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THE UP-REGULATED EXPRESSION OF BOTH PHOSPHOLIPASE A2 AND CYCLOOXYGENASE-2 IS INVOLVED IN RENAL INJURY IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

Y.L. Lu, L. Ye, H. Wu, F.Z. Xia, J. Yu, L.Z. Yang*

Shanghai Ninth People's Hospital affiliated Shanghai Jiaotong University School of Medicine - Division of Endocrinology and Metabolism, Shanghai, China

Abstract

Context. Recent evidence has stressed that many proinflammatory factors are particularly conducive to the progression of diabetic nephropathy, but the mechanisms underlying the changes are poorly understood.

Objective. The purpose of this study was to investigate if up-regulated expression of both phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2) is involved in renal damage and micro-inflammatory state in streptozotocin-induced diabetic rats.

Animals and methods. Sixteen Sprague Dawley rats were randomly divided into 2 groups: control group and diabetes group. Animals in diabetes group were treated with intraperitoneal injection of streptozotocin. Eight weeks later, rat renal tissue was studied with light and transmission electron microscopes, and PLA2 and COX-2 and their mRNA expression were examined by immunohistochemistry and reverse transcription polymerase chain reaction, respectively.

Results. The renal pathological lesions in diabetes group were obvious, including increased amounts of mesangial matrix, thickening of the glomerular and tubular basement membranes and fusion and effacement of the adjacent podocyte foot processes. Infiltrating inflammatory cells were observed in the tubules. Compared with control group, the expression of cytosolic PLA2 and COX-2 was significantly increased in diabetes group.

Conclusions. It uncovers that the PLA2-COX-2 pathway may lead to renal inflammation associated with renal damage in streptozotocin- induced diabetic rats.

Key words: phospholipase A2, cyclooxygenase-2, diabetes, inflammation, kidney.

INTRODUCTION

Diabetic nephropathy is a diabetic microvascular disease. The pathogenesis is very complicated. In recent years, much attention has been paid to inflammatory mechanism of diabetes mellitus. It is recognized that renal inflammation is an early manifestation of diabetic vascular disease (1). However, the role of inflammation in diabetic nephropathy is still unclear.

Both PLA2 and COX-2 are important regulatory substances, which are two activators of the inflammatory

*Correspondence to: Dr. Li-Zhen Yang, Shanghai Ninth People's Hospital affiliated Shanghai Jiaotong University School of Medicine, Division of Endocrinology and Metabolism, 639 Zhizaoju Road, Shanghai, 200011, China, E-mail: dryanginsh@yahoo.com

reaction (2-3). We hypothesized that one source of pathogenesis in diabetic renal injury may be associated with up-regulated or over-activated expression of both PLA2 and COX-2. Hence, properly inhibiting the expression of PLA2 and COX-2 will be a potentially attractive way to prevent and treat diabetic nephropathy.

Although many reports (4-7) showed that high glucose may cause up-regulation of cyclooxygenase-2, to our knowledge, no experiments have yet been conducted on the association between the PLA2 - COX-2 pathway and renal inflammation directly, especially in diabetes mellitus. Therefore, in the study, we investigated if the PLA2-COX-2 pathway leads to renal inflammation in streptozotocin - diabetic rats, and if this micro-inflammatory state can be associated with renal damage in streptozotocin - diabetic rats.

MATERIALS AND METHODS

Animals and drug administration

Sixteen Sprague Dawley male rats, aged 10 weeks and weighing between 250-280 g, were obtained from the Animal Center of Shanghai Laboratory affiliated Chinese Academy of Sciences. Sixteen fasted rats were randomly divided into control group (C group, n = 8) and diabetes group (D group, n = 8). D group was treated with intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO, USA) 60 mg/kg in 0.1 M sodium citrate buffer at pH 4.5 once in each rat. The rats in C group were injected with sodium citrate buffer alone. In addition, rats with blood glucose levels greater than 16.7 mmol/L were considered diabetic in D group.

All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the guidelines of the Animal Welfare Act.

Tissue preparation

Eight weeks following the establishment of diabetes, the rats in both C and D groups were sacrificed by intraperitoneal administration of ketamine (35mg/kg body weight). Immediately, all right kidneys were carefully excised and immersed in 4% paraformaldehyde. The fixed kidneys were dehydrated through a graded series of ethanol solutions, embedded in paraffin waxes, sectioned at 4-μm thickness, and placed onto glass slides, which made preparations for the further light microscopic evaluation and immunohistochemical analysis. All left kidneys were removed, decapsulated, and divided into cortical and medullary portions.

The cortex tissue (1 mm^3) was immersed in primary fixative (2.5% glutaraldehyde, phosphate buffer, pH 7.2) for the further electron microscopic evaluation.

Histopathology and immuno-histochemistry

Renal tissue sectioned at 4-μm thickness was stained with haematoxylin and eosin for light microscopic evaluation. Immunohistochemistry was performed as follows:

1. The renal tissue sections were blocked with the goat serum, and then incubated with rabbit anti-rat PLA2 and

COX-2 antibodies (Cayman Chemical, USA) diluted 1:100 and stayed overnight at 4°C respectively. As control, phosphate buffered saline (PBS) was used in place of the primary antibodies.

2. Immunocomplexes were visualized using diaminobenzidine tetrahydrochloride.

3. The handling tissue sections were observed under the light microscope, and the PLA2 and COX-2 positive proteins were expressed as brown particles in the cytoplasm.

Electron microscopy

The cortical tissues were taken and fixed with a 2.5% glutaraldehyde fixative solution (pH 7.2~7.4). After rinsing with 0.1 M PBS three times for 5 minutes, tissues were post-fixed with 1% osmium tetroxide for 2 hours.

Dehydration was accomplished by gradual ethanol series (30%, 50%, 70%, 80%, 90% and 100%), and tissues were embedded in epoxy resin. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate. Sections were then viewed and photographed with a transmission electron microscope (JEM1200, Japan).

Cytosolic PLA2 and COX-2 mRNA expression

The total RNA of the renal tissue was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The OD values and concentrations were measured by an ultraviolet spectrophotometer.

The integrity of the RNA was identified using RNA electrophoresis. cDNA was acquired by reverse transcription as Oligo dT - Adaptor used as a random primer. The gene-specific primer was designed and the target gene was amplified by PCR.

The primers for cytosolic PLA2 (cPLA2) were 5' - GTCACCAA CTTGTTCTCA AACCCAT - 3' (sense) and 5' - CAACTCCACCAGAATCTCACT-3' (antisense), and the determined peptide was 497 bp. The primers for COX - 2 were 5'-CAACAAAGTGAGCAAGTCCGT-3' (sense) and 5' - ACACTCTATCACTGGC ATCCG - 3' (antisense), and the determined peptide was 124 bp. The primers for GAPDH were 5' - TCCCTCAAGATTGTCA GCAA - 3' (sense) and 5' - AGATCCACAAACGGATACATT - 3' (antisense), and the determined peptide was 308 bp.

The results were analyzed by Tianneng gelatin analysis software (GIS-2800, Shanghai Tanon Science and Technology Co. Ltd.).

Statistical analyses

The Statistical Package for the Social Sciences (SPSS version 11.0) was used to analyze data. All results were expressed as mean ± SD and differences between groups were evaluated by Student's t-tests. A P value of less than 0.05 was considered to indicate significance.

RESULTS

Body weight and blood glucose

There were no significant differences in rat body weight and blood glucose between two groups before the experiment. We next monitored body weight and blood glucose in two groups

before and at the end of the experiment.

Three rats died in D group during the study. All five surviving rats in D group presenting higher plasma glucose levels (20.66 ± 0.75 mmol/L) were considered diabetic and then included in the study.

The rats in D group had lower body weight and higher blood glucose levels ($p < 0.01$) than the control animals in C group (Table 1).

Pathological analyses

Under light microscope (400 - times magnification), the renal lesions in D group were obvious, which showed increased amounts of mesangial matrix and thickened glomerular basement membranes. Both glomeruli and tubules became dilated. In addition, infiltrating inflammatory cells could be seen in the tubules. Under transmission electron microscope, the ultrastructure

Table 1. Rat body weight and blood glucose before and at the end of the experiment

Group	body weight (g)		blood glucose (mmol/L)	
	before	end	before	end
C	200.88±4.75(8)	331.24±28.90 (8)	4.60±0.60 (8)	4.06±0.44 (8)
D	202.34±8.48*(8)	268.90±47.67#(5)	4.39±0.74* (5)	20.66±0.75# (5)

Data were expressed as mean ± SD. # P<0.01, * P> 0.05, D group compared with C group. The results show that the diabetic rats had lower body weight (268.90±47.67g) and higher glucose level (20.66±0.75 mmo/L) than the control rats at the end of the experiment. n, number of rats.

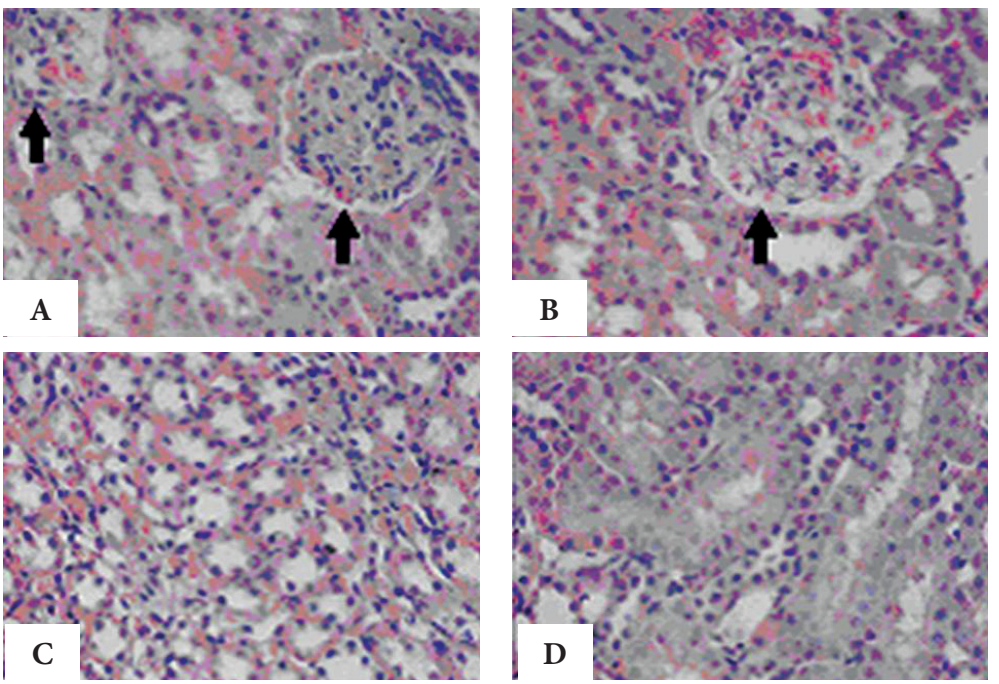

Figure 1. The histopathology of the rat renal glomeruli and tubules under light microscope. The renal lesions in diabetes group were obvious. The results showed that the glomerular volume and the amounts of mesangial matrix (A: Control group; B: Diabetes group) were increased and both glomeruli and tubules became dilated (C: Control group; D: Diabetes group) (magnification, ×400).

of the kidney was intact and distinct in C group. The capillary basement membrane was uniform. Nevertheless, in D group, the irregular thickness of the glomerular filtration membrane was evident. Tubular basement membranes were also thick. Moreover, the fusion and effacement of the foot processes could be observed (Figs. 1 and 2).

Immunohistochemical analyses

We first analyzed both COX-2 and cPLA2 in C and D groups by immunohistochemical analyses. Our results showed that the expression of cPLA2 and COX-2 proteins was negative in C group. In the D group, it showed positive staining in renal tubules and interstitial areas (Fig. 3). These results suggested that hyperglycemia increased the expression of PLA2 and COX-2.

cPLA2 and COX-2 mRNA expression

The RT-PCR amplification of cPLA2 and COX-2 mRNA in the D group showed strongly positive bands, whereas, in the C group, it showed moderate or weak bands (Fig. 4), which revealed that the mRNA expression

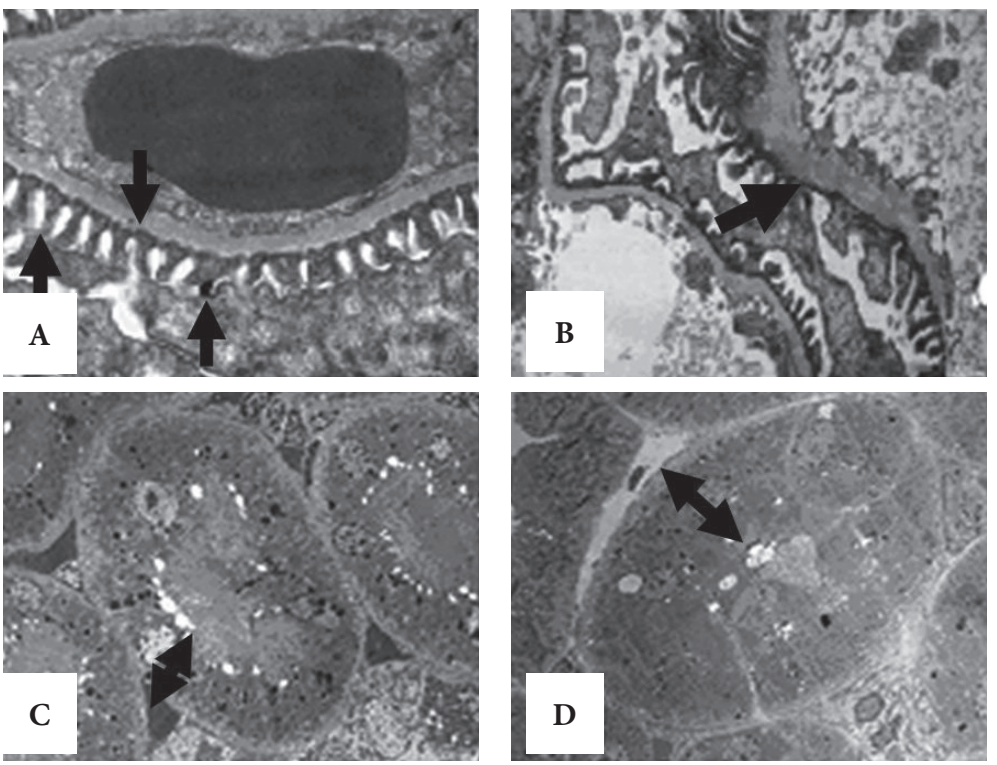

Figure 2. The ultrastructure of the rat renal tissue under transmission electron microscope.The glomerular basement membrane (GBM), the foot process of the podocyte (A: Control group; B: Diabetes group, magnification, ×33000) and the tubular basement (C: Control group; D: Diabetes group, magnification, ×1850) are indicated by arrows. In control group, the ultrastructure of the renal tissue was intact and distinct. In addition, the capillary basement membrane was uniform. In diabetes group, the irregular thickness of the GBM was evident. The tubular basement membranes were thick. Moreover, fusion and effacement of the foot processes could be seen.

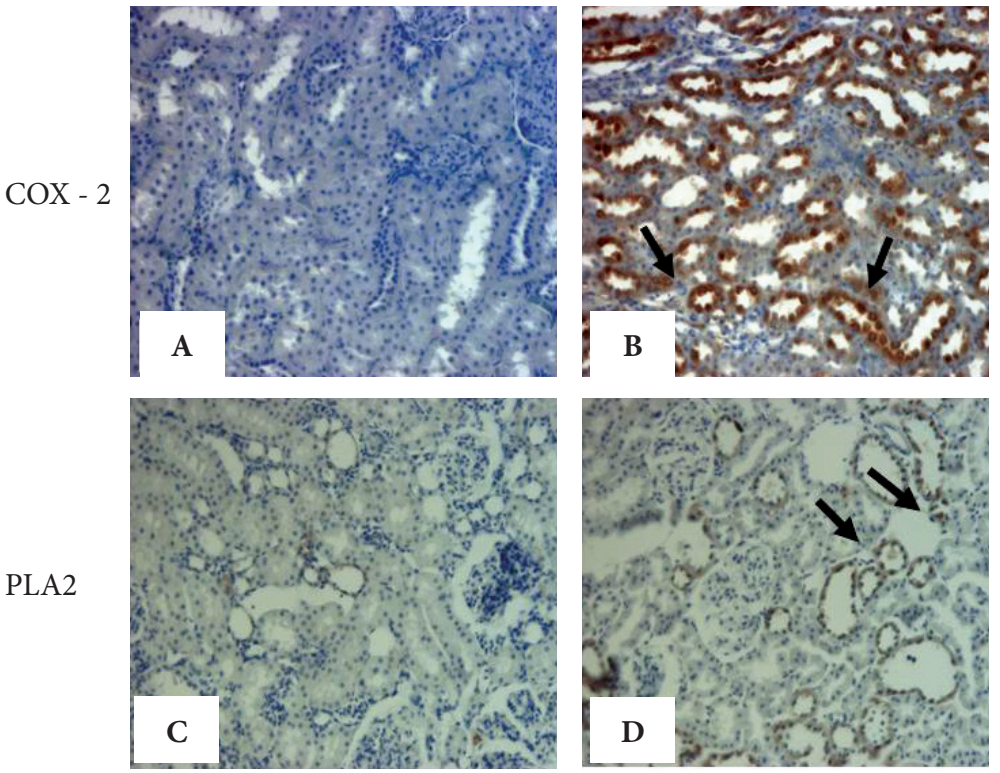

Figure 3. The immunohistochemical results of PLA2 and COX-2 in tubules and interstitial areas in different groups. The expression of COX-2 (A: Control group; B: Diabetes group) and PLA2 (C: Control group; D: Diabetes group) proteins was negative in control group. Nevertheless, it was strongly positive in tubules and interstitial areas in diabetes group (magnification, ×200).

levels of cPLA2 and COX-2 were significantly increased in the diabetic rats compared with that in the normal control group (Fig. 4 and Table 2).

DISCUSSION

Diabetes mellitus is a metabolic disorder that results in hyperglycemia and the development of complications including microvascular (retinopathy and nephropathy) and macrovascular (atherosclerosis) diseases. As a severe microvascular complication of diabetes mellitus is associated with the highest mortality (8), diabetic nephropathy is one of the most important causes for chronic renal failure and the end-stage renal disease, which may need renal replacement therapy worldwide. Although both glomerular hyperfiltration and renal hypertrophy are considered important in the development of diabetic nephropathy, the pathogenesis of diabetic nephropathy remains unclear.

The Kimmelstiel – Wilson nodule, an expansion of the glomerular mesangium, is positively associated with diabetic nephropathy. Some reports showed that hyperglycemia contributes to an increase in the deposition of advanced glycation endproducts in glomeruli leading to mesangial expansion even early on in diabetes (9-11).

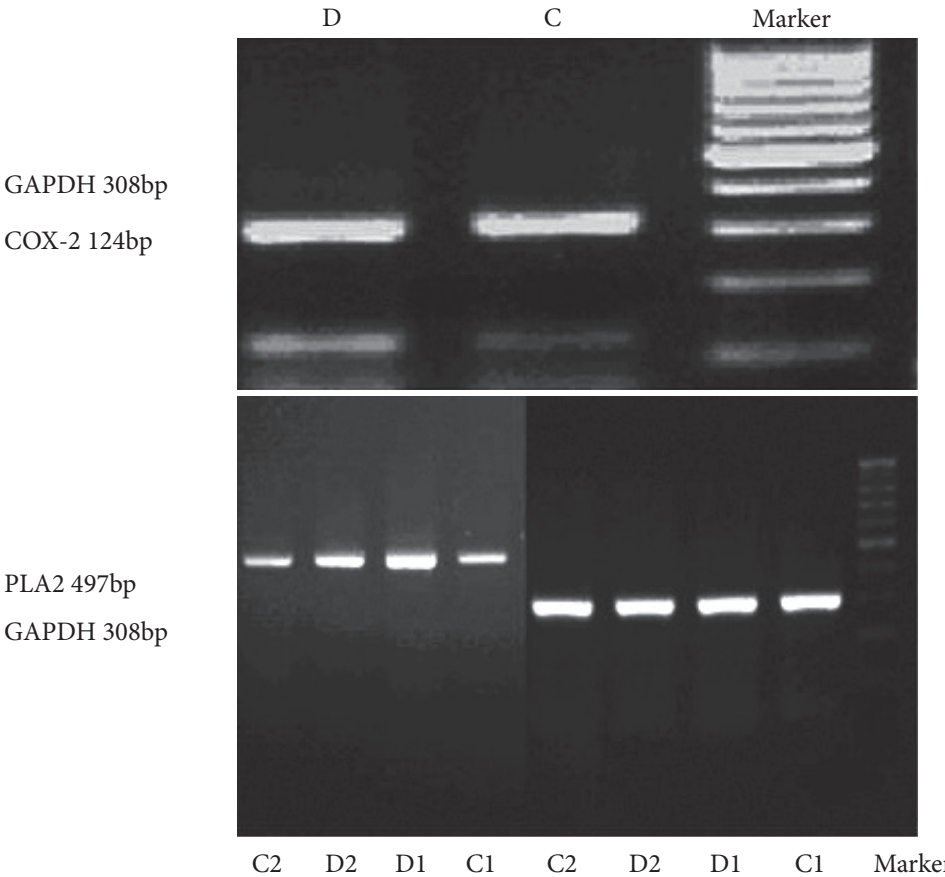

Figure 4. The mRNA expression levels of cPLA2 and COX-2 in the different rat groups. Total RNA of the renal tissue was extracted from control group and diabetes group. Relative amounts of cPLA2 and COX-2 mRNA were measured by semi-quantitative RT - PCR, and GAPDH was used as an internal reference. It showed that the mRNA expression levels of cPLA2 and COX-2 in the rat kidneys were higher in diabetes group than in control group (C = Control group; D = Diabetes group).

Table 2. The cPLA2 and COX-2 mRNA expression by semi-quantitative analysis

Group	COX-2 mRNA (n)	cPLA2 mRNA (n)
C	0.342±0.016 (8)	0.819±0.069 (4)
D	0.474±0.012 # (5)	0.969±0.076* (4)

Data were expressed as mean ±SD of four to eight independent samples for each group. C group compared with D group, *P<0.05, #P<0.01. n = number.

According to our findings, in diabetic rats, both glomeruli and tubules became dilated. In addition, thickening of the glomerular and tubular basement membranes, mesangial proliferation and infiltrating inflammatory cells could be observed. All these reflect a possible association between renal damage and inflammation in diabetic rats. Of course, the pathological changes observed in induced diabetic rats may not represent what happens in human diabetic nephropathy.

Recent evidence has stressed

that inflammation is particularly conducive to the progression of diabetic micro- and macro-angiopathy. Renal pathologic changes consist of renal blood vessel media hypertrophy, focal and segmental glomerulosclerosis, tubular atrophy and interstitial inflammation and fibrosis, which are associated with greater levels of transforming growth factor (TGF)-β1, nuclear transcription factor (NF-κB) and cytosolic phospho-IκB-α, and inflammatory markers expression such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and Mphi chemoattractant monocyte chemoattractant protein-1 (MCP-1) (12).

A number of proinflammatory factors that occur in diabetes appear to play a role, but the mechanisms leading to glomerular hyperfiltration in diabetes are poorly understood (13). Previous studies showed that mesangial cells from diabetic rats cultured with high glucose exhibited higher activity levels of cPLA2 and secreted phospholipase A2 (sPLA2) than those cultured with physiologic level of glucose (14,15). Moreover, cPLA2 could act together with sPLA2 to liberate arachidonate (16-18). All these suggest that increased activity levels of glomerular cPLA2 and sPLA2 may promote the progression of early diabetic glomerular hyperfiltration in the course of diabetic nephropathy. Nevertheless, the role of sPLA in promoting arachidonic acid release in mammalian cells is much less clear than for cPLA2 and is under active investigation (18).

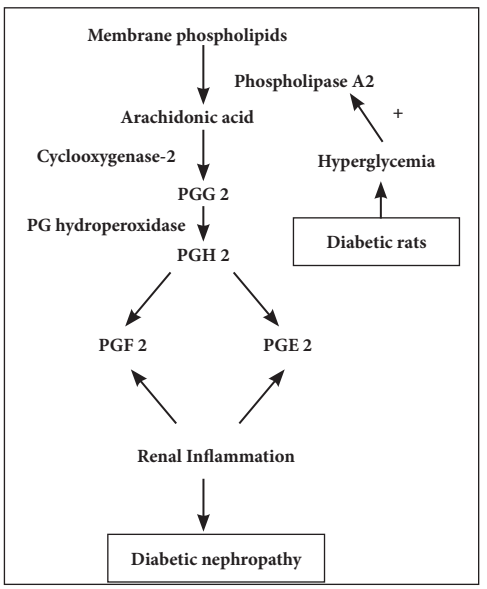

Figure 5. The coordinate activation of phospholipase A2 and cyclooxygenase-2 may induce renal inflammation in streptozotocin-diabetic rats. Prostaglandin E2 (PGE2), prostaglandin G2 (PGG2), prostaglandin H2 (PGH2), prostaglandin F2 (PGF2).

Furthermore, it was reported that various cytokines such as interleukin-1b, tumor necrosis factor-α can induce the expression of COX-2 (19), resulting in damage to renal blood vessels and glomerular filtration membranes. Treatment with the selective COX-2 inhibitor can attenuate the development of diabetic nephropathy in rats (3). Hence, it is considered that COX-2, a downstream enzyme of cPLA2 and secreted phospholipase A2 (sPLA2), plays an important role in the inflammatory process of diabetic nephropathy.

Under a normal condition, COX-2 is expressed at low or undetectable levels but is readily up-regulated by a wide range of inflammatory mediators (20), physical stimuli (21) and high blood glucose (4-7). It is widely known that PLA2 catalyses the liberation of arachidonic acid from cell membrane phospholipids, and COX-2 uses arachidonic acid as a substrate

to generate inflammatory mediators like prostaglandins (prostaglandin E2, prostaglandin F2 alpha) and thromboxane A2 (22-24). In addition, PLA2 activates or is activated by some inflammatory factors, such as NF-κB and signal transducers and activators of transcription-3 (STAT3) (25). Moreover, over-expression of both PLA2 and COX-2 in cells can lead to produce high levels of prostaglandins, because a coordinate activation of PLA2 and COX-2 is necessary to produce significant amounts of prostaglandins (26).

In this study, the expression of both cPLA2 and COX-2 in kidney was increased, and moderate immunoreactivity was observed in renal tubules and interstitial areas of streptozotocin - diabetic rats. These hint that elevated cPLA2 and COX-2 - mediated inflammatory response contributes to the development of diabetic nephropathy (Fig. 5).

In conclusion, in the study the renal tissue of the diabetic rats had significant destruction including increased extracellular matrix, thickened basement membranes, fused foot processes and infiltrating inflammatory cells, which may be induced through the cPLA2 – COX - 2 pathway.

Acknowledgement

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Conflict of interest

The authors declare that there are no conflicts of interest.

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