibition treatment include genital mycotic infections, urinary tract infections (UTI) [215]. The tubular hypothesis has been elucidated in experimental models of diabetic mellitus demonstrating that administration of SGLT2 inhibitor leads to reduced renal hyperfiltration and histological evidence of DN [216,217]. Glomerular hyperfiltration (increased GFR, also called hyperfiltration) is a proposed mechanism for renal injury in several clinical conditions [218]. At the single-nephron level, hyperfiltration is hypothesized to be prelude of intraglomerular hypertension leading to albuminuria. Elevated glomerular capillary hydrostatic pressure may be due to changes in systemic arterial pressure and/or changes in efferent and afferent arteriolar resistances. In the absence of therapeutic interventions, GFR then falls progressively in parallel with a further rise in albuminuria, in the long run, to ESRD [218].

Study in T1DM mice showed that 15 week empagliflozin treatment lowered blood glucose level together with improved DN lesion as indicated by reduced glomerular hypertrophy, renal inflammation, urinary albumin excretion along with eGFR [51]. Moreover, empagliflozin also prevented the increase in blood pressure and renal gluconeogenesis [51]. These results suggested that empagliflozin could modulate eGFR via restored tubuloglomerular feedback (TGF) mechanism, and attenuated development or progression of DN. In streptozotocin (STZ)-induced diabetic rats, empagliflozin treatment suppressed expression levels of receptor for advance glycation end product (RAGE), 8-hydroxydeoxyguanosine (8-OHdG), and F4/80, markers of oxidative stress and macrophages, and thereby preventing the expressions of inflammatory and fibrotic genes in the kidneys although the serum creatinine and urine albumin was not improved. These findings suggest that empagliflozin could inhibit oxidative, inflammatory and fibrotic reactions partly via suppression of the AGE-RAGE axis [219].

Previous studies regarding the antihyperglycemic effect and non-glycemic effects of SGLT2 inhibitors are limited to diabetic animals or patients. Especially, effect of SGLT2 inhibition on kidney function in pre-diabetic or metabolic syndrome is unknown. In the present study, we investigated the renoprotective effect of dapagliflozin in obese-insulin-resistant rat.

Materials and methods

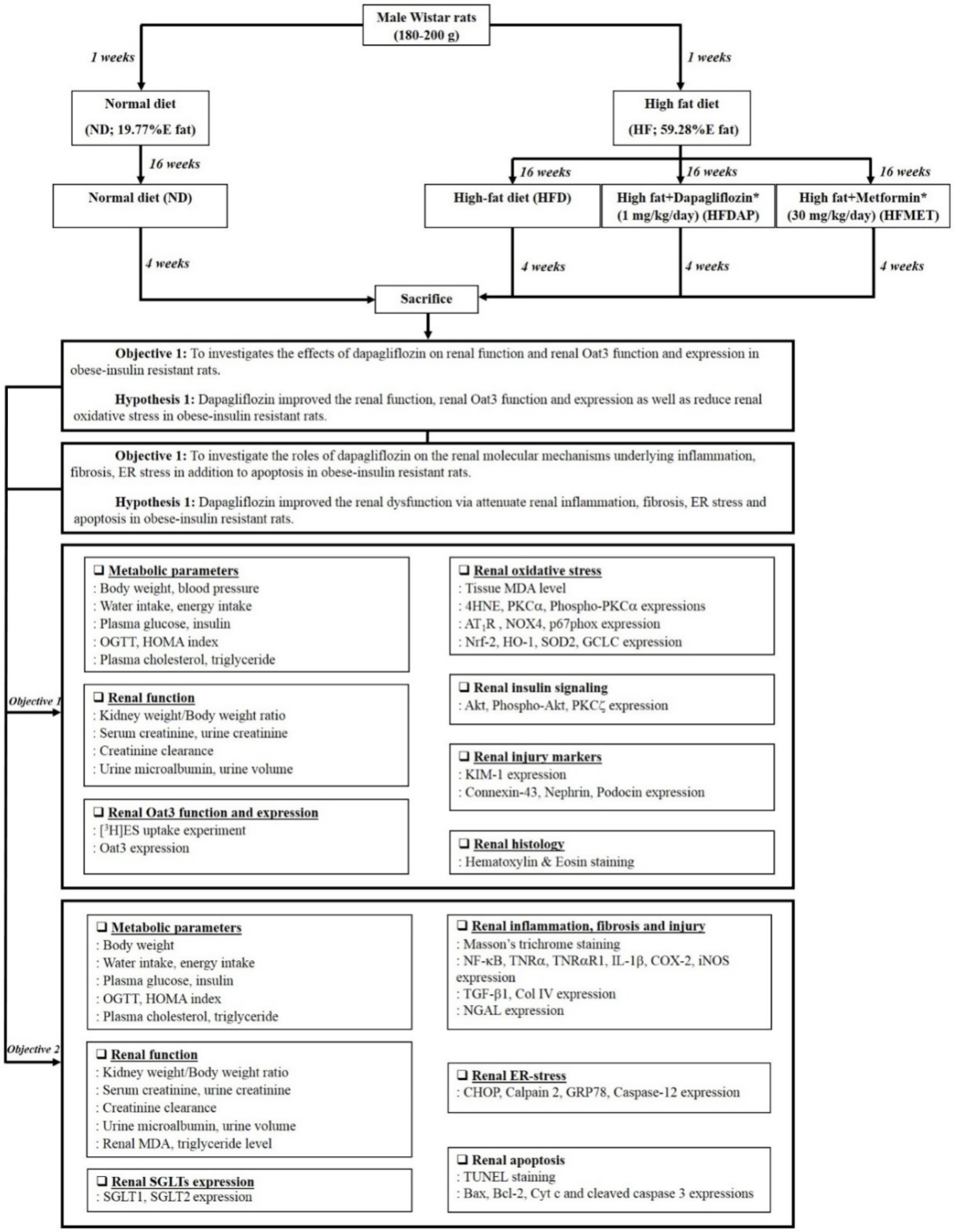
1. Animal preparation and treatment

Male Wistar rats (180-200 g) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. The animal facilities and protocols were approved by the Laboratory Animal Care and Use Committees at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Dapagliflozin (SGLT2 inhibitor) was obtained from AstraZeneca (Thailand) Limited, Bangkok, Thailand. All experimental rats were housed under controlled temperature (25±1°C) and lighting in a 12:12-hrs-light/dark cycle (lights on 06.30-18.30) with food and water ad libitum. The rats were allowed to acclimatize for 7 days before the beginning of the experiment.

2. Experimental design

Animals were divided randomly into two dietary groups: normal diet and high-fat diet groups. The animals in normal-diet (ND) group (n=6) were received standard rat chow (C.P. Mice Feed Food No. 082; energy content 4.02 kcal/g) containing fat for 19.77 % of total energy (%E) while the animals in high-fat diet (HFD) group (n=18) were consumed a high-fat diet which had energy content of 5.35 kcal/g and fat for 59.28 %E [220]. The composition of the normal diet and high-fat diet are shown in Table 1-1 and Table 1-2, respectively. After 16 weeks, the animals in high-fat diet group were divided into four groups (six rats per group). (1) high-fat diet (HFD); (2) high-fat diet plus dapagliflozin (HFDAP*);* and (3) high-fat diet plus metformin (HFMET) groups. After 16 weeks, the HFDAP and HFMET groups received either dapagliflozin (AstraZeneca (Thailand) Limited, Bangkok, Thailand)at dose of 1.0 mg/kg/day [221] or metformin (Glucophage, Merkserono, Bangkok, Thailand) at dose of 30 mg/kg/day [222] while the ND and HFD groups were given just saline as a control vehicle. Saline and the drugs were given by oral gavage for the final 4 weeks of the experimental period. All animals were allowed free access to water and food which freshly provided every day and body weight were daily recorded. Blood sampling, 24-hour urine collection, and oral glucose tolerance test (OGTT) were performed at 16^{th} and 20^{th} weeks. At the end of the study, the animals were sacrificed, blood and kidney tissue samples were collected for subsequent experiments.

3. Research process flow diagram



*Dapagliflozin and Metformin were dissolved in normal saline that were administered by gastric gavage

Note : Blood sampling and 24 hour urine collection, and oral glucose tolerance test were performed at 16^{th} and 20^{th} weeks.

Table 1.1. Composition of normal diet

Composition	Normal diet		
	g	**kcal**	**%E**
Carbohydrate	495.30	1981.20	51.99
Fat	83.70	753.30	19.77
Protein	269.00	1076.00	28.24
Vitamins	65.40	-	-
Fiber	34.30	-	-
Total	947.70	3810.50	100
Kcal/g	4.02 kcal/g		

Table 1.2. Composition of high fat diet

Compositions	High fat diet		
	g	**kcal**	**%E**
Carbohydrate	190.76	763.04	14.27
Fat	342.24	3080.16	57.60
Protein	353.60	1414.40	26.45
Cholesterol	10	90	1.68
Vitamins	85.19	-	-
DL-Methionine	3	-	-
Fiber	13.21	-	-
Yeast powder	1	-	-
Sodium chloride	1	-	-
Total	1000	5347.60	100
kcal/g	5.35 kcal/g		

Note: Diet ingredients and nutrient analyses were modified from Srinivasan *et al* [220]. Energy (kcal) per gram: carbohydrate 4; fat 9; Protein 4.

5. Animal sacrifice

At the end of experiment, the animal was anesthetized with isoflurane inhalation, the abdominal cavity was opened and the blood sample was withdrawn from inferior vena cava.

After that, the kidneys were immediately removed, decapsulated and weighted. One kidney was kept for determination of renal Oat3 transport function, western blot analysis and measurement of malondialdehyde (MDA). The other kidney was cut in longitudinal line into two half; one half was kept for morphological analysis while the other half of renal cortex was sliced for evaluating renal Oat3 uptake experiment. Then, the blood serum, plasma and renal tissue were placed in liquid nitrogen and stored at-20 or -80 ºC for further studies.

Methodological approach for research

1. Study of oral glucose tolerance test and HOMA index

After overnight fasting, a 0-minute blood sample (0.5 ml) were collected by cutting the tail tip as baseline value, and then glucose solution (2 g/kg body weight) was administrated by gavage feeding. Thereafter, the blood samples (0.5 ml) was taken at 30, 60 and 120 minutes after glucose loading. All blood samples were collected in microcentrifuge tubes containing sodium fluoride (NaF) or ethylenediaminetetraacetic acid (EDTA) and kept on ice until being centrifuged to separate the plasma. The plasma samples were frozen at -20 °C for measurement of plasma glucose and insulin concentrations. The increment of plasma glucose following the glucose loading was expressed in term of the area under the curve (AUCg) for glucose, using the trapezoidal rule.

To determine insulin resistance, the fasting plasma glucose and the plasma insulin concentrations were used to calculate the Homeostasis Model Assessment (HOMA) [223]. The increase of HOMA index represents a higher degree of insulin resistance.

$$\textbf{HOMA-IR} = \left[\text{Fasting insulin level } (\mu\text{U/ml}) \text{ x Fasting glucose level (mmol/L)}\right]/22.5$$

2. Biochemical analysis

Plasma glucose, triglyceride and cholesterol concentrations were determined by the enzymatic colorimetric method using a commercial kit (Erba Diagnostics Mannheim GmbH, Mannheim, Germany). Plasma insulin concentration was measured using a Sandwich ELIZA (Millipore, MI, USA). Serum and urine creatinine levels were measured by following enzymatic colorimetric methods using commercial kits (Diasys Diagnostic Systems GmbH, Holzheim, Germany), and the data were expressed as mg/dl. Urine microalbumin was detected with commercial assay kit (Roche Diagnostics, Mannheim, Germany), and the data were expressed as mg/g creatinine.

3. Determination of creatinine clearance or glomerular filtration rate (GFR)

To determine renal function, the GFR was calculated using the following equation:-

GFR (ml/min) = [Urine creatinine (mg/dl) x Urine flow rate (ml/min)] / Serum creatinine (mg/dl)

4. Histopathological studies

4.1 Hematoxylin and Eosin (H&E) staining

The kidney was fixed in fresh 4% % paraformaldehyde in PBS for 24 hours and embedded in paraffin. Each slide was cut into 5 μm thick sectionsand stained with Hematoxylin and Eosin (H&E). Bright-field microscopic evaluation was used to determine morphological changes in the tissues. The semiquantitative determination of renal lesions was assessed by a method modified from previous studies [177,199]. Renal injury score was defined as the infiltration ofmononuclear cells, renal tubular desquamation, the existance of pyknotic nuclei and renal fibrosis. A total of five fields were scored from each sample and an average calculated. Scores from different sections were then summed up to obtain an average score per field for each experimental group.

4.2 Masson's trichrome staining

After being fixed with 4% paraformaldehyde in PBS,embedded in paraffin, and serially sectioned (5 μm), to evaluatecollagen deposition or fibrosis in the kidney was stained with Masson's trichromesolution (Millipore, MA, USA). Histological evaluation was performed in the central zone of the renal cortex. Images were viewed under a bright-field microscopic system. Two histological fields for each kidney tissues were analyzed. The observer performed semiquantitative morphometric analysis in a blinded manner. The semiquantitative determination renal fibrosis was classified as follows: 0 (nil), 1 (<25%), 2 (25-50%), 3 (50-75%), and 4 (>75% of interstitium). All the histological protocols were in accordance with the standard procedures demonstrated previously [224].

5. Renal lipid extraction and accumulation

Kidney triglyceride was determined as described previously [225] with some modifications. Briefly, kidney tissue (50 mg) was homogenized and extracted with 1 ml of ice-cold isopropanol and centrifuged (8,000 rpm, 4°C) for 15 minutes then the supernatant contents of total trilglyceride in the kidneys were determined through commercial kits (Erba Diagnostics Mannheim GmbH, Mannheim, Germany).

6. Determination of renal oxidative stress

Measurement of malondialdehyde (MDA) level, a marker of oxidative stress was carried out in the renal cortical tissue. Briefly, the renal cortical tissues were cut and suspended in CellLyticMT mammalian tissue lysis/extraction reagent (Sigma Aldrich, MO, USA) containing 1% complete protease inhibitor cocktail (Roche Applied Science, IN, USA) according to the manufacturer’s protocol. The tissue was homogenized and centrifuged at 1,600 g for 10 minutes at 4°C. Supernatants were collected and protein contents were measured by the Bradford method [25]. Tissue MDA concentrations were measured according to the manufacturer’s instructions (Cayman Chemical Company, Ann Arbor, MI, USA). The amount of MDA was expressed as nmol/mg protein [25,226].

7. Determination of renal apoptosis by Terminal deoxynucleotidyltransferased UTP nick end labeling (TUNEL) assay

. To identify apoptosis, TUNEL assay was performed on paraffin embedded kidney tissue sections to assess DNA fragmentation, according to the manufacturer's instructions (Millipore, Billerica, MA, USA) as previously described [43]. The number of TUNEL-positive cells were manually counted under the light microscopy in a blinded manner. The data are presented as number of TUNEL-positive cells [227].

8.Determination of renal Organic anion transporter 3 (Oat3) function

The renal Oat3 function assessment was performed using renal cortical slice uptake assay [25,226]. The kidneys were removed, decapsulated and placed into phosphate buffer saline (PBS). Thin renal cortical slices ($\leq$0.5 mm; 5-15 mg, wet weight) were cut with a Stadie-Riggs microtome and maintained in ice-cold oxygenated modified Cross and Taggart buffer containing 95 mM NaCl, 80 mM mannitol, 5 mM KCl, 0.74 mM $CaCl_2$, and 9.5 mM

Na_2HPO_4(pH 7.4). The renal cortical slices were pre-incubated for 10 min in 1 ml Cross and Taggart buffer and then moved to 0.5 ml of Cross and Taggart buffer containing 50 nM [^{3}H]estrone sulfate (ES) for 30 min at room temperature, samples being gently shaken. To prevent further reaction, the renal cortical slices were washed in ice-cold 0.1 M $MgCl_2$ solution. After that, the slices were blotted on filter paper, weighed, then put in the scintillation vial containing 0.4 ml of 1 M NaOH and incubated overnight whilst being gently shaken. The preparation was neutralized using 0.6 ml of 1 M HCl, and the radioactivity was measured using a Liquid Scintillation Analyzer (PerkinElmer Life Sciences, MA, USA). To determine renal Oat3 function, the [^{3}H]ES uptake was calculated using disintegrations per unit time (dpm) as the following equation:-

Tissue to medium ratio (T/M) = (dpm/g tissue) / (dpm/ml medium)

9. Western blotting assay

9.1 Tissue preparation and Western blot analysis+

To determine protein expressions in each cellular component, subcellular fractions were extracted from renalcortical tissues using differential centrifugation as described previously [25,43]. Briefly, tissue from the renal cortex was gently cut from the outer part of the kidney, in sections extending down for approximately 3-4 mm, using a microtome and tissues were homogenized in CellLyticMT/extraction reagent (Sigma Aldrich, MO, USA) with a protease cocktail inhibitor (Roche Applied Science, IN, USA).

The homogenate was centrifuged at 5,000 g for 10 min at 4ºC and the supernatant was designated as whole cell lysate. The supernatant was then centrifuged at 100,000 g for 2 hour at 4ºC to obtain a membrane (pellet) and a cytosolic enriched (supernatant) fraction. The 5,000 g pellet was resuspended and centrifuged at 10,000 g for 10 min at 4ºC. The supernatant was designated as the nuclei enriched fraction. All fraction collected were stored at -80ºC until used.

9.2 Protein quantification

The quantitative total protein concentration was determined by colorimetric Bradford protein assay using commercially available kits (Bio-Rad, PA, USA). To determine total protein concentration, bovine serum albumin was used as a standard. The protein solution was quantified in duplicate in 96 well plates and the absorbance was measured at a wavelength of 595 nm using a microplate reader (Wallac 1410; Pharmacia, WallacOy, Turku, Finland).

9.3 Preparation of SDS-polyacrylamide gel

The protein in each fraction was separated using polyacrylaminde gel (10-15% gel) and transferred onto a polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA, USA). The preparation of polyacrylaminde gel; firstly, glass plate was cleaned with 70% ethanol and then assembled onto a setting ring. Separating gel was prepared with 30% acrylaminde/bis, 1.5 M Tris-HCl (pH 8.8), 10% SDS, and deionized water. Then the solutions were mixed together and degassed for 15 minutes. 10% ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added and mixed well. After that, the separating gel solution was poured into glass plate and deionized water was added on top of the gel to make the surface of gel smooth. Gel was set in about 30-45 minutes. Once setting, the isopropanol was poured off and the 4% stacking gel was made of using all of the above ingredients except the Tris-HCl buffer was 0.5 M with pH 6.8. Again, the solution was added with 10% APS and TEMED, respectively. Stacking gel solution was poured till the top of the glass plate. The comb was placed on gel and then kept the gel into 4 ºC overnight.

9.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The polyacrylamide gel was removed from the setting rig, placed in the running chamber, and then poured the running buffer 1X into the chamber. The standard marker volume 5 μl and each sample (50 or 100 μg) volume 20 or 40 μl was loaded into each lanes of gel and run in running buffer at 120 volts for 60 min by using a BioRad PowerPac 300 (BioRad Laboratories Ltd., Hemel Hemstead, UK).

9.5 Blotting/Transfer

The polyvinylided fluoride (PVDF) membrane was incubated in methanol for 5-10 minutes. The sponges and filter papers were incubated in transfer buffer until used and then PVDF was incubated with transfer buffer for 5 minutes. Once the gel had finished running, it was then removed from the running chamber and glass plates. The stacking gel was removed. Then, gel was packed together with sponge, filter paper and PVDF on gel, respectively in the cassette and then it was closed and placed in the blotting chamber. Transfer butter was filled into the blotting chamber. Ice pack was put into the chamber. The voltage was set at 100 volts to transfer the protein for 60 minutes with a BioRad PowerPac 300 (BioRad Laboratories Ltd., Hemel Hemstead, UK).

After 60 minutes, the PVDF membrane was removed from the cassette and blocked with 5% nonfat dry milk in PBS (Phosphate Buffer Saline) containing 0.1% Tween-20 (PBS-T) or TBS (Tris-Buffer Saline) containing 0.1% Tween-20 (TBS-T) buffer for 60 min at room temperature. Then, the solution was removed and PVDF membrane was washed with PBS-T or TBS-T buffer three times for 5 minutes. After washed, the PVDF membrane was incubated with primary antibody concentration overnight at 4ºC and then it was washed with PBS-T or TBS-T 5 minutes for three times. After that, PVDF membrane was incubated with secondary antibody concentration for 60 min at room temperature. Then, the membrane was washed with PBS-T and TBS-T 5 min for three times. After washing, the membrane was incubated in an enhanced chemiluminescent (ECL) reagent (BioRad Laboratories Ltd., Hemel Hemstead, UK) for 5 minutes). The ChemiDoc touch imaging system (Bio-Rad Laboratories, Hercules, CA, USA) was used to expose the membrane using the Chemiluminescence mode. Each membrane was stripped and re-probed with housekeeping protein or another antibody for further protein expression detection. Finally, the ImageJ program was used to determine the density which was normalized with housekeeping protein[25,43].

The primary or secondary antibody in the current research were purchased from Cosmo Bio (Cosmo Bio Co. Ltd., Tokyo, Japan), Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Millipore(Millipore, Billerica, MA, USA), Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA), Abcam (Abcam, Cambridge, MA, USA), and Thermo Fisher Scientific (Thermo Fisher Scientific,IL, USA), respectively. Table shows antibodies for protein detection in each signaling pathways in this experiment.

Table 2. Protein detection in each signaling pathways

Primary antibody	
Target pathway	**Target protein**
Renal insulin signaling	Akt, phopho-Akt and PKCζ
Renal gluconeogenesis and transporters	Oat3, SGLT1 and SGLT2
Renal injury markers	NGAL, Kim 1, Nephrin, Podocin and Connexin 43
Renal oxidative stress	4-HNE, NOX4, $p67^{phox}$, AT_1R, SOD2, PKCα, phospho- PKCα, Nrf2, Keap1, GCLC and HO-1
Renal ER stress	GRP78, CHOP, Calpain 2 and Caspase-12
Renal inflammation and fibrosis	NF-κB p65, TNFα, TNFαR1, IL-1β, COX-2, iNOS, TGF-β1 and Col IV
Renal apoptosis	Bax, Bcl-2, Cytochrome c and Cleaved caspase-3
Others	β-actin, GAPDH, Na-K ATPase and Lamin b1
Secondary antibody	
HRP conjugated	1. Goat anti-Rabbit IgG, HRP conjugated 2. Goat anti-Mouse IgG, HRP conjugated 3. Goat anti-Rat IgG, HRP conjugated 4. Rabbit anti-Goat IgG, HRP conjugated

9. Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. All data were presented as means ± SEM. Differences among groups were analyzed by a one-way ANOVA followed by a Fisher's Least significant difference test (LSD). p value < 0.05 was considered significant.

Results

1. Metabolic and renal changes after high-fat diet (HFD) consumption for 16 weeks

The initial body weights of the rats were in the range of 180-200 g. As shown in Table 3, consumption of HFD in rats for 16 weeks produced significant increases in body weight, energy intake, plasma cholesterol level, and total area under the curve for glucose (TAUCg) compared with those observed in the normal diet fed rat group (ND) ($p < 0.05$). Regarding renal function, serum creatinine level, urinary albumin excretion, and 24-hr urine volume were significantly higher in HFD, HFDAP and HFMET rats than those of the ND rats ($p < 0.05$). However, 16 weeks of HFD consumption did not lead to any significant changes in plasma glucose and triglyceride levels in all groups. These results indicated that long-term consumption of a high-fat diet produced obesity, insulin-resistance and impaired renal function.

Table 3. Characteristics of obese-insulin resistance in rats fed with HFD for 16 weeks

Parameters		***Experimental groups***		
	ND	**HFD**	**HFDAP**	**HFMET**
Body weight (g)	495.00 ± 9.92	647.50 ± 28.06*	603 ± 28.94*	623.33 ± 51.20*
Energy intake (kCal/day)	90.60 ± 0.23	153.38 ± 1.43*	148.38 ± 2.07*	148.97 ± 4.14*
Water intake (ml/day)	32.83 ± 3.11	35.83 ± 6.11	33.33 ± 3.57	34.17 ± 3.27
Plasma glucose (ml/dl)	100.83 ± 4.61	103.09 ± 11.98	114.39 ± 9.17	110.41 ± 5.20
Plasma glucose AUC (AUCg)(mg/dl × min × 10^4)	1.92 ± 1.10	2.21 ± 0.08*	2.16 ± 0.04*	2.25 ± 0.05*
Plasma cholesterol (ml/dl)	74.21 ± 2.93	107.94 ± 5.96*	105.46 ± 13.12*	116.19 ± 6.34*
Plasma triglyceride (ml/dl)	61.65 ± 2.59	68.47 ± 4.07	72.64 ± 10.06	65.14 ± 3.35
Serum creatinine (ml/dl)	0.27 ± 0.02	0.39 ± 0.02*	0.034 ± 0.01*	0.35 ± 0.01*
Microalbuminuria (mg/g creatinine)	17.52 ± 4.50	43.43 ± 7.72*	42.14 ± 4.25*	53.93 ± 7.80*
24-hr urine volume (ml/day)	15.17 ± 0.65	24.00 ± 2.88*	25.33 ± 3.64*	24.33 ± 2.94*

Values are mean ± standard error of the mean (SEM, n = 6 per group). $^*p < 0.05$ vs ND. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

2. Effect of dapaglifozin and metformin treatments on metabolic status in obese-insulin resistant rats

At week 20th, the body weight, energy intake, plasma cholesterol, insulin levels, HOMA index and TAUCg together with the visceral fat weight/body weight ratio, and mean arterial pressure, were significantly elevated in HFD rats compared with the ND rats ($p < 0.05$) (Table 4). Interestingly, dapagliflozin treatment led to significant decreases in the body weight, visceral fat weight/body weight ratio, mean arterial pressure, plasma insulin as well as cholesterol levels, HOMA index and TAUCg while metformin administration significantly reduced plasma insulin as well as cholesterol levels, HOMA index and TAUCg when compared with HFD rats ($p < 0.05$). Moreover, the mean arterial pressure, visceral fat weight/body weight ratio, and TAUCg were significantly lower in HFDAP rats than HFMET rats ($p < 0.05$). These results suggested that dapagliflozin treatment not only markedly reduced systemic hypertension but also effectively improved metabolic disturbance, and insulin sensitivity to a greater level than metformin in obese-insulin resistant rats.

Table 4. Effect of dapaglifozin and metformin treatments on metabolic status in obese-insulin resistant rats

Parameters	ND	Experimental groups		
		HFD	**HFDAP**	**HFMET**
Body weight (g)	531.33 ± 11.45	776.66 ± 44.11*	656.66 ± 66.90*†	698.33 ± 49.62*
Energy intake (kcal/day)	90.90 ± 0.88	153.80 ± 3.52*	149.36 ± 5.77*	149.30 ± 9.36*
Water intake (ml/day)	28.33 ± 4.08	33.33 ± 5.16	40.00 ± 8.90*	32.33 ± 6.38
Visceral fat/body weight ratio (× 10^{-2})	5.66 ± 2.02	8.78 ± 0.91*	5.94 ± 0.36†	8.93 ± 1.41*‡
Mean arterial pressure (mmHg)	88 ± 7.02	140 ± 16.12*	110 ± 2.99*†	137.93 ± 2.24*‡
Plasma glucose (mg/dl)	97.10 ± 16.20	116.46 ± 6.00	114.17 ± 7.24	107.74 ± 14.31
Plasma insulin (ng/ml)	2.25 ± 0.61	7.91 ± 0.61*	3.50 ± 1.93†	3.34 ± 0.83†
Plasma glucose AUC (TAUCg) (mg/dl × min × 10^4)	1.75 ± 0.10	2.25 ± 0.07*	1.81 ± 0.10†	1.96 ± 0.13*†‡
HOMA index	10.01 ± 3.36	41.71 ± 11.23*	16.04 ± 9.11†	15.74 ± 5.15†
Plasma cholesterol (mg/dl)	86.27 ± 17.85	111.87 ± 6.59*	89.23 ± 17.08†	91.12 ± 13.40†
Plasma triglyceride (mg/dl)	96.45 ± 21.74	103.87 ± 25.67	100.70 ± 27.88	96.45 ± 19.19

Values are mean ± standard error of the mean (SEM, n = 6 per group). *p<0.05 vs ND, †p<0.05 vs HFD and ‡p<0.05 vs HFDAP.

ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

3. Effect of dapagliflozin and metformin treatments on renal function

A decreased relative kidney weight (kidney weight/body weight ratio) accompanied by impaired renal function as shown by marked increases in serum creatinine level and urine albumin excretion presented in the HFD rats when compared with ND rats ($p < 0.05$) (Table 5). Moreover, kidney triglyceride content was significantly greater in HFD rats than ND rats ($p < 0.05$). Creatinine clearance in HFD rats was statistically increased when compared with ND rats ($p<0.05$). Dapagliflozin treatment led to significant increases in the kidney weight/body weight ratio and urine glucose excretion along with apparently reduced serum creatinine and microalbuminuria in comparison with HFD rats ($p < 0.05$). Both dapagliflozin and metformin treatments could significantly restore creatinine clearance and decreased kidney triglyceride content when compared with HFD rats ($p < 0.05$). However, metformin treatment had no effect on serum creatinine and urine albumin excretion in comparison with HFD rats. The kidney weight/body weight ratio, 24-hr. urine volume, and urine glucose excretion were significantly lower in HFMET rats than those of HFDAP rats ($p < 0.05$). These findings suggested that dapagliflozin treatment exerted a greater efficacy on preventing impairment of kidney function compared with metformin treatment in obese-insulin resistant rats.

Table 5. Effect of dapaglifozin and metformin treatments on renal function in obese-insulin resistant rats.

Parameters		Experimental groups		
	ND (n=6)	HFD (n=6)	HFDAP (n=6)	HFMET (n=6)
Kidney/body weight ratio	2.40 ± 0.09	1.63 ± 0.06*	2.10 ± 0.10†	1.52 ± 0.15*‡
Serum creatinine (mg/dl)	0.38 ± 0.02	0.45 ± 0.02*	0.38 ± 0.07†	0.40 ± 0.03
Microalbuminuria (mg/g creatinine)	14.42 ± 1.34	44.42 ± 10.15*	19.85 ± 2.98†	31.28 ± 6.15*
Creatinine clearance (ml/min)	3.14 ± 0.22	5.58 ± 1.39*	2.82 ± 0.31†	2.91 ± 0.47†
24-hr. urine volume (ml/day)	16.34 ± 1.51	20.67 ± 6.15	26.67 ± 8.82*	18.67 ± 5.89‡
Urinary glucose excretion (mg/dl)	43.16 ± 5.33	92.30 ± 15.94	4459.83 ± 199.72*†	65.57 ± 10.51‡
Kidney triglyceride content (mg/g tissue)	1.09 ± 0.39	17.31 ± 1.28*	15.16 ± 0.07*†	14.06 ± 0.19‡

Values are mean ± standard error of the mean (SEM, n = 6 per group). *p<0.05 vs ND, †p<0.05 vs HFD and ‡p<0.05 vs HFDAP.

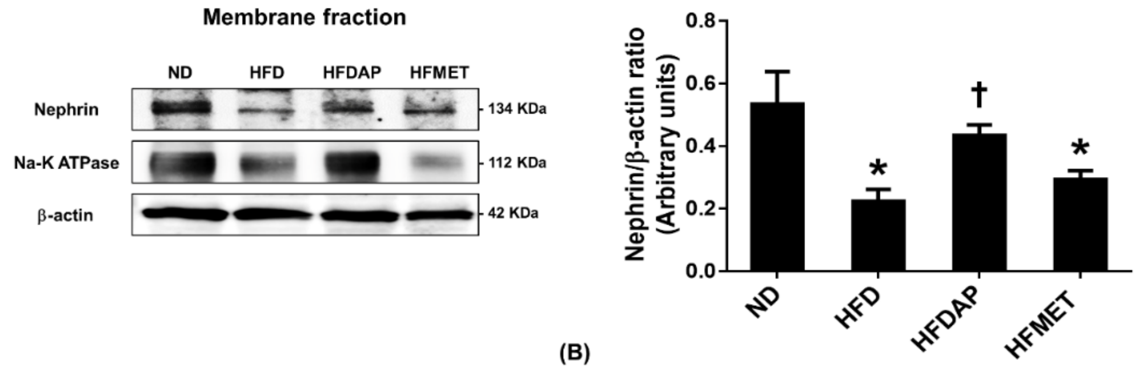
ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

4**. Effects of dapagliflozin and metformin treatments on glomerular barrier markers, tubular injury markers and renal morphology in obese-insulin resistant rats**

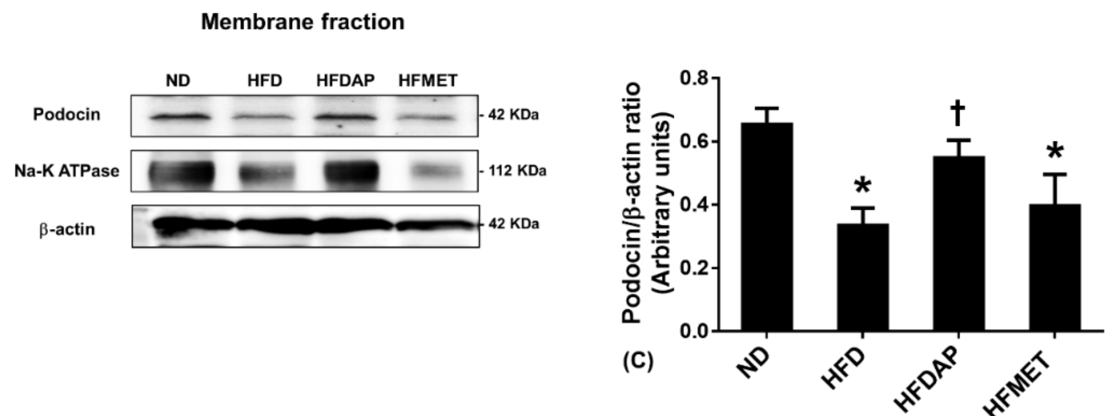
It has been shown that proteinuria and impaired renal function in HFD-induced obese rats are correlated with the decreased expression of the podocyte-specific proteins, nephrin and podocin [177]. As shown in Figure 9A, B, C, D, and E, there were significant decreases in renal expression of Nephrin, Podocin, and Connexin 43 in HFD rats when compared with ND rats ($p < 0.05$). Meanwhile, the renal expressions of Kim 1 and NGAL, the markers of renal tubular injury, were apparently higher in HFD rats than those of ND rats ($p < 0.05$). Dapagliflozin treatment led to significantly increased expressions of Nephrin and Podocin, as well as Connexin 43, and decreases in the expression of Kim 1 and NGAL in comparison with HFD rats ($p < 0.05$). However, the metformin treatment normalized only the expression of Connexin 43 and Kim 1 when compared with HFD rats ($p < 0.05$).

The representative images of Hematoxylin and Eosin (H&E) staining of the kidney showed interstitial mononuclear cell infiltration, pyknoptic nulcei and renal fibrosis which were consistent with the significant increase in kidney injury score in HFD rats in comparison with ND rats ($p < 0.05$) (Figure 9A and B). The level of histopathology as well as the scores of renal injury were reduced in HFDAP and HFMET rats when compared with HFD rats ($p < 0.05$). However, the kidney injury score was lower in HFDAP rats than that of HFMET rats ($p < 0.05$). These findings suggested that in this study dapagliflozin exerted a greater efficacy than metformin on ameliorating kidney injury in obese insulin-resistant rats.

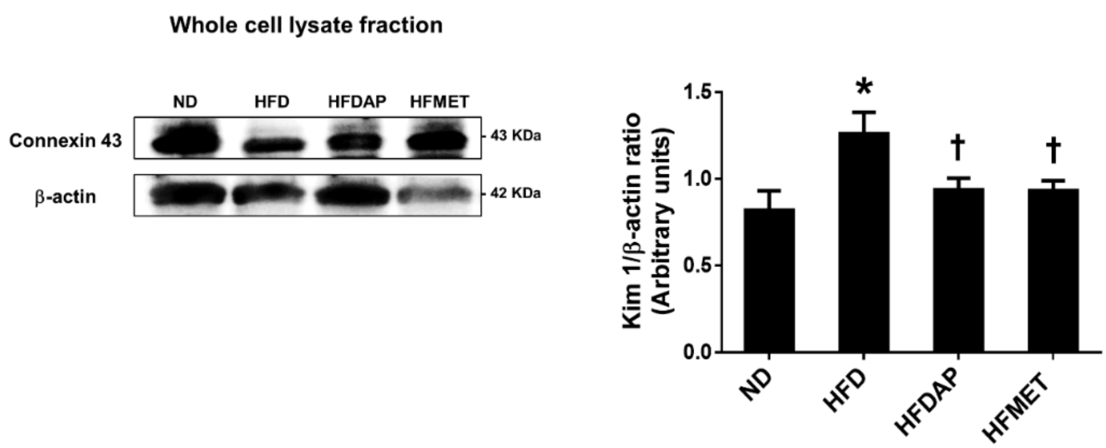
(A)


Membrane fraction
ND
HFD
HFDAP
HFMET
Nephrin
Na-K ATPase
β-actin
134 KDa
112 KDa
42 KDa
Nephrin/β-actin ratio (Arbitrary units)
0.0
0.2
0.4
0.6
0.8

(B)


Membrane fraction
ND
HFD
HFDAP
HFMET
Podocin
Na-K ATPase
β-actin
42 KDa
112 KDa
42 KDa
Podocin/β-actin ratio (Arbitrary units)
0.0
0.2
0.4
0.6
0.8
(C)

(C)


Whole cell lysate fraction
ND
HFD
HFDAP
HFMET
Connexin 43
β-actin
43 KDa
42 KDa
Kim 1/β-actin ratio (Arbitrary units)
0.0
0.5
1.0
1.5

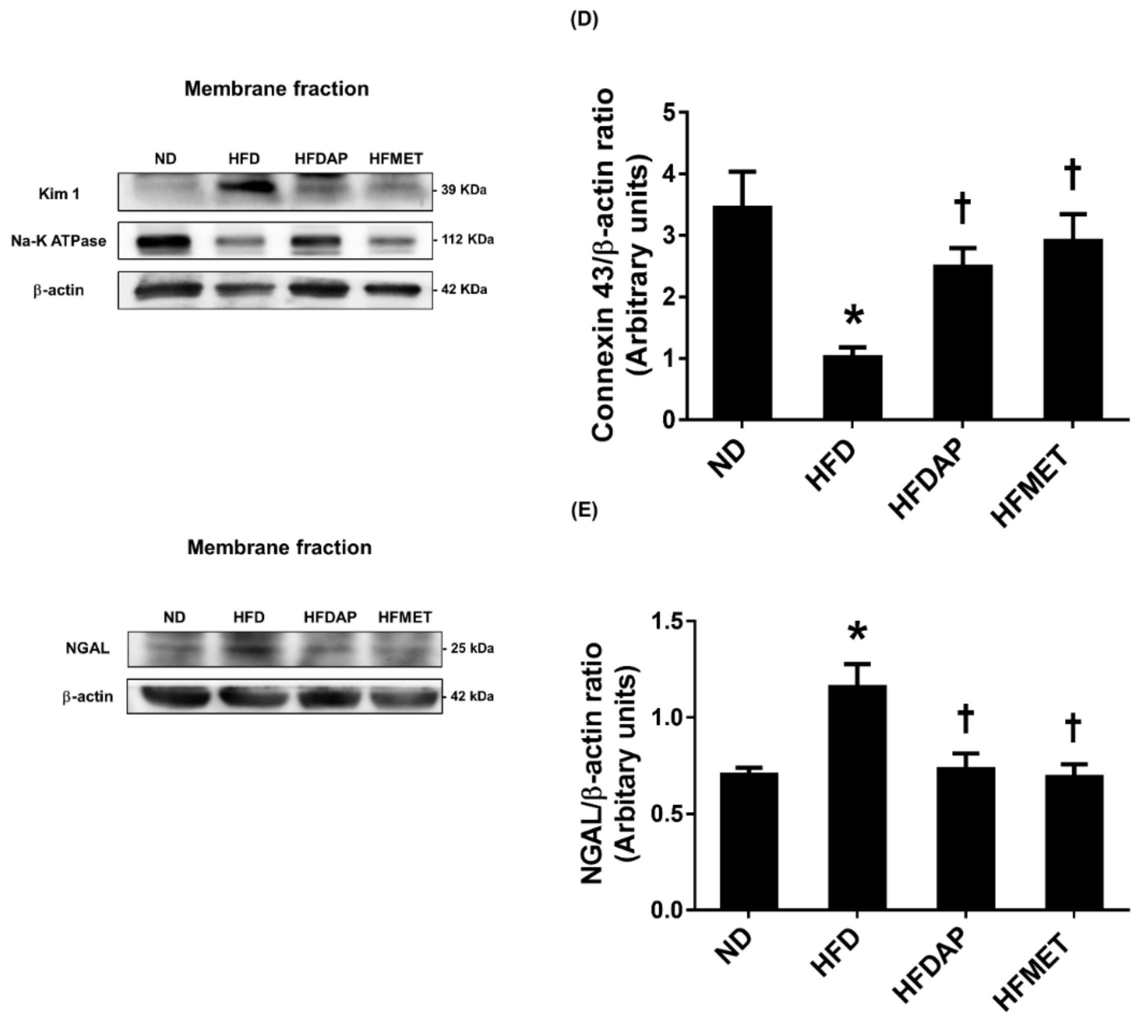


Figure 9. Effects of dapagliflozin and metformin treatments on renal expression of Nephrin (A), Podocin (B), Connexin 43 (C), Kim 1 (D) and NGAL (E) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND and $^{\dagger}p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

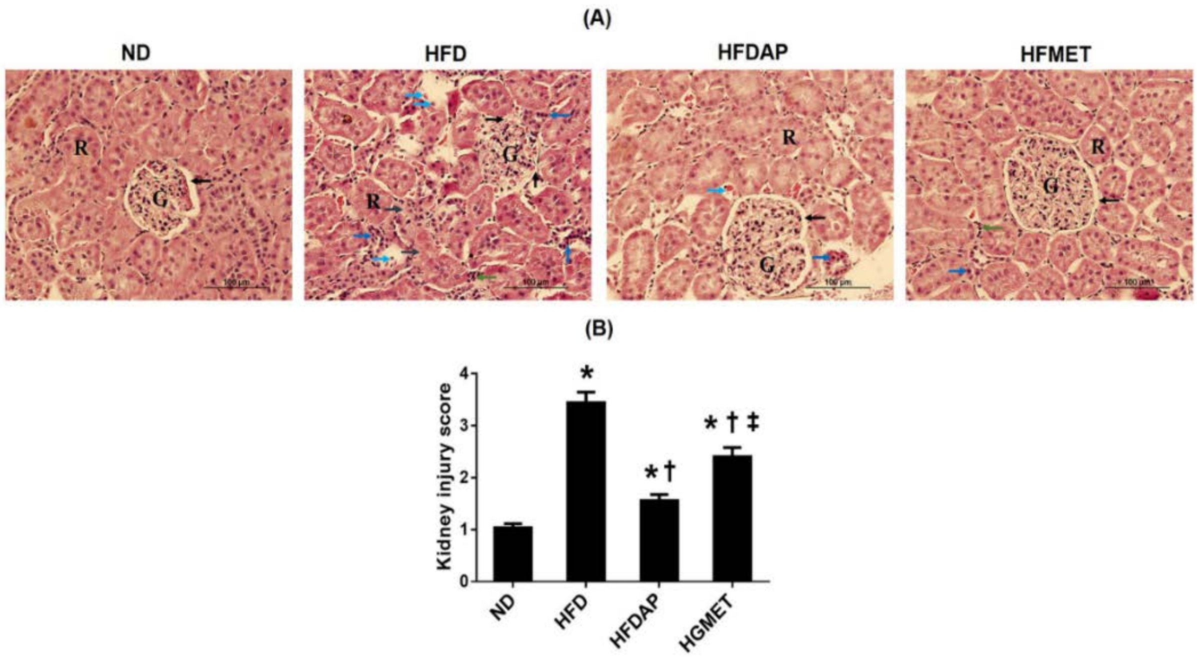


Figure 9. Effects of dapagliflozin and metformin treatments on renal photomicrographs (A) and kidney injury score (B) in obese-insulin resistant rats. Mononuclear cells infiltration (green arrow), fibrosis (grey arrow), tubular desquamation (light blue arrow) and pyknotic nuclei (blue arrow). Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND, $^{\dagger}p<0.05$ vs HFD and $^{\ddagger}p<0.05$ vs HFDAP. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups; G: glomerulus; R: renal tubule.

6. Effects of dapagliflozin and metformin treatments on renal Oat3 function and expression in obese-insulin resistant rats

Down-regulation of renal Oat3 activity concomitant with a decrease in membrane expression of renal Oat3 have been reported previously in diabetic condition [50]. To investigate renal Oat3 function, the uptake of [^{3}H]ES into renal cortical slices was measured. As demonstrated in Figure 10, the decrease in renal Oat3 function occurred in the HFD rats when compared with ND rats ($p < 0.05$). This was shown by a significant reduction in [^{3}H]ES uptake into renal cortical slices accompanied by a marked reduction in renal Oat3 expression in the membrane fraction. However, there was no significant difference in renal Oat3 expression in the whole cell lysate fraction among the experimental groups. Dapaglifozin or metformin treatment significantly restored both the uptake of [^{3}H]ES into renal cortical slices and the renal Oat3 expression when compared with HFD rats ($p < 0.05$). The renal cortical [^{3}H]ES uptake was higher in the HFMET group than that of the HFDAP rats ($p < 0.05$). However, the membrane expression of Oat3 in the HFMET group was significantly lower than that of the HFDAP rats ($p < 0.05$). These findings suggested that dapagliflozin or metformin treatment could ameliorate the decreased renal Oat3 function and expression in the obese-insulin resistant rats in this study.

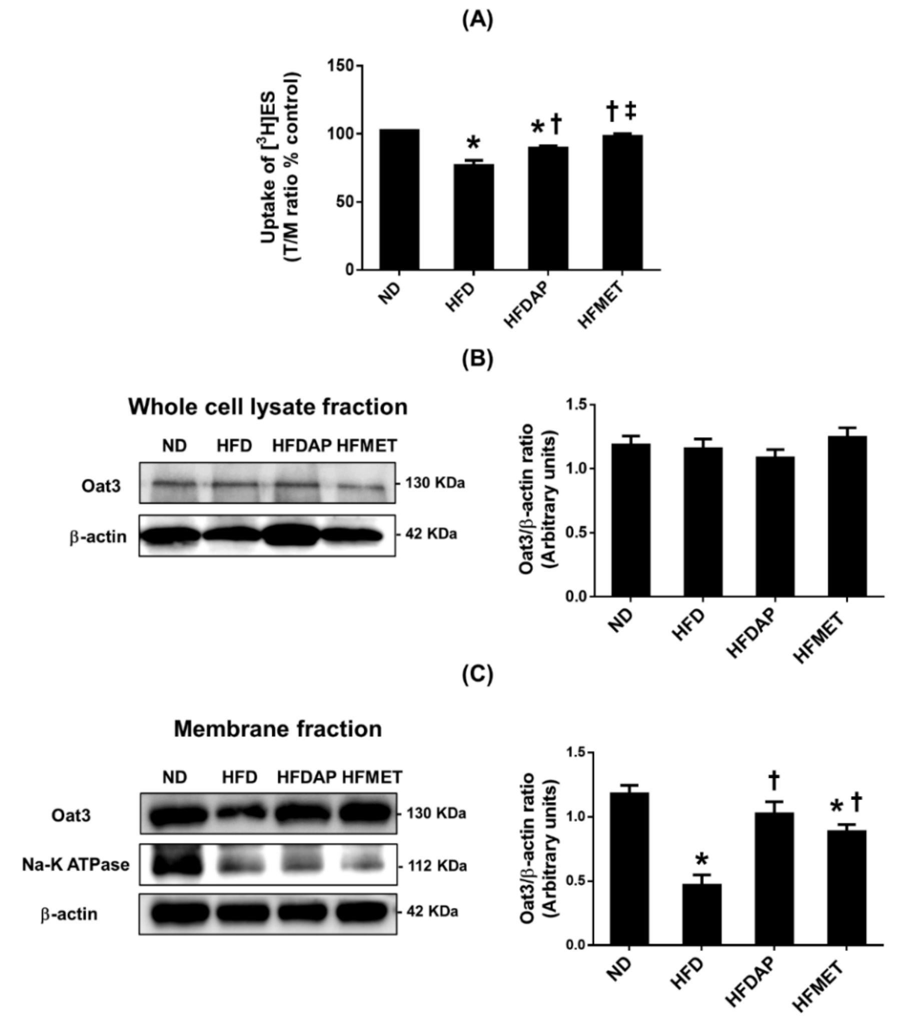


Figure 10. Effect of dapagliflozin and metformin treatments on [^{3}H]ES uptake into renal cortical slices (A), renal Oat3 expression in whole cell lysate fraction (B), and renal Oat3 expression in membrane fraction in obese-insulin resistant rats (C). Values are mean ± standard error of the mean (SEM, n = 6 per group). $^*p<0.05$ vs ND, $^\dagger p<0.05$ vs HFD and $^\ddagger p<0.05$ vs HFDAP. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

7. Effects of dapagliflozin and metformin treatments on renal oxidative stress in obese-insulin resistant rats

Previous studies demonstrated the association of down-regulated renal Oat3 expression and oxidative stress under conditions of diabetes and gentamicin-induced nephrotoxicity [24,25,50,202]. As presented in Figure 11A and B, the oxidative stress in the obese-insulin resistant condition was observed as indicated by the significant increases in both the renal cortical MDA level and 4-HNE expression in HFD rats when compared with ND rats ($p < 0.05$). These indicators of HFD-induced oxidative stress correlated with the increased renal cortical expression of NOX4, $p67^{phox}$ and AT_1R in the membrane fraction in comparison with ND rats ($p < 0.05$) (Figure 12A, B and C). For renal antioxidant, our data showed that the protein expression of renal cortical SOD2 was not different among groups (Figure 12D). Interestingly, the renal cortical MDA and 4-HNE levels were lower in rats treated with dapagliflozin or metformin than in HFD rats ($p < 0.05$) although a significant difference was noted only the renal cortical MDA level. Also, dapagliflozin or metformin treatment led to significantly decreased membrane expression of NOX4 and AT_1R in the renal cortical tissues in comparison with HFD rats ($p < 0.05$). The membrane expression of $p67^{phox}$ was lower in HFDAP and HFMET rats than in HFD rats, however, significant difference was observed only between the HFD and HFDAP rats ($p < 0.05$). Previous studies reported that the overproduction of ROS could activate PKCα, which in turn inhibited Oat3 translocation to the membrane leading to decreased Oat3 function [24,25,202,226]. As shown in Figure 12E and F, both total PKCα and activated PKCα (p-PKCα) expressions in renal cortical whole cell lysate were significantly increased in HFD rats as compared with ND rats ($p < 0.05$) and both of these were significantly reversed with dapagliflozin treatment ($p < 0.05$). Although the total PKCα and p-PKCα expression in renal cortical whole cell lysate were lower in HFMET rats than those of HFD rats, only the expression of p- PKCα was significantly different ($p < 0.05$). These results suggested that dapagliflozin or metformin treatment restores the impaired renal Oat3 expression and function by modulating renal oxidative stress in this study.

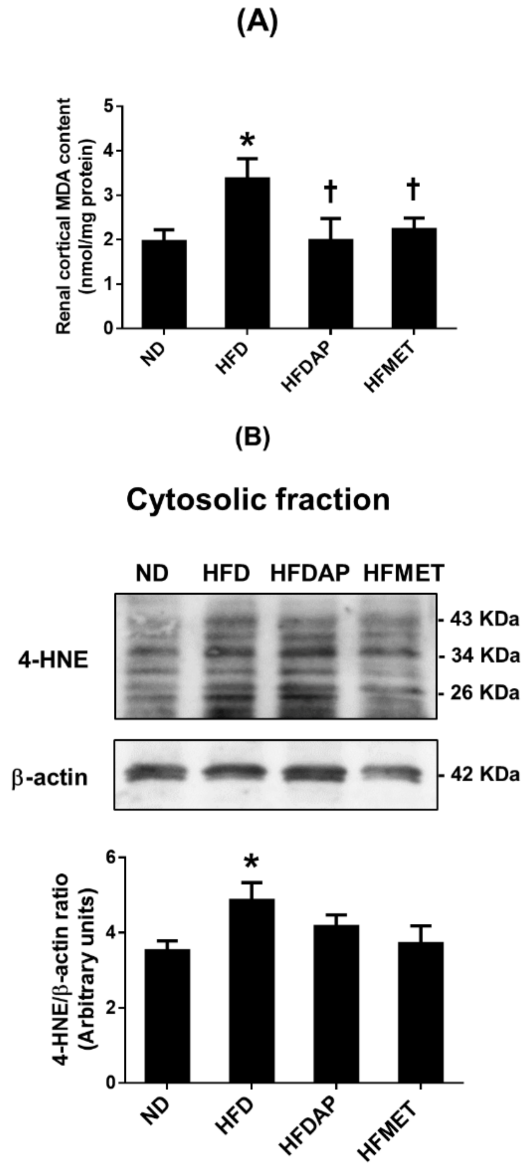
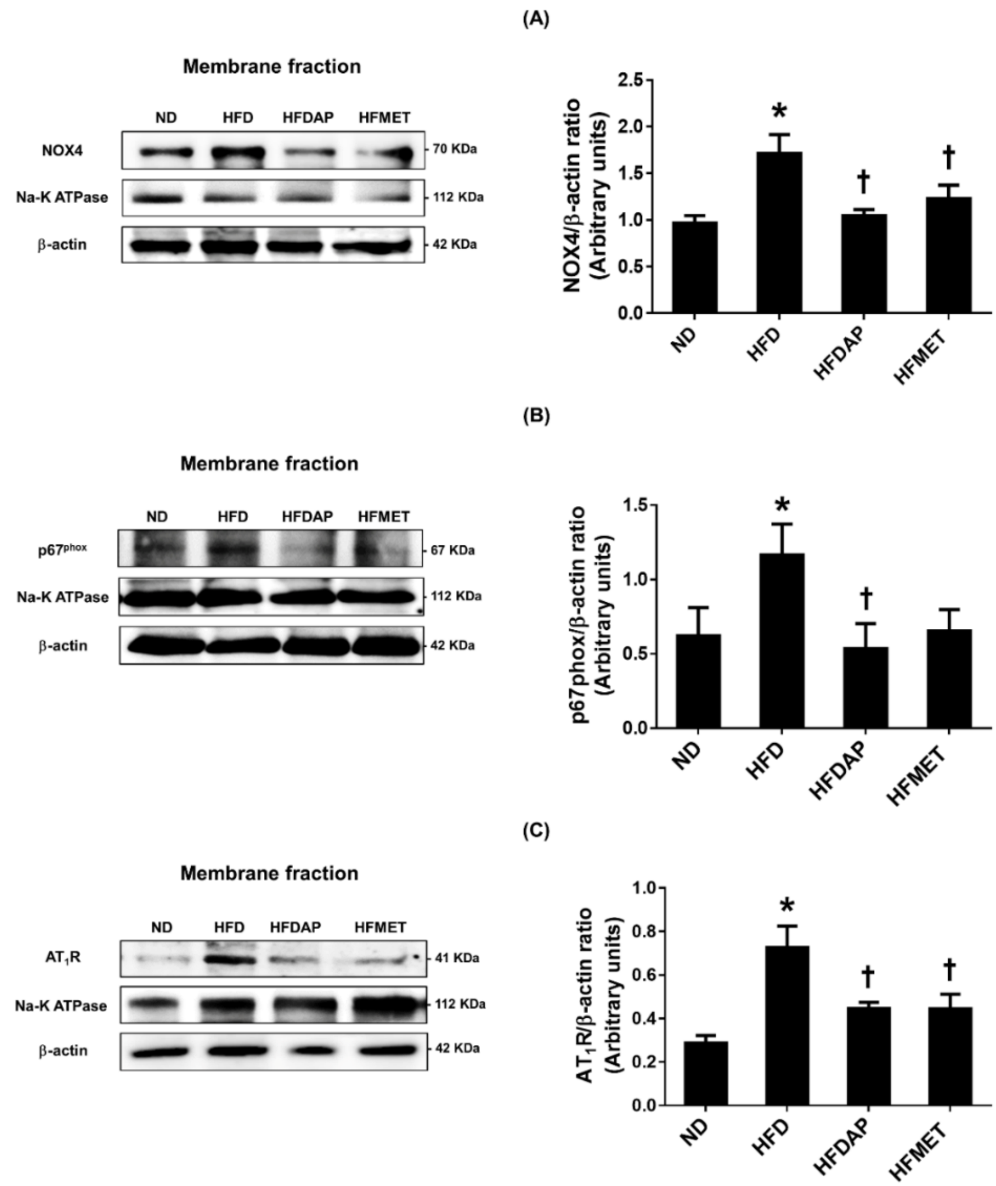


Figure 11. Effect of dapagliflozin and metformin treatments on renal cortical MDA content (A), and 4-HNE expression (B) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND and $^{\dagger}p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.


(A)
Membrane fraction
ND HFD HFDAP HFMET
NOX4
Na-K ATPase
β-actin
70 KDa
112 KDa
42 KDa
NOX4/β-actin ratio (Arbitrary units)
(B)
Membrane fraction
ND HFD HFDAP HFMET
p67phox
Na-K ATPase
β-actin
67 KDa
112 KDa
42 KDa
p67phox/β-actin ratio (Arbitrary units)
(C)
Membrane fraction
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42 KDa
AT1R/β-actin ratio (Arbitrary units)

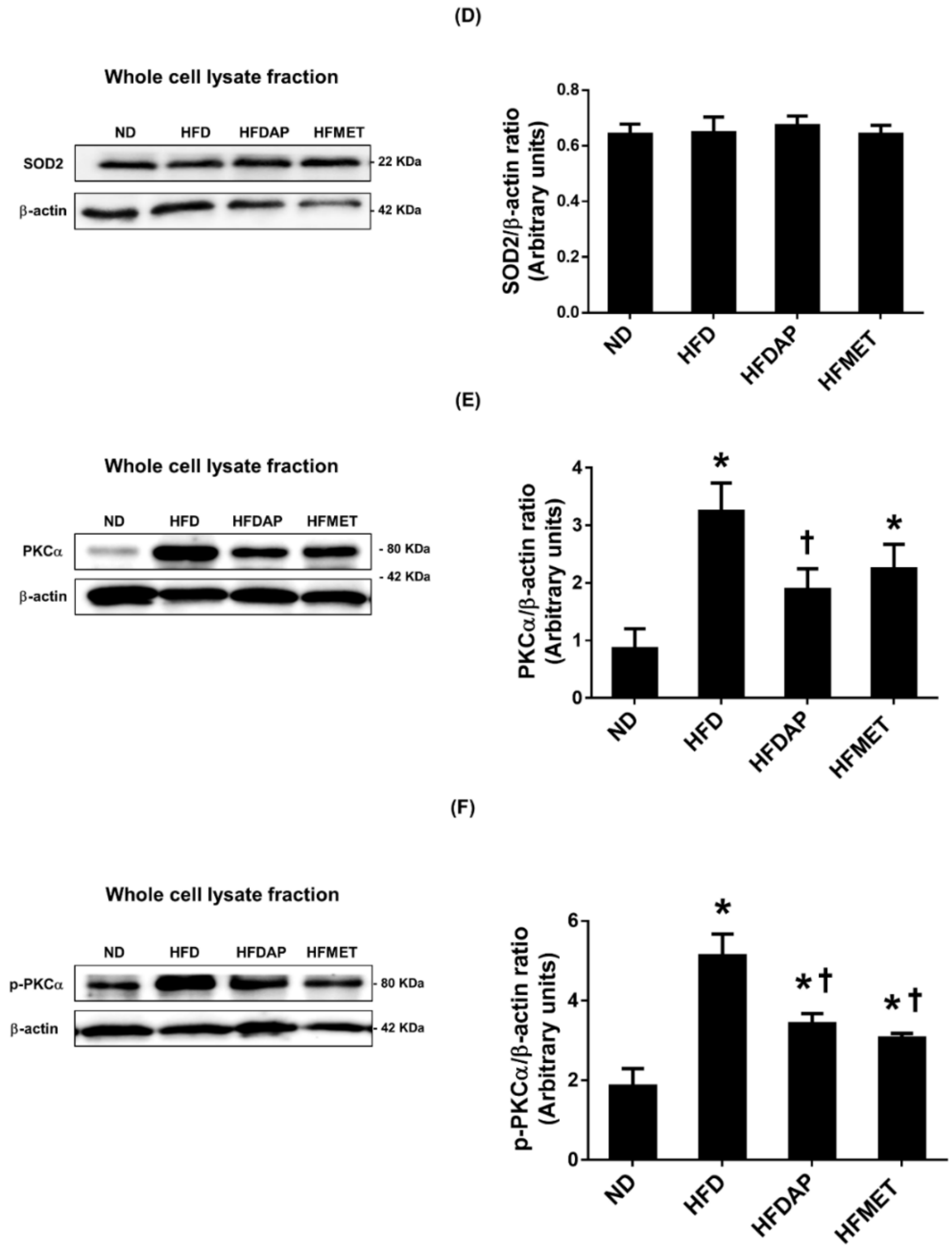
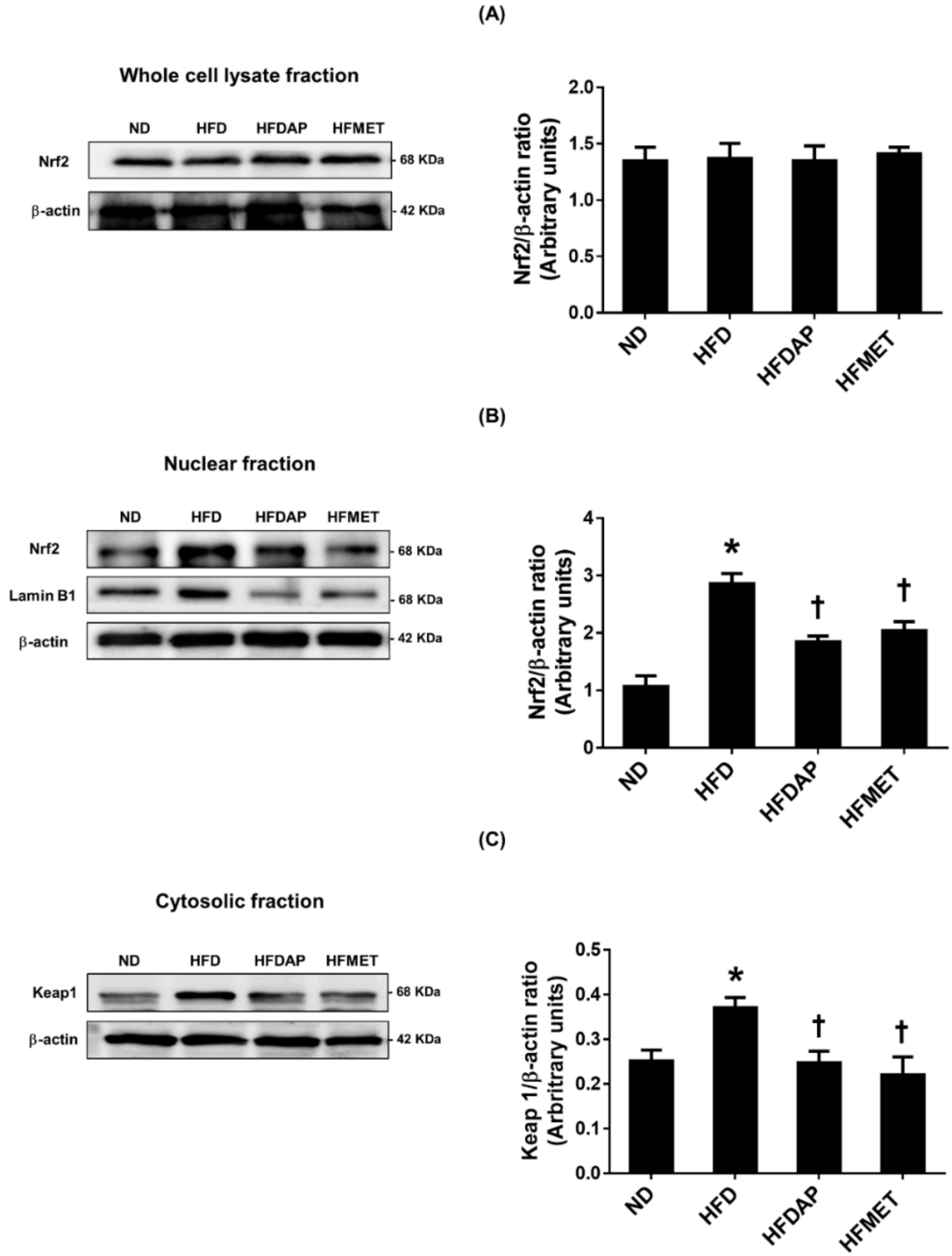


Figure 12. Effect of dapagliflozin and metformin treatments on renal expression of NOX4 (A), p67^{phox} (B), AT_1R (C), SOD2 (D), PKCα(E) and p-PKCα (F) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND and $^{\dagger}p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

8. Effect of dapagliflozin and metformin treatments on renal expression of Nrf2/Keap1 in obese insulin-resistant rats

The Nrf2/Keap1 signaling pathway is the major regulator of the antioxidants essential for cellular protection [174]. Progressing from the report that activation of PKC mediates the stimulation of Nrf2 in response to oxidative stress [228], the effect of dapagliflozin treatment on Nrf2/Keap1 system in the kidneys was next in line for analysis. The protein levels of Nrf2 in the nuclei-enriched fraction along with the expression of cytosolic Keap1, whole cell lysate GCLC and HO-1 protein levels were significantly increased in HFD rats when compared with the ND rats ($p < 0.05$) (Figure 13). These findings indicate that the translocation of Nrf2 into the nucleus leads to up-regulation of antioxidant enzymes. Interestingly, dapagliflozin and metformin treatments led to a reversal in both the nuclear expression of Nrf2 and the expression of Keap1, GCLC and HO-1 in comparison with HFD rats ($p < 0.05$). The results confirmed that in this study dapagliflozin or metformin treatment causes a decrease in renal oxidative stress probably via modulation of the Nrf2/Keap1 system in obese insulin-resistant rats.


(A)
Whole cell lysate fraction
ND HFD HFDAP HFMET
Nrf2
68 KDa
β-actin
42 KDa
Nrf2/β-actin ratio (Arbitrary units)
2.0
1.5
1.0
0.5
0.0
ND
HFD
HFDAP
HFMET
(B)
Nuclear fraction
ND HFD HFDAP HFMET
Nrf2
68 KDa
Lamin B1
68 KDa
β-actin
42 KDa
Nrf2/β-actin ratio (Arbitrary units)
4
3
2
1
0
*
†
†
ND
HFD
HFDAP
HFMET
(C)
Cytosolic fraction
ND HFD HFDAP HFMET
Keap1
68 KDa
β-actin
42 KDa
Keap 1/β-actin ratio (Arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
*
†
†
ND
HFD
HFDAP
HFMET

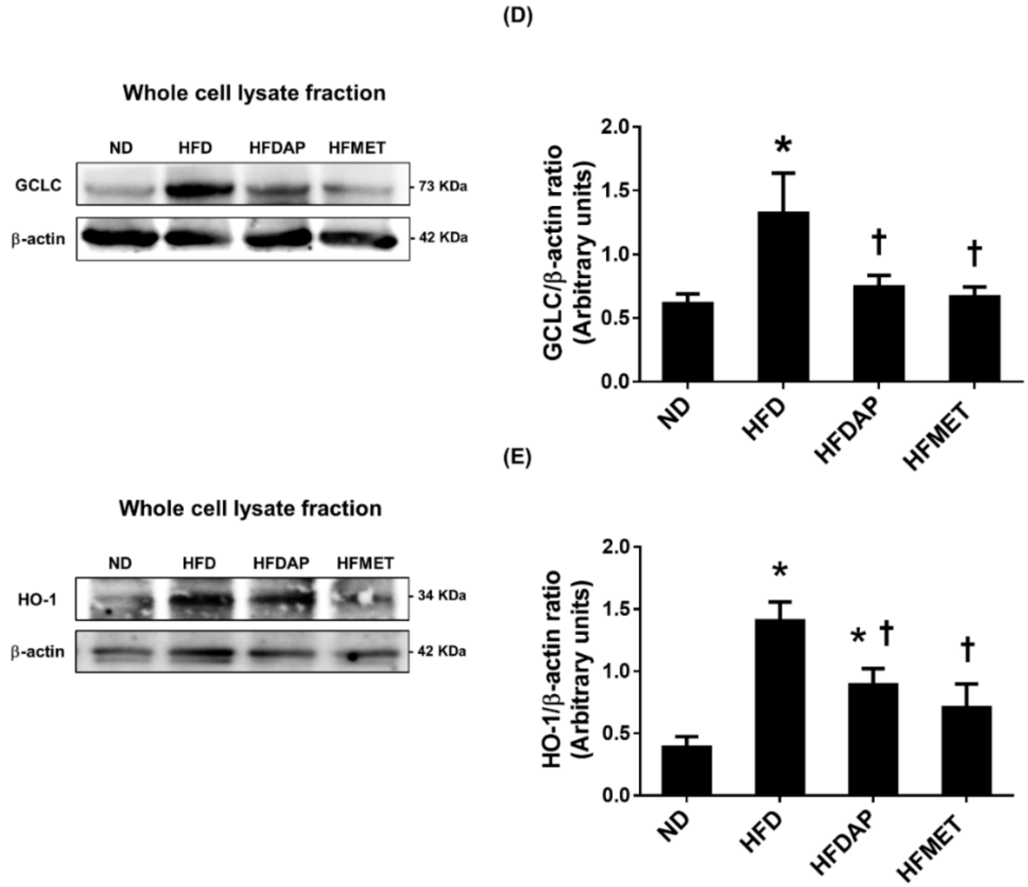


Figure 13. Effect of dapagliflozin and metformin treatments on renal expression of Nrf2 (A and B), Keap1 (C), GCLC (D) and HO-1 (E) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND and $^{\dagger}p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

9. Effects of dapagliflozin and metformin treatments on renal expression of Akt, p-Akt and PKCζ in obese-insulin resistant rats.

Previous studies in diabetic rats showed that insulin treatment could reverse the decreased renal expression and function of Oat3 via modulation of insulin signaling cascades [50,202]. To investigate whether the improved renal Oat3 function instigated by dapagliflozin treatment in obese-insulin resistant rats also involved the modulation of insulin signaling, the renal cortical expression of PKCζ, Akt and activated Akt (p-Akt) were determined. The p-Akt and PKCζ expressions were significantly decreased in HFD rats when compared with ND rats ($p < 0.05$) (Figure 14B and C) indicating an impairment in renal insulin signaling in obese-insulin resistant rats. In comparison with HFD rats, dapagliflozin treatment led to significantly restored PKCζ and p-Akt expressions ($p < 0.05$) while metformin treatment instigated a significant increase only in p-Akt expression ($p < 0.05$). These findings suggest that dapagliflozin or metformin treatment improves renal Oat3 function and expression in part via the modulation of the Akt/PKCζ signaling pathway in obese-insulin resistant rats in this study. The data also demonstrated that dapagliflozin had greater efficacy for improving renal insulin signaling and renal Oat3 function than those of metformin.

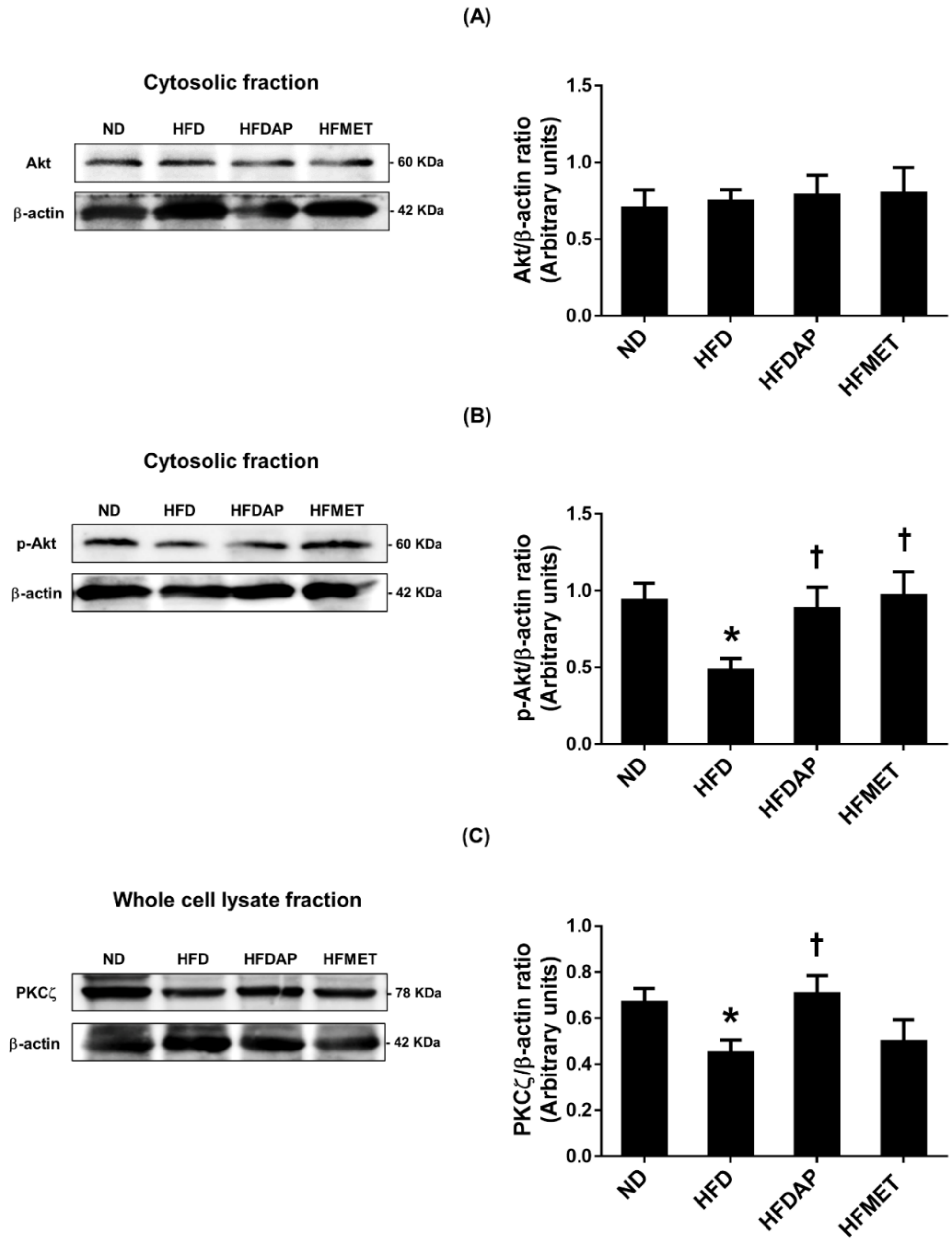
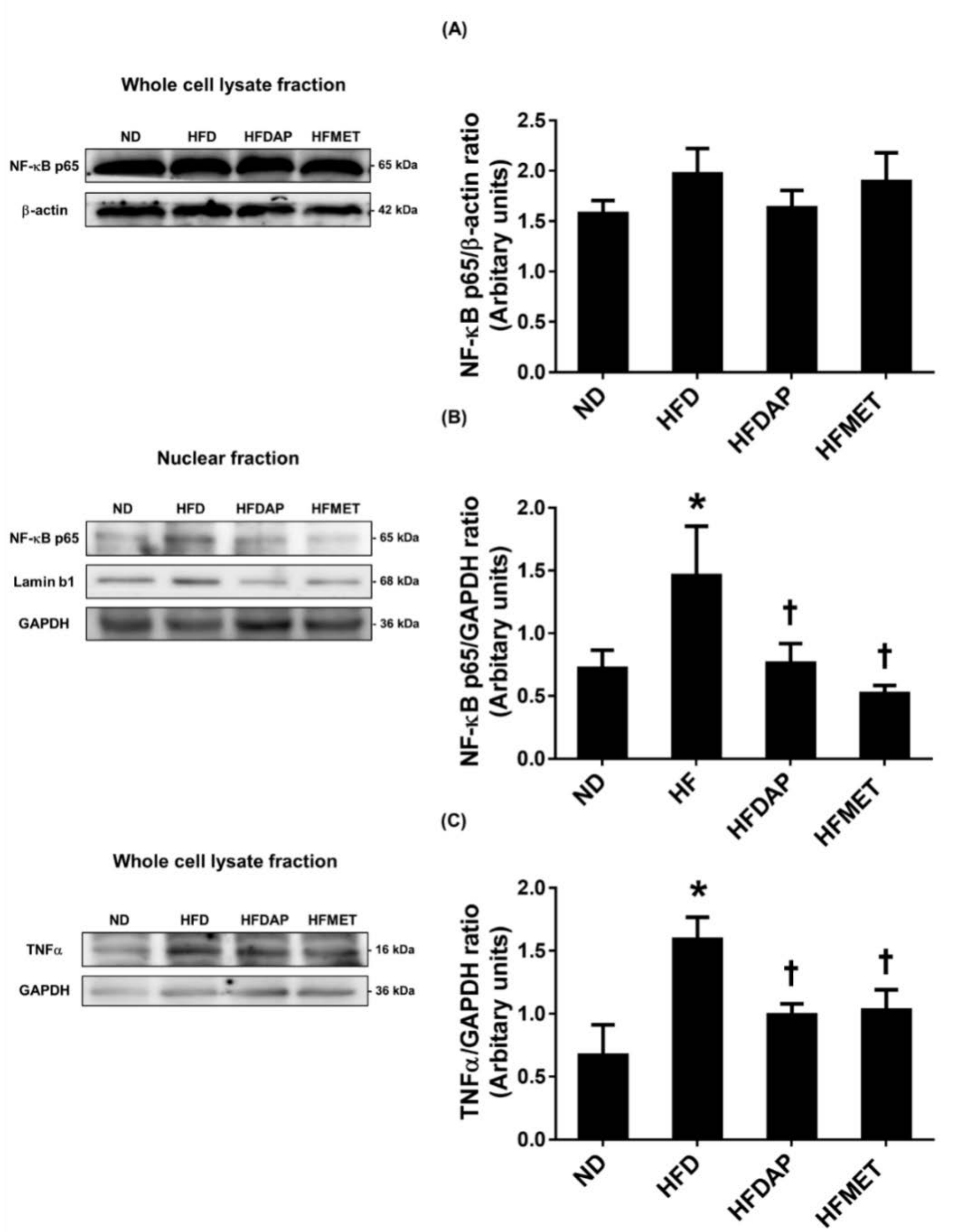


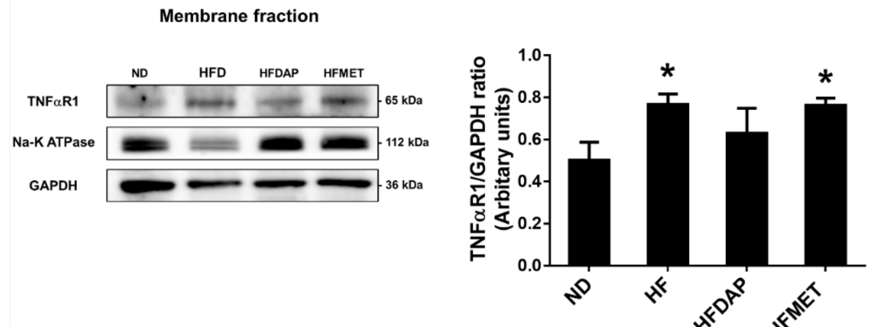
Figure 14. Effect of dapagliflozin and metformin treatments on renal expression of Akt (A), p-Akt (B) and PKCζ (C) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND and $^{\dagger}p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

10. Effect of dapagliflozin and metformin treatments on renal inflammation in obese-insulin resistant rats

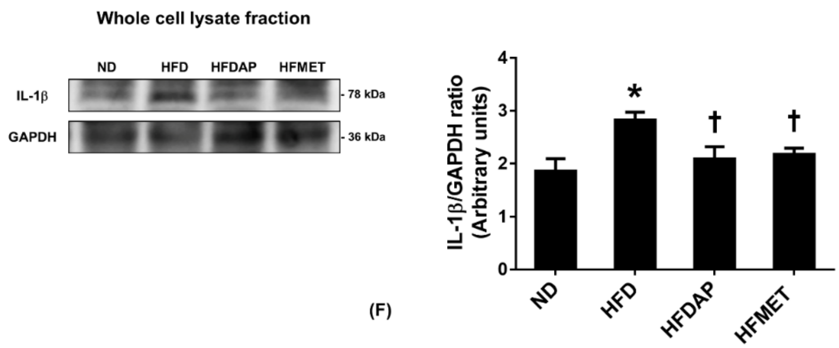
Renal inflammation via up-regulate NF-κB signaling pathway has been recently considered as a key contributor to the onset and progression of obesity-induced renal disorders [229]. As compared with the ND rats, not only the NF-κB expression in nuclear fraction of renal cortical tissues but also the protein expressions of inflammation-related factors including TNFαR1, TNFα, IL-1β, COX-2 and iNOS were significantly elevated in HFD rats ($p < 0.05$) (Figure 15). Interestingly, there were marked reduction of the NF-κB, TNFα, IL-1β, COX-2 and iNOS expressions in HFDAP and HFMET rats in comparison with HFD rats ($p < 0.05$). Dapagliflozin treatment tended to decrease the expression of TNFαR1 in relative to HFD rats, whereas metformin treatment had no effect. These data suggested that dapagliflozin and metformin effectively suppressed renal inflammation, subsequently decreasing obesity-related renal injury.


(A)
Whole cell lysate fraction
ND HFD HFDAP HFMET
NF-κB p65
65 kDa
β-actin
42 kDa
NF-κB p65/β-actin ratio (Arbitary units)
2.5
2.0
1.5
1.0
0.5
0.0
ND HFD HFDAP HFMET
(B)
Nuclear fraction
ND HFD HFDAP HFMET
NF-κB p65
65 kDa
Lamin b1
68 kDa
GAPDH
36 kDa
NF-κB p65/GAPDH ratio (Arbitary units)
2.0
1.5
1.0
0.5
0.0
*
†
†
ND HF HFDAP HFMET
(C)
Whole cell lysate fraction
ND HFD HFDAP HFMET
TNFα
16 kDa
GAPDH
36 kDa
TNFα/GAPDH ratio (Arbitary units)
2.0
1.5
1.0
0.5
0.0
*
†
†
ND HFD HFDAP HFMET

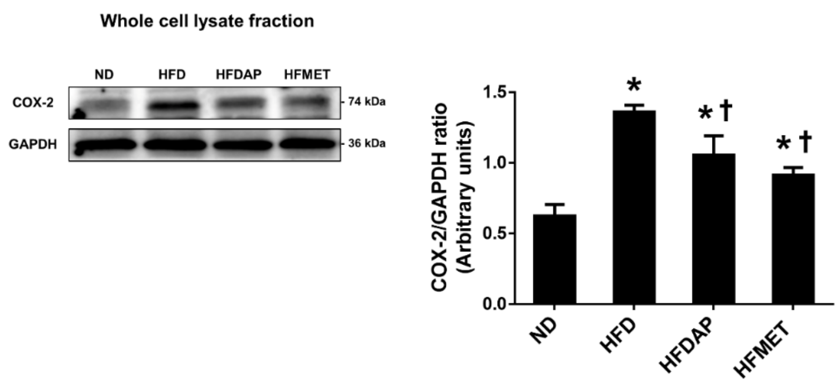
(D)


Membrane fraction
ND HFD HFDAP HFMET
TNFαR1
Na-K ATPase
GAPDH
65 kDa
112 kDa
36 kDa
TNFαR1/GAPDH ratio (Arbitary units)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
ND
HF
HFDAP
HFMET

(E)


Whole cell lysate fraction
ND HFD HFDAP HFMET
IL-1β
GAPDH
78 kDa
36 kDa
IL-1β/GAPDH ratio (Arbitrary units)
4
3
2
1
0
*
†
†
ND
HFD
HFDAP
HFMET

(F)


Whole cell lysate fraction
ND HFD HFDAP HFMET
COX-2
GAPDH
74 kDa
36 kDa
COX-2/GAPDH ratio (Arbitrary units)
1.5
1.0
0.5
0.0
*
*†
*†
ND
HFD
HFDAP
HFMET

(G)

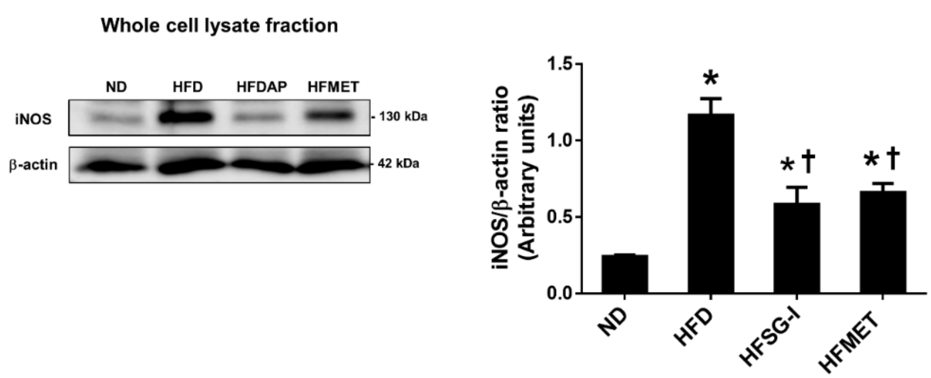

Whole cell lysate fraction
ND HFD HFDAP HFMET
iNOS
β-actin
130 kDa
42 kDa
iNOS/β-actin ratio (Arbitrary units)
1.5
1.0
0.5
0.0
*
*†
*†
ND
HFD
HFSG-I
HFMET

Figure 15. Effect of dapagliflozin and metformin treatments on renal expression of NF-κB (A and B), TNFα (C), TNFαR1 (D), IL-1β (E), COX-2 (F) and iNOS (G) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). *p<0.05 vs ND and †p<0.05 vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

11. Effect of dapagliflozin and metformin treatments on renal fibrosis in obese-insulin resistant rats

The correlation of profibrogenic cytokine production with the activity of the transcription factors NF-κB signaling pathwayhas been demonstrated [230]. In present study, collagen deposition in the kidneys was assessed by Masson's trichrome staining. Renal tubulointerstitium fibrosis was greater in HFD rats than ND rats (p < 0.05) (Figure 16A and B). Consistently, the expression of TGF-β1 and Col IV was significantly elevated in HFD rats as compared with ND rats (p < 0.05) (Figure 16C and D). These renal fibrotic changes in the HFD-fed were remarkably abrogated by dapaglifozin or metformin treatment as shown by less collagen deposition in comparison with the HFD rats. These data indicated that dapagliflozin and metformin effectively prevented renal injury via suppression of renal fibrosis in obese-insulin resistant rats.

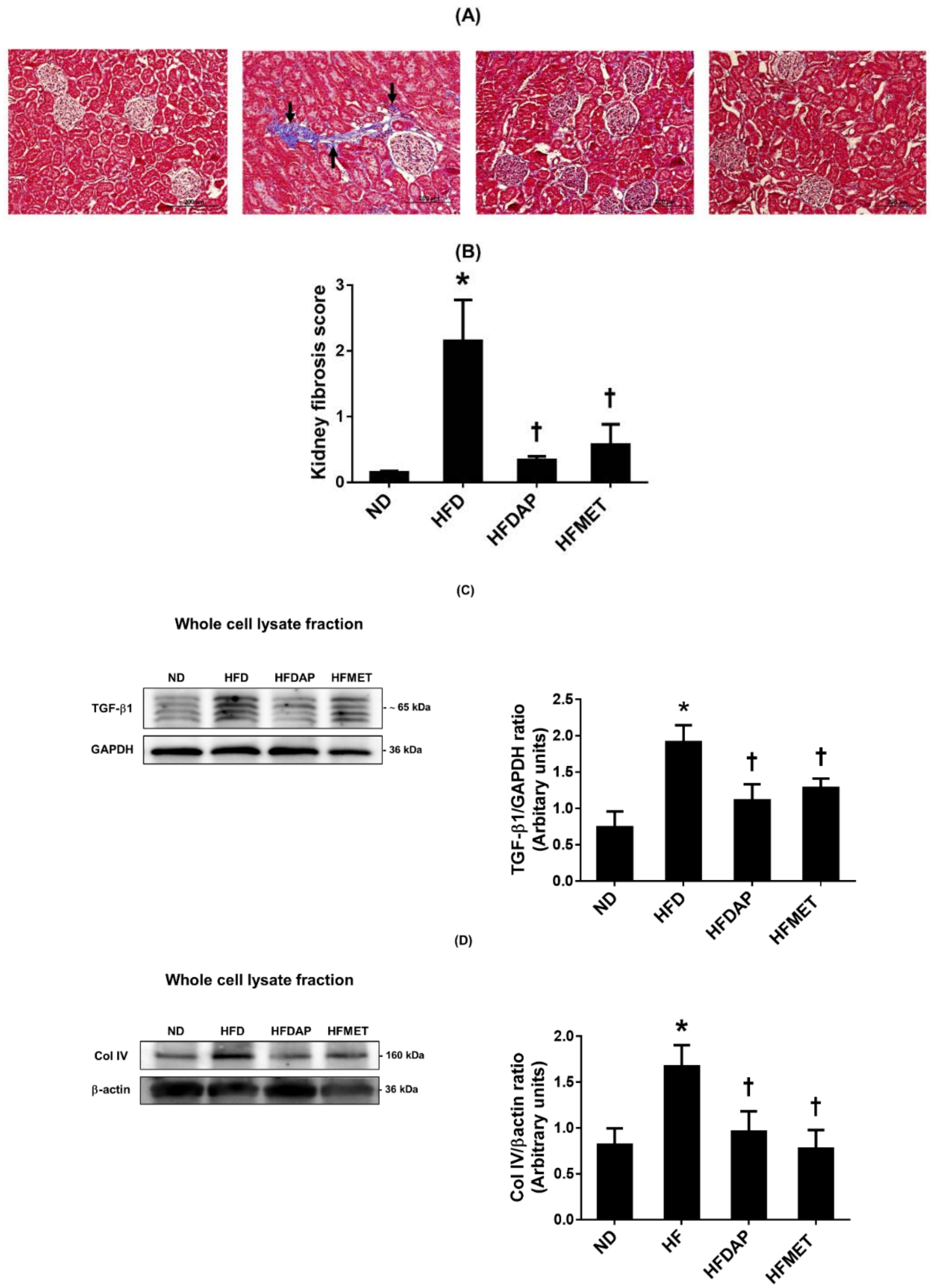


Figure 16. Effect of dapagliflozin and metformin treatments on renal fibrosis by Masson's trichrome staining (A and B), renal expression of TGF-β1 (C) and Col IV (D)in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^*p<0.05$ vs ND and $^\dagger p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

12. Effect of dapagliflozin and metformin treatments on renal ER stress in obese-insulin resistant rats

ER stress has been reported to contribute to not only the pathogenesis in acute renal diseases, but also exacerbating renal damage [43,231-233]. To investigate the effect of obese-insulin resistance rats on ER stress, the expression of protein involving ER stress pathway by western blotting was assessed. As presented in Figure 17A, B, C and D, the expressions of renal GRP78, CHOP, Calpain 2, and Caspase-12were significantly up-regulated in HFD rats as compared with ND rats, indicating that the function of ER was disrupted and leading to ER stress ($p < 0.05$). Both dapagliflozin and metformin treatments substantially suppressed the expressions of those proteins involving ER stress pathway in relative to HFD rats ($p < 0.05$). These results suggested that dapagliflozin had potent protective effects against renal ER stress and apoptosis in obese-insulin resistant rats.

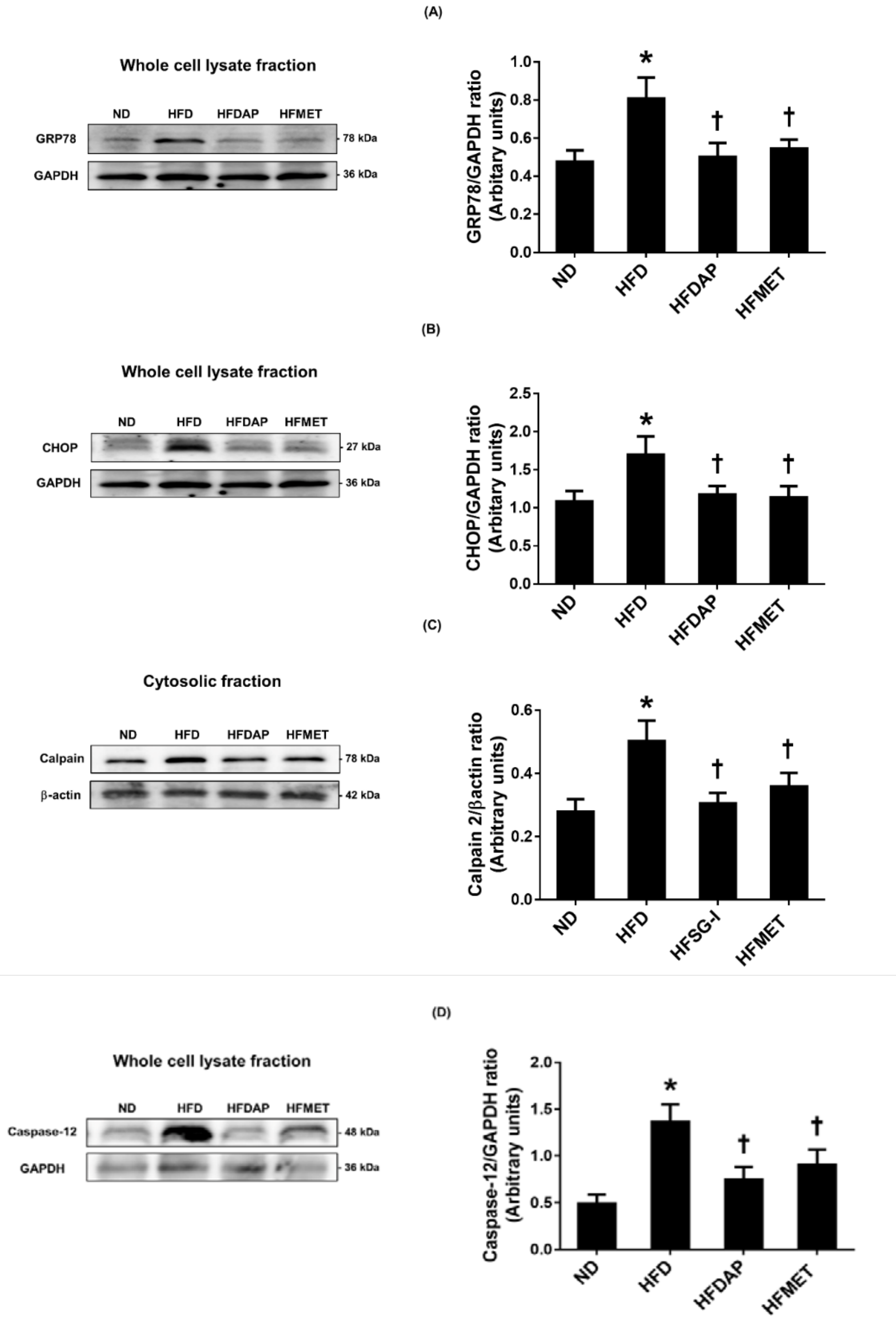
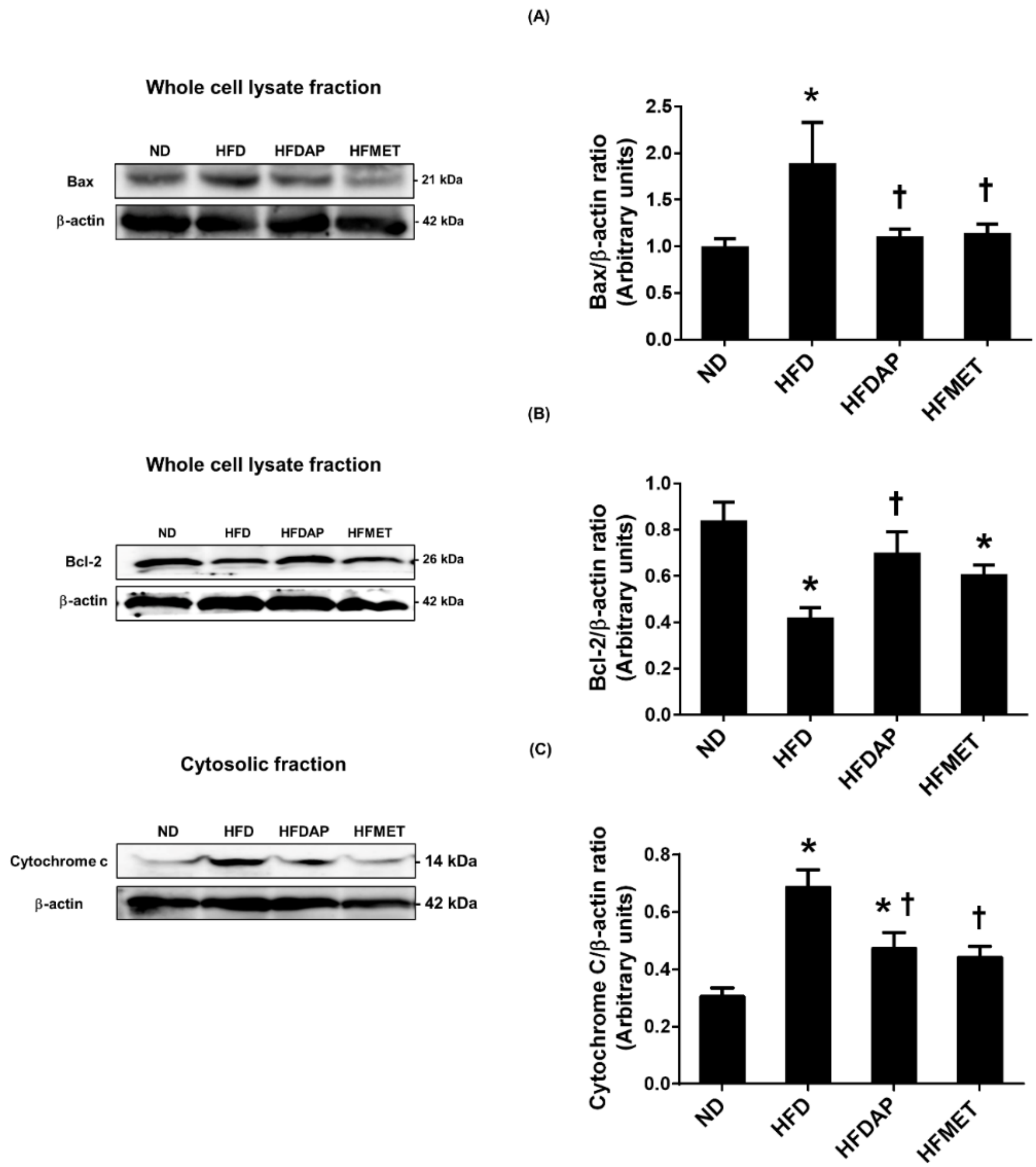


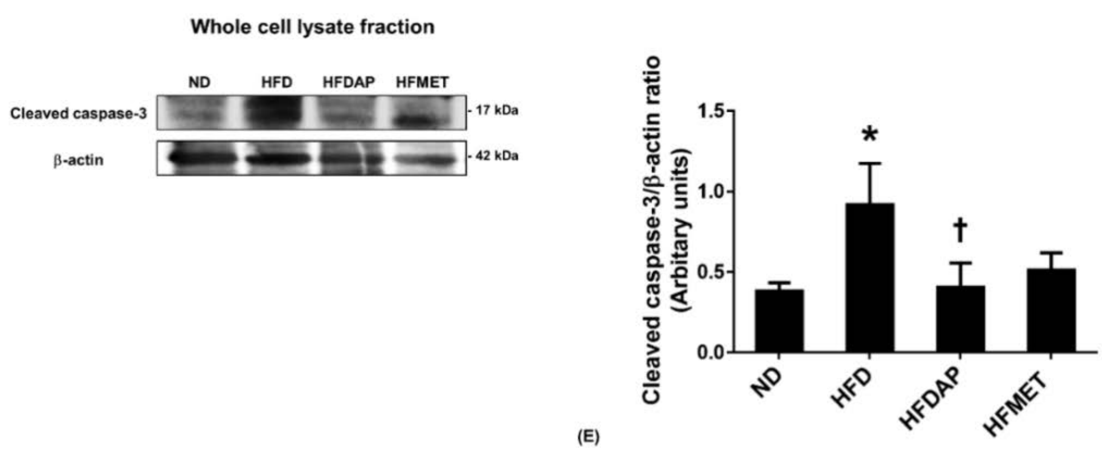
Figure 17. Effect of dapagliflozin and metformin treatments on renal expression of GRP78 (A), CHIOP (B), Calpain 2 and Caspase-12 (C) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). $^{*}p<0.05$ vs ND and $^{\dagger}p<0.05$ vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP: high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

12. Effect of dapagliflozin on renal apoptosis in obese-insulin resistant rats

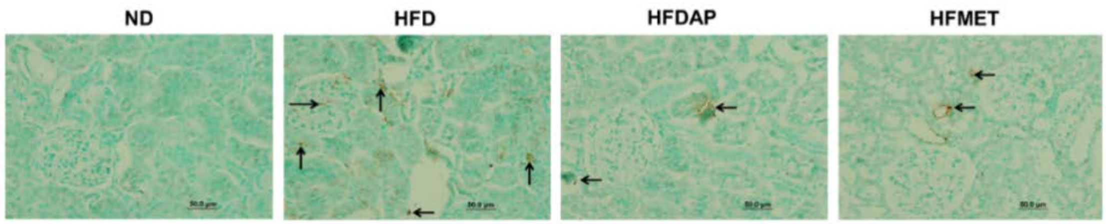
Previous studies demonstrated that ER stress and apoptosis always presented in diabetic individuals with kidney dysfunction, leading to the elevation of proteinuria [44,234]. Accordingly, the kidney apoptosis was determined. HFD rats had significant up-regulations of renal pro-apoptotic protein Bax, Cytochrome c and Cleaved caspase-3 expressions but down-regulation of anti-apoptosis protein, Bcl-2 expression in comparison with ND rats ($p < 0.05$) (Figure 18A, B, C and D). These results were further corroborated by performing TUNEL assay in the renal section. Renal sections of HFD rats showed significant increase of number of apoptotic cells compared with those of ND rats ($p < 0.05$) (Figure 18E and F). Dapagliflozin or metformin treatment not only restored the expressions of Bax, Cytochrome c, Cleaved caspase-3, and Bcl-2 but also decreased number of apoptotic cells in comparison with HFD rats ($p < 0.05$). However, metformin treatment had no effect on Bcl-2 and cleaved caspase-3 expression when compared with HFD rats. These results suggested that dapagliflozin had a potent effect on the reduction of renal apoptosis in obese-insulin resistant rats.


(A)
Whole cell lysate fraction
ND
HFD
HFDAP
HFMET
Bax
β-actin
21 kDa
42 kDa
Bax/β-actin ratio (Arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
*
†
†
(B)
Whole cell lysate fraction
Bcl-2
26 kDa
Bcl-2/β-actin ratio (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
(C)
Cytosolic fraction
Cytochrome c
14 kDa
Cytochrome C/β-actin ratio (Arbitrary units)
* †

(D)



(E)



(F)

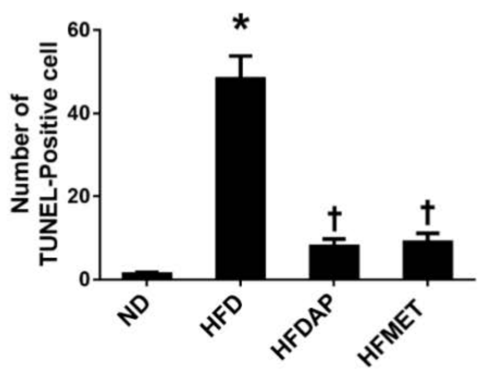


Figure 18. Effect of dapagliflozin and metformin treatments on renal expression of Bax (A), Bcl-2 (B), Cytochrome c (C), Cleaved caspase-3 (D) and TUNEL positive cell (E and F) in obese-insulin resistant rats. Values are mean ± standard error of the mean (SEM, n = 6 per group). [*]p<0.05 vs ND and [†]p<0.05 vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

13. Effect of dapagliflozin on renal expression of SGLT1 and SGLT2

Inhibition of renal SGLT2 has been found to increase glycosuria and reduce hyperglycemia in T2DM. However, there are controversial reports on the impact of obesity/diabetes on the expression of renal SGLT-mediated transport [219,235-237]. Therefore, we next sought to examine the changes of renal SGLTs and whether inhibition of SGLT2 affected the occurrence of renal SGLT-mediated transport in the obese-insulin resistant condition. As shown in Figure 19A and B, there was a significantly increased membrane expression of SGLT1, but not SGLT2 in HFD rats compared with ND rats ($p < 0.05$). Dapagliflozin or metformin treatment did not affect the expression of SGLT2in HFD rats. Metformin induced a down-regulation of SGLT1($p < 0.05$) while dapagliflozin had no effect on SGLT1 expression in this study.

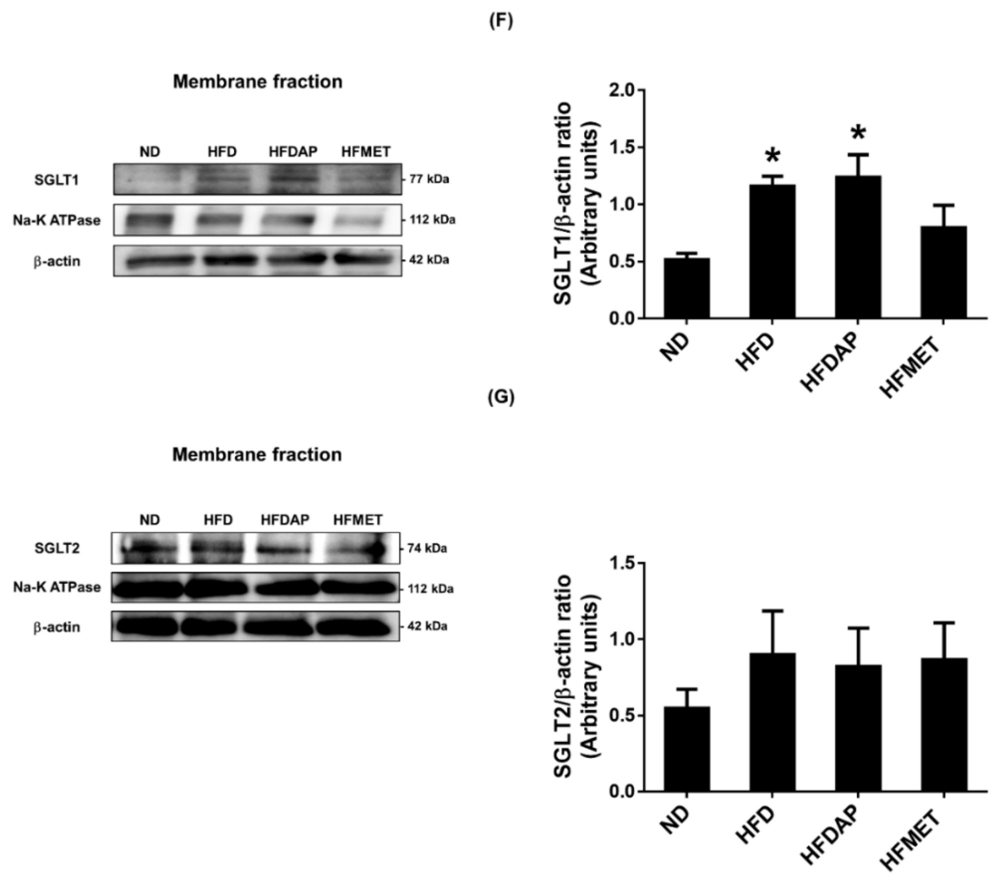


Figure 23. Effect of dapagliflozin and metformin treatments on renal expression of SGLT2 (A) and SGLT2 (B) in obese-insulin resistant rats. Values are mean ± standard error of the mean

(SEM, n = 6 per group). *p<0.05 vs ND and †p<0.05 vs HFD. ND: normal diet; HFD: high-fat diet; HFDAP high-fat diet plus dapagliflozin; HFMET: high-fat diet plus metformin groups.

Discussion

Current knowledge acknowledges that insulin resistance is an important and independent risk factor for chronic kidney disease (CKD) [238]. Therapeutic strategies to address kidney disease related to obese-insulin resistance are of immediate importance. In this study, a model using high-fat diet (HFD)-induced obese rats to create an insulin resistant state was used to assess the nephroprotective effect of dapagliflozin, a selective and potent SGLT2 inhibitor. We have shown that dapagliflozin treatment: 1) decreased body weight and peripheral insulin resistance; 2) reduced blood pressure, renal hyperfiltration and microalbuminuria; 3) decreased renal injury associated with renal lipid accumulation, oxidative stress, ER stress, inflammation, fibrosis and apoptosis; 4) not only decreased insulin resistance but also restored renal insulin signaling and modulation of renal PKCα/Nrf2 signaling pathways leading to improved renal function and renal Oat3 function in obese-insulin resistant rats.

In this study, long-term HFD fed rats developed obese-insulin resistance. These findings was consistent with our previous report [27]. Recently, various beneficial effects of a SGLT2 inhibitor on glucose homeostasis and reduced insulin resistance in the whole body have been indicated [239-241]. The improvement in metabolic dysfunction in obese insulin-resistant rats following dapaglifozin treatment was in agreement with several previous studies in diabetic rats and type 2 diabetic patients which had demonstrated a decrease in body weight and improved glycemic control [239-241]. Moreover, dapaglifozin treatment in this study also promoted urine glucose excretion, increased 24-hour urine volume and decreased visceral fat accumulation leading to reduced body weight and hypertension. Excessive caloric intake of dietary fat led to significant increase of not only visceral fat mass but also renal triglyceride contents in HFD rats. The results of renal oxidative stress, along with impaired renal function were elevated in HFD-fed rats suggested that lipid-mediated oxidative stress caused by increased renal lipid accumulation (lipotoxicity) implicated in renal dysfunction [242]. Also, previous studies in diet-induced obese mice found that hyperinsulinemia up-regulated renal expression of SREBP-1 which were associated with increased renal triglyceride accumulation and the development of glomerulosclerosis, renal cell death (lipoapotosis) and renal dysfunction [26,44,243]. Dapagliflozin treatment decreased not only renal triglyceride accumulation and MDA level but also glomerular hyperfiltration as well as microalbuminuria, a sign of glomerular damage, leading to improved renal function.

Animal models of diet-induced obesity demonstrated increased intra-abdominal pressure of visceral obesity and direct physical compression of the kidneys (decreased renal mass) that may affect renal function and hypertension. The increasing of abdominal fat compression around kidney might decrease tubular flow through the distensible loop of Henle, which could ultimately result in greater fluid or sodium reabsorption and increased GFR [244]. Our study has demonstrated that serum creatinine level and microalbuminuria were observed along with an increased and GFR and visceral fat accumulation in obese-insulin resistant rats. These findings suggested that glomerular hyperfiltration contributed to the progression of impaired renal function in obese-insulin resistant rats. In metabolic syndrome, hypertension and renal hemodynamic changes are associated with increased renal blood flow (RBF) and renal perfusion leading to the glomerular capillary hypertension [131]. Previous studies demonstrated that hyperinsulinemia and oxidative stress up-regulated vascular endothelial growth factor A (VEGF-A) expression leading to an increase in glomerular microvascular density and these alterations accounted for the elevation of RBF and GFR in metabolic syndrome [18,19]. In this study the obese-insulin resistant rats consistently demonstrated not only hyperinsulinemia but also oxidative stress as shown by the increased expression of NOX4, p67^{phox}, and 4-HNE along with an elevation of MDA level in the renal cortical tissues. It has been demonstrated NOX4 plays a role in the basal production of reactive oxygen species (ROS) in the kidney and the up-regulation of NOX4 or NOX2 has been found in pathologic conditions such as diabetic nephropathy (DN) and CKD [10,11].

To overcome oxidative stress, the cell is also equipped with a redox sensitive transcription factor, nuclear factor E2-related factor2 (Nrf2). Nrf2 antioxidant response pathway is considered as one of the major cellular defenses against the cytotoxic effects of oxidative stress. Under basal conditions, Nrf2is sequestered in the cytosol part by a Kelch like-ECH-associated protein 1 (Keap1) homodimer which facilitates the ubiquitination and proteasomal degradation of Nrf2. When the cell is faced with an insult such as chemical or oxidative stress, a conformational change in Keap1 mediated via its reactive cysteine residues results in the release of Nrf2 from one Keap1 molecule. This disruption of the these complex allows Nrf2 translocation into the nucleus, leading to the activation of the antioxidative response element (ARE) and up-regulation of antioxidant-related enzymes expression [245]. Therefore, to counteract the increased renal oxidative stress, Nrf2 was activated as shown by the significant elevations of nuclear Nrf2 and cytosolic Keap1 expression in the renal cortical tissues in obese-insulin resistant rats. These findings indicated that Nrf2 was dissociated from Keap1 and translocated into the nucleus with subsequently led to the transcriptional up-regulation of the antioxidant defense system as indicated by the increased renal GCLC and HO-1 expressions. Dapagliflozin-treatment in obese-insulin resistant rats caused down-regulation in the activation of the Nrf2/Keap1 system in concurrent with the observed reduction in renal oxidative stress. It therefore could be suggested that dapagliflozin possibly exerted an antioxidant action via the modulation of the Nrf2/Keap1 signaling pathway.

The significant increase in mean arterial blood pressure was observed along with the activation of renal cortical angiotensin II type-1 receptor (AT_1R) expression in obese-insulin resistant rats in this study. These results were correspondent with the report that oxidative stress played a role in the pathogenesis of obesity-related hypertension which was associated with an up-regulation of renal AT_1R [151]. Angiotensin II (Ang II) is also a potent activator of the NOX complex and augments ROS production [22]. In this study, the binding of Ang II to AT_1R stimulated PKCα and subsequently activated PKCα-mediated NOX4 expression to increase the generation of ROS in obese-insulin resistant rats [246,247]. Also, hyperinsulinemia has been found to increase in the expression of AT_1R in a dose-dependent manner [161,162]. Moreover, the up-regulation of AT_1R magnifies the Ang II response/signaling leading to overstimulation of Na-K ATPase, and Na/H exchanger 3 and subsequently contributes to increases in not only efferent arteriolar resistance, intraglomerular pressure, and glomerular hyperfiltration but also sodium reabsorption, retention and hypertension [32,248]. These results cause the decreases in sodium delivery to the macular densa and adenosine which deactivate tubuloglonerular feedback (TGF). This can then lead to enhanced afferent arteriolar vasodilatation, and subsequently increased renal supranormal rise in GFR causing glomerular hyperfiltration [143,249]. It was noteworthy that dapagliflozin treatment led to a marked reduction of mean arterial pressure accompanied with the lowered glomerular hyperfiltration in obese-insulin resistant rats in this study. Previous study demonstrated that an inhibition of renal tubular glucose uptake by empagliflozin resulted in attenuated renal hyperfiltration and intraglomerular pressure in diabetic condition [250]. These effects could be due to an increased sodium delivery to the macula densa resulting in an increased adenosine release to reduce renal blood flow/glomerular pressure via TGF mechanism [251,252]. It has also been suggested that JNJ-39933673, a potent and selective SGLT2 inhibitor, could activate TGF mechanism by increasing cluster of differentiation 73 (CD73) expression which commonly serves to convert adenosine monophosphate to adenosine in the macula densa and to restore level of adenosine receptors in renal diabetic mice [249]. Therefore, an enhanced sodium excretion secondary to a decreased glucose reabsorption in the proximal tubule and the down-regulation of renal renin-angiotensin system led to an increased fluid loss as indicated by increased 24-hour urine volume in obese-insulin resistant rats treated with dapagliflozin. These diuresis effects of dapagliflozin were associated with its antihypertensive action. The current results also demonstrated that the decreased expression of renal cortical AT_1R was linked with the decreased renal oxidative stress. Therefore, treatment with dapagliflozin attenuated renal hyperfiltration, decreasing renal oxidative stress and improved insulin resistance through the inactivation of renal cortical AT_1R/PKCα/NOX expression and the decreased plasma insulin level in obese rats.

Histologically, mononuclear infiltration, tubular desquamation, pyknoptic nuclei and fibrosis as shown in the sections of the kidneys from obese-insulin resistant rats were in agreement with the previous reports which demonstrated that PKCα/NOX4-mediated oxidative stress contributed to renal damage and apoptosis and subsequently led to impaired renal function [24,177]. Glomerular injury was also observed as shown by the decreased expression of glomerular filtration barrier proteins including Nephrin, Podocin and Connexin 43 in addition to tubular damage as indicated by the increased renal Kim and NGAL expression in obese-insulin resistant rats. Recently, a study in diabetic *db/db* mice demonstrated the increased glomerular hyperfiltration and albuminura in parallel with a high urinary Kim 1 level [60]. Interestingly, dapagliflozin treatment in obese-insulin resistant rats could ameliorate renal injury as indicated by the reduced kidney injury score, expression of Kim 1 and NGAL as well as the increased expression of glomerular filtration barrier proteins in this study. Our findings also showed a correlation between the decreases in glomerular hyperfiltration and microalbuminuria. However, no evidence reporting the effect of metformin treatment on renal function involving TGF mechanism in obese-insulin resistant condition was available. Nonetheless, metformin exerts pleiotropic actions on the kidney beyond its effects as glucose-lowering agents by attenuating DN associated with its ability to improve insulin resistance, lipid metabolism, antioxidative, and anti-inflammatory functions [27,253,254]. Based on the previous reports, the increased GFR and elevated blood pressure observed in obesity were found to cause glomerularcapillary hypertension. These changes subsequently increased glomerular capillary overstretching leading to renal injury via inflammation and fibrosis. The sustained elevation of blood pressure in metformin-treated rats led to sustained glomerular capillary hypertension and subsequent glomerular barrier injury and microalbumin leakage [118,255,256]. Compared with metformin treatment, dapagliflozin had a greater efficacy in decreasing renal damage leading to an improvement of renal function in obese-insulin resistant rat.

Giving weight to the concept that obesity is recognized as a chronic low-grade systemic inflammatory disease with accumulation of pro-inflammatory cytokines. In onset and progress of obesity and insulin resistance, inflammation and oxidative stress play important roles in the pathogenesis of renal injury [27,177]. A recent study has found that inflammation could induce renal cluster of differentiation 36 (CD36) expression leading to eventually increased cellular uptake of free fatty acid (FFA), as well as the deposition of lipid, and also initiates cellular stress response [29]. Previous researches had revealed the role of inflammation in kidney disease in the development of kidney lipid accumulation in obese mice. The resulting in turn triggers inflammation and the release of multiples inflammatory factors causing oxidative injury and structural changes which further aggravate kidney dysfunction [30,31]. In the current study, renal inflammation in HFD rats was shown by an increase in nuclear NF-KB p65 protein expression. This induced the up-regulation of TNFα and TNFαR1 along with pro-inflammatory cytokines, IL-1β, COX-2, and iNOS in renal cortical tissues. In addition, the morphological observations revealed by Masson's trichrome staining indicated tubulointerstitial fibrosis which was confirmed by the increased expressions of Kim 1 and NGAL, renal tubular injury marker, and TGF-β1 and Col IV, the downstream targets of NF-KB p65 in the renal cortical tissues of HFD rats. In insulin resistance, hyperinsulinemia has been shown to promote not only the expression of TGF-β1 and Col IV but also the increased glomerular capillary proliferation, associated with the elevation of renal hyperfiltration related to renal fibrosis [18,19,58,238]. Interestingly, the improved renal injury observed in the HFD rats treated with dapagliflozin showed a correlation with the decreased renal cortical expression of NF-KB p65, proinflammatory cytokines and TGF-β1, Col IV, Kim 1, and NGAL. It has been found the decreased hyperglycemia and the slowed progression of renal injury, evidenced by reduced hyperglycemia-induced oxidative stress, inflammation and fibrosis in *db/db* mice treated with dapagliflozin [257]. Taken together, it might be suggested that the anti-inflammatory and anti-fibrotic effects of dapagliflozin were mediated through improved hyperinsulinemia along with the decreases in renal lipid accumulation and renal hyperfiltration.

Based on previous studies, many reports focused on the effects of inflammatory stress on lipid accumulation in the kidney. Some evidence from animal studies has demonstrated that abnormal tissue lipid metabolism in obesity or insulin resistance leads to an accumulation of unfolded proteins in the endoplasmic reticulum (ER) lumen. These changes may participate in the unfolded protein response (UPR), subsequently triggering ER stress [37-39]. If, however protein aggregation is persistent and the stress cannot be resolved, signaling switches from pro-survival to pro-apoptosis through up-regulated pro-apoptotic protein Bax and down-regulated anti-apoptotic protein Bcl-2, leading to activation of apoptotic caspases cascades [41-43]. Previous studies also demonstrated that the elevations of tubulointerstitial fibrosis, renal apoptosis, tubular vacuolization, and lipid accumulation were the pathological factors involved in the HFD-induced nephropathy in animal models [258,259]. Moreover, studies in obese rats demonstrated the obesity-related renal dysfunction through ER-mediated stress mechanisms supporting the correlation of lipid accumulation, ER stress, cellular apoptosis[37,44,260-263]. In our study, ER stress in HFD rats was shown by the elevation of GRP78, CHOP, Calpain 2, and Caspase-12 expression in renal cortical tissues. Activation of the apoptotic pathway in HFD rats was demonstrated by the up-regulated pro-apoptotic protein Bax and down-regulated anti-apoptotic protein Bcl-2 with the subsequent release of Cytochrome c leading to the activation of Caspase-3. These findings in agreement with previous studies [43,177], suggest a correlation between the increase in transcriptional CHOP protein and the activation of the apoptotic pathway. Alternatively, the increased expression of renal Calpain 2 and Caspase-12 in HFD rats also led to ER-calcium leak-induced apoptosis through direct activation of Caspase-3 [264]. These observations corresponded with the increased number of TUNEL-positive cells in the renal sections of HFD-fed rats. Interestingly, dapagliflozin has been shown to mitigate renal lipid accumulation correlating with a reduction in SREBP-1c mRNA [26]. Consistent with the results of the observed decrease in renal triglyceride accumulation, the TUNEL-positive cells, the expression of GRP78, Calpain 2 and Caspase-12 and the CHOP-mediated apoptotic signal pathway were diminished in HFD-fed rats with dapagliflozin treatment. This study is the first to demonstrate the protective effects of dapagliflozin treatment against renal injury related to renal

ER stress and apoptosis in HFD rats. The present study also demonstrated that metformin treatment ameliorated ER stress-induced apoptosis in HFD rats; however, the restoration of anti-apoptotic protein Bcl-2 was lowered to a greater extent in HFMET rats than in HFDAP rats. It might be suggested that the reduced renal lipotoxicity observed with dapagliflozin treatment contributed to the effective prevention of obesity-related renal ER stress and apoptosis. However, whether dapagliflozin plays a direct role in these effects remain unclear and will require further investigation.

The normal renal function is one of the determinants of the elimination of endogenous metabolic products as well as balance of the excretion of exogenous drugs and environmental exposures [265]. The tubular secretion and reabsorption are mainly mediated by numerous transporters in the basolateral or apical membranes of renal proximal tubule cells, which is totally different from the passive transport during glomerular filtration [266]. It has been known that proximal tubular injury might affect the function and expression of renal organic anion transporter (Oat3) which could affect the elimination of numerous organic anion substances from blood circulation into the urine [182]. Previously, the function in remote interorgan communication by regulating levels of signaling molecules and key metabolites in tissues and body fluids called "Remote Sensing and Signaling Hypothesis" of Oats has been reported [48]. The knockouts of *Oat1* and *Oat3* showed a significant increase in the level of uremic toxins and solutes, including those derived from the gut microbiome (e.g., furan fatty acid metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), phenylsulfate, indole-3-acetic acid). Many of these molecules are involved in interorgan and interorganismal communication [267]. CMPF has recently been shown to have a potential link to glucose intolerance as it effects on pancreatic β-cells leading to reduce insulin biosynthesis [198]. In this study, we also found the decrease in renal Oat3 function and expression in the membrane fraction in obese-insulin-resistant rats. This phenomenon would decrease the excretion of toxic endogenous metabolite such as creatinine and CMPF [268]. Thus, renal Oat3 dysfunction might be the one factor affect to increased serum creatinine and cause of glucose intolerance in the present study.

Previous studies in gentamicin-induced nephrotoxicity and in type 1 diabetic rats reported that the decreased renal Oat3 function and expression were related with PKCα activation induced by oxidative stress [25,50,202]. The internalization of renal Oat3 into the cytoplasm via the activation of PKCα affected to the decreases in renal Oat3 expression and function [25,50]. Moreover, insulin signaling is a major regulator of renal Oat3 trafficking, impaired insulin signaling as indicated by the down-regulation of renal PKCζ and Akt expression led to the decreased renal Oat3 trafficking to the basolateral membrane of the tubular cells in this study [50,202,269]. It is noteworthy that dapagliflozin therapy effectively improved the function and expression of renal Oat3 in obese-insulin resistant rats which correlated with the decrease in renal oxidative stress together with the restoration of renal insulin signaling. To support these findings, previous studies demonstrated that a decrease in renal PKCα and a reversal in Akt/PKCζ activation in the diabetic rats subsequently led to the up-regulation of renal Oat3 expression and function[50,202]. However, the degree of renal Oat3 function produced by metformin was greater than that seen in rats treated with dapagliflozin. One would assume that the increased insulin sensitivity might be involved in augmented transporter affinity observed in HFD rats treated with metformin. Therefore, the increased renal Oat3 function in dapagliflozin or metformin treatment might affect to increase the excretion of toxic endogenous metabolites leading to restore renal function and improved glucose tolerance in this study.

Inhibition of proximal tubular glucose uptake via SGLTs, particularly SGLT2, is one of the most effective approaches for treatment of hyperglycemia and/or obesity in patients with type 2 diabetes [270]. To date, there has been no investigation into whether renal tubular SGLTs levels in the obese-insulin resistant condition are altered as a result of SGLT2 inhibition. The current results demonstrated an increased membrane expression of SGLT1, but not SGLT2 in HFD rats, and either dapagliflozin or metformin treatment did not affect the expression of SGLT2 in HFD rats. Previous studies indicated that the activation of protein kinase C (PKC) decreased the transport maximum rate of SGLT1 accompanied by reduced the number of SGLT1 at the plasma membrane, indicating that PKC regulated endocytosis of the vesicles containing the transporter [271]. In agreement with this concept, the increased AMPK activity has been shown to down-regulate SGLT1 translocation to the apical membrane of the small intestine through PKC activation [272]. Therefore, the increased activation of PKC in obese rats might induce the down-regulation of SGLT1 in the tubular cells. These data are in line with our results showing that metformin, an AMPK activator, induced a down-regulation of SGLT1 while dapagliflozin had no effect on SGLT1 expression.

Conclusion

In conclusion, this study highlights the potential role of dapagliflozin in alleviating obese-insulin resistance-induced renal dysfunction in rats. The results obtained from this study are the first to provide evidence that dapagliflozin exerts nephroprotective effects by improving renal function and renal Oat3 function in obese-insulin resistant rats. These effects are achieved by effectively reducing insulin resistance, renal oxidative stress, glomerular and tubular injury in addition to ameliorating histological changes to a greater efficacy than metformin. Moreover, dapagliflozin also attenuates obesity-induced inflammation, fibrosis, ER stress, apoptosis as well as lipid accumulation in the kidneys of HFD-fed rats. Overall, our findings may provide new insights of the mechanisms by which SGLT2 inhibition in the prevention of obesity related kidney injury in obese-insulin resistant condition. Therefore, it is suggested that the antidiabetic drug, dapagliflozin, should be evaluated in clinical studies in the obese-insulin resistant or pre-diabetic condition and may be a novel therapeutic option through the modulation of the early features of renal injury

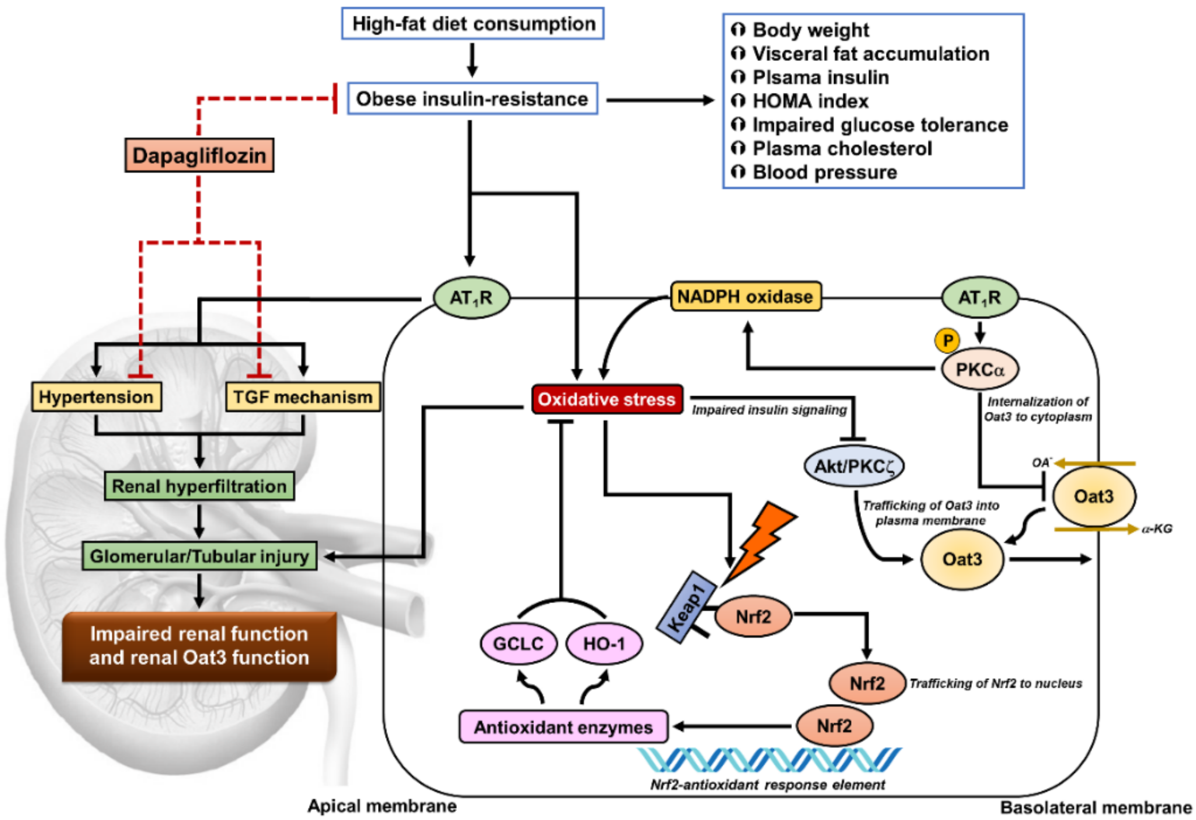


Figure 20. Shematic diagram shows propose mechanism of obese-insulin resistance-induced renal injury and Oat3 dysfunction via oxidative stress ans insulin signaling and the treatment of dapagliflozin to attenuate this condition.

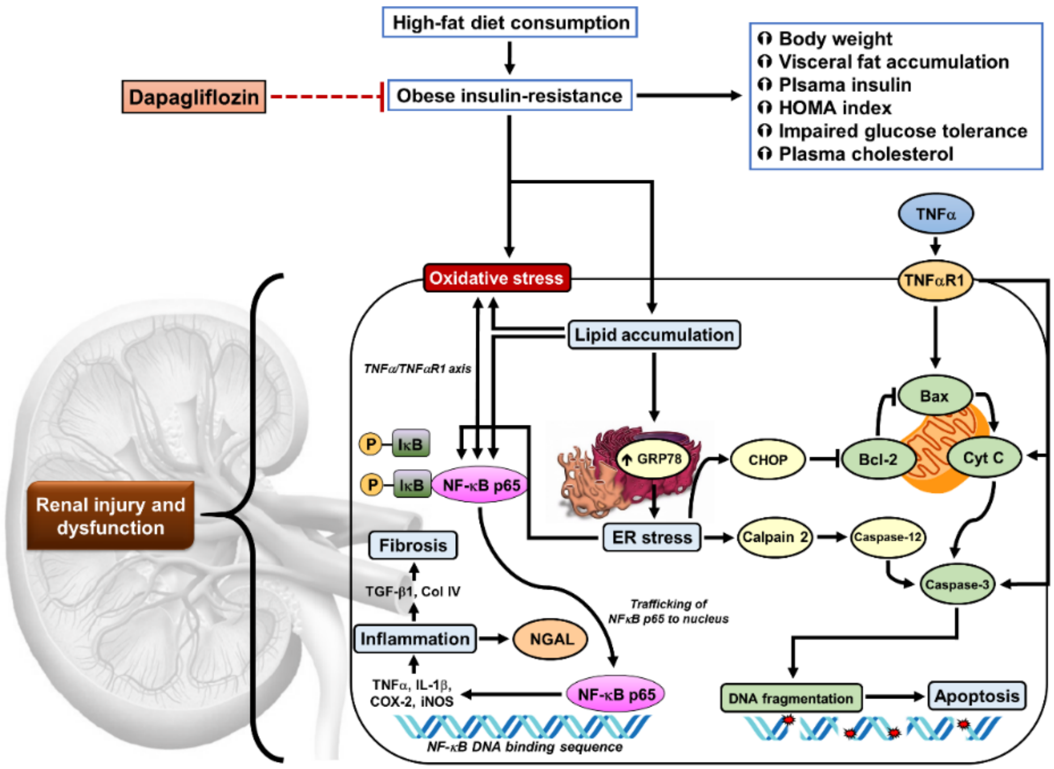


Figure 21. Shematic diagram shows propose mechanism of obese-insulin resistance-induced renal dysfunction via inflammation, fibrosis, ER stress, apoptosis, and by reduce the accumulation of lipids in the kidneys and the treatment of dapagliflozin to attenuate this condition.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

1.1 Thongnak L, Chatsudthipong V, Kongkaew A, **Lungkaphin A**. Effects of dapagliflozin and statins attenuate renal injury and liver steatosis in high-fat/high-fructose diet-induced insulin resistant rats. Toxicol Appl Pharmacol. 2020 Apr 4:114997. doi: 10.1016/j.taap.2020.114997. (2018: IF 3.585 ISI: Q1)

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1.9 Jaikumkao K, Pongchaidecha A, Chueakula N, Thongnak L, Wanchai K, Chatsudthipong V, Chattipakorn N, **Lungkaphin A**. Renal outcomes with sodium glucose cotransporter 2 (SGLT2) inhibitor, dapagliflozin, in obese insulin-resistant model. Biochim Biophys Acta. Molecular basis of disease 2018 Mar 20;1864(6 Pt A):2021-2033. (2018: IF 4.328 ISI: Q1)

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1.11 Nuttawud Chueakula, Krit Jaikumkao, Phatchawan Arjinajarn, Anchalee Pongchaidecha,Varanuj Chatsudthipong, Nipon Chattipakorn, **Anusorn Lungkaphin**. Diacerein alleviates kidney injury through attenuating inflammation and oxidative stress in obese insulin-resistant rats. Free Radic Biol Med. 2018 Feb 1;115:146-155. doi: 10.1016/j.freeradbiomed.2017.11.021. Epub 2017 Nov 28. (2018; IF 5.657 ISI: Q1)

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

โดยมีการนำองค์ความรู้จากผลงานวิจัยที่ตีพิมพ์ผลงานตีพิมพ์ในวารสารระดับนานาชาติไปใช้เป็นประโยชน์ด้านวิชาการ การเรียนรู้ การเรียนการสอน ในระดับปริญญาตรี และระดับบัณฑิตศึกษาทั้งปริญญาโทและเอก ในภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ และผู้สนใจด้านวิชาการ และมีการนำผลงานวิจัยไปทำการวิจัยต่อยอดเพื่อศึกษาถึงกลไกการเกิดภาวะความผิดปกติของการทำงานของไตในภาวะเบาหวานหรือในภาวะดื้อต่อฮอร์โมนอินซูลิน และศึกษาถึงกลไกการออกฤทธิ์ของยาและสารอื่นๆ ในการต้านต่อการเกิดภาวะแทรกซ้อนของไตที่เกิดจากภาวะเบาหวานและภาวะอ้วนและดื้อต่ออินซูลิน เพื่อประโยชน์ในการนำสารสกัดหรือสมุนไพรที่มีฤทธิ์ต้านต่อการเกิดภาวะเครียดออกซิเดชั่นและการอักเสบ เช่น การศึกษาการใช้โพรไบโอติกหรือพรีไบโอติกในภาวะอ้วนและดื้อต่ออินซูลิน เพื่อการพัฒนาไปสู่การเกิดนวัตกรรมการผลิตผลิตภัณฑ์ที่สามารถใช้เสริมกับยารักษาเบาหวานในการลดภาวะแทรกซ้อนดังกล่าวต่อไป และได้มีความร่วมมือกับนักวิจัยจาก คณะเภสัชศาสตร์และคณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ และคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล เพื่อศึกษาต่อยอดในการศึกษาและสร้างสรรผลิตภัณฑ์ที่มีฤทธิ์ในการรักษาภาวะเบาหวานต่อไป นอกจากนี้ยังได้ผลิตบัณฑิตนักวิจัยรุ่นใหม่ระดับปริญญาโท นางสาวลอองดาว ทองนาค และระดับปริญญาเอก นายกฤช ใจคุ้มเก่า จากโครงการวิจัยนี้ซึ่ง นางสาวลอองดาว ทองนาค ขณะนี้กำลังศึกษาในระดับปริญญาเอก

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 Swe MT, Phengpol N, Thongnak L, Pongchaidecha A, **Lungkaphin A**. The effects of high-fat high-fructose diet on glucose metabolism in rat model. International conference on Innovation of Functional Foods in Asia (IFFA), January 22-24, 2018. University of Phayao, Phayao Province, Thailand. (Poster presentation)

3.2 Phengpol N, Pongchaidecha A, Wanchi K, Jaikumkao K, Thongnak L, Swe MT, **Lungkaphin, A**. High-fat high-fructose diet induces renal dysfunction in rat. International conference on Innovation of Functional Foods in Asia (IFFA), January 22-24, 2018. University of Phayao, Phayao Province, Thailand. (Poster presentation)

3.3 Thongnak L, Phengpol N, Swe MT, Jaikumkao K, Pongchaidecha A, **Lungkaphin, A**. The effect of high-fat high-fructose diet on lipid accumulation in rat.

International conference on Innovation of Functional Foods in Asia (IFFA), January 22-24, 2018. University of Phayao, Phayao Province, Thailand. (Poster presentation)

3.4 Swe MT, Jaikumkao K, Thongnak L, Pongchaidecha A, **Lungkaphin A**. Effect of dapagliflozin on glucose metabolism and renal and hepatic PEPCK expression in obese rats. 9th Federation of the Asian and Oceanian Physiological Societies (FAOPS), March 28-31, 2019. Kobe, Japan. (Poster presentation) (Young Scientist Travel Award)

3.5 Thongnak L, Swe MT, Jaikumkao K, Pongchaidecha A, **Lungkaphin A**. Protective effects of dapagliflozin and atorvastatin on renal function in insulin-resistant rats. 9th Federation of the Asian and Oceanian Physiological Societies (FAOPS), March 28-31, 2019. Kobe, Japan. (Poster presentation) (Young Scientist Travel Award)

3.6 Sasivimon Promsan, Rada Chenwelling, Anchalee Pongchaidecha1 and **Anusorn Lungkaphin**. Protective Effects of Agomelatine on Inflammation in Obesity-Induced Kidney Injury. 9th Federation of the Asian and Oceanian Physiological Societies (FAOPS), March 28-31, 2019. Kobe, Japan. (Poster presentation)

ภาคผนวก

เอกสารแนบหมายเลข 2

บทคัดย่อ

รหัสโครงการ : RSA6080015

ชื่อโครงการ : ผลของยายับยั้ง sodium-glucose cotransporter 2 (SGLT2 inhibitor) ต่อการปรับปรุงภาวะดื้อต่อฮอร์โมนอินซูลิน โปรตีนขนส่งกลูโคสที่ไตและการทำงานของไตในหนูอ้วนที่มีภาวะดื้อต่อฮอร์โมนอินซูลิน

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ระยะเวลาโครงการ : 3 ปี

การศึกษาครั้งนี้ใช้หนูขาวเพศผู้พันธุ์ Wistar แบ่งเป็นสองกลุ่มโดยได้รับอาหารต่างชนิดกันเป็นเวลา 16 สัปดาห์ ได้แก่ กลุ่มที่ได้รับอาหารปกติ (ND) และกลุ่มที่ได้รับอาหารไขมันสูง (HFD) หลังจากครบกำหนด 16 สัปดาห์ หนูในกลุ่มที่ได้รับอาหารไขมันสูงจะถูกแบ่งเป็น กลุ่มที่ได้รับอาหารไขมันสูง กลุ่มที่ได้รับอาหารไขมันสูงร่วมกับยาดาพากลิโฟลซิน (HFDAP) และกลุ่มที่ได้รับอาหารไขมันสูงร่วมกับยาเมทฟอร์มิน (HFDMET) โดยการป้อนทางปากเป็นระยะเวลา 4 สัปดาห์ หลังจากนั้นทำการประเมินการเปลี่ยนแปลงทางเมตาบอลิก การทำงานของไต การทำงานและการแสดงออกของโปรตีนขนส่งสารอินทรีย์ประจุลบชนิดที่ 3 การสะสะสมของไขมันในเนื้อไต ภาวะเครียดออกซิเดชั่น การอักเสบพังผืดในไต ภาวะเครียดต่อเอ็นโดพลาสมิคเรติคูลัม และการตายของเนื้อเยื่อไตแบบอะพอพโตซิส

พบว่าในหนูกลุ่มที่ได้รับยาดาพากลิโฟลซินมีประสิทธิภาพในการลดน้ำหนักตัว การสะสมของเนื้อเยื่อไขมันในช่องท้อง ความดันโลหิต รวมไปถึงระดับของครีตินินในเลือดและโปรตีนอัลบูมินในปัสสาวะ ซึ่งบ่งบอกถึงการปรับปรุงการทำงานขอไตได้ดีกว่ากลุ่มที่ได้รับยาเมทฟอร์มิน นอกจากนี้พบว่าในหนูกลุ่มที่ได้รับยาดาพากลิโฟลซินหรือยาเมทฟอร์มินสามารถช่วยปรับปรุงประสิทธิภาพการทำงานและการแสดงออกของโปรตีนขนส่งสารอินทรีย์ประจุลบชนิดที่ 3 ที่ผนังเซลล์ท่อไต ซึ่งมีความสอดคล้องกับการลดลงของภาวะเครียดออกซิเดชั่น รวมถึงการปรับปรุงการส่งสัญญาณของฮอร์โมนอินซูลินที่เนื้อเยื่อไตที่ดีขึ้น ลดการสะสะสมของไขมันในเนื้อเยื่อไต การอักเสบ การเกิดพังผืดในไต ภาวะเครียดต่อเอ็นโดพลาสมิคเรติคูลัม รวมถึงการตายของเนื้อเยื่อไตแบบอะพอพโตซิสได้

การศึกษาในครั้งนี้ได้แสดงให้เห็นถึงผลของยาดาพากลิโฟลซินในการช่วยปรับปรุงประสิทธิภาพการทำงานของไต และการทำงานของโปรตีนขนส่งสารอินทรีย์ประจุลบชนิดที่ 3 ในภาวะอ้วนและดื้อต่อฮอร์โมนอินซูลินโดยพบว่ากลไกที่เกี่ยวข้องกับการปรับปรุงการทำงานและการลดบาดเจ็บของไตนั้นผ่านทางการส่งสัญญาณของฮอร์โมนอินซูลินในไตได้ดีขึ้น รวมไปถึงการลดภาวะเครียดออกซิเดชั่น การอักเสบและพังผืดในไต ภาวะเครียดต่อเอ็นโดพลาสมิคเรติคูลัม และนำมาสู่การลดลงของการตายของเนื้อเยื่อไตแบบอะพอพโตซิส

คำหลัก : ยาดาพากลิโฟลซิน; ภาวะดื้อต่อฮอร์โมนอินซูลิน; โปรตีนขนส่งสารอินทรีย์ประจุลบชนิดที่ 3 ที่ไต; ภาวะเครียดออกซิเดชั่น; ภาวะเครียดต่อเอ็นโดพลาสมิคเรติคูลัม

Abstract

Project Code : RSA6080015

Project Title : Effects of sodium-glucose cotransporter 2 (SGLT2) inhibitor on the improvement of insulin resistance, renal glucose transporters and renal function in obese-insulin resistant rats

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Project Period : 3 years

In this study, to work towards a resolution of these issues, we evaluate the renoprotective roles of dapagliflozin in high-fat diet fed rats, a model of obese-insulin resistance. Male Wistar rats were divided into two groups, and received either a normal diet (ND) or a high-fat diet (HFD) for 16 weeks. After that, the HFD-fed rats were subdivided into three subgroups and received either a vehicle (HFD), dapagliflozin (HFDAP) or metformin (HFMET), by oral gavage for four weeks. Metabolic parameters, renal function, renal organic anion transport 3 (Oat3) function, renal lipid accumulation, renal oxidative stress, renal inflammation, renal fibrosis, renal ER stress, and renal apoptosis, and renal morphology were determined. Dapagliflozin or metformin treatment decreased insulin resistance, hypercholesterolemia, creatinine clearance and renal oxidative stress leading to improved renal function. However, dapagliflozin treatment decreased body weight, visceral fat accumulation, blood pressure, serum creatinine, urinary microalbumin and increased glucose excretions than metformin. Importantly, dapagliflozin and metformin effectively improved the function and expression of renal Oat3 in obese-insulin resistant rats which correlated with the decrease in renal oxidative stress together with the restoration of renal insulin signaling. Meanwhile, dapagliflozin and metformin had equal effect regarding the suppression of renal triglycerides levels, subsequently leading to a decrease in renal inflammation and fibrosis. Renal ER stress and apoptosis were increased in HFD rats, which were effectively reduced following administration of dapagliflozin. Collectively, these findings indicate that dapagliflozin exerts renoprotective effects by alleviating obesity-induced renal dysfunction and renal Oat3 function at least in part by reducing renal oxidative stress, renal inflammation, fibrosis, ER stress, apoptosis and modulating renal insulin signaling, and hence ameliorating renal injury.

Keywords : Dapagliflozin; Insulin resistance; Renal Oat3; Oxidative stress; ER stress