Baicalin attenuates fibrogenic process in human renal proximal tubular cells (HK-2) exposed to diabetic *milieu*

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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(Directed by professor Chul Woo Ahn)

Baicalin is a flavonoid glycoside substance extracted from *Scutellaria baicalensis*, which has been suggested to exhibit anti-fibrotic effect owing to multiple therapeutic properties as well as effect on glucose metabolism. Diabetes mellitus is characterized by chronic hyperglycemic condition, inducing inflammation and oxidative stress, thereby, it can promote renal fibrosis and kidney failure. Although baicalin has been reported that it has anti-fibrogenic effects in lung and liver, no study has investigated its roles and mechanism in the kidney. In this study, we examined protective effect of baicalin in human kidney proximal tubular epithelial cells (HK-2) induced by diabetic *milieu* and suggested mechanism of the effect. According to the results, baicalin attenuated NF-*κ*B and STAT3 activation level via inhibiting I*κ*B and JAK2 phosphorylation*, respectively*, induced by oxidative stress and inflammation. Consequently, baicalin also significantly reduced transactivation of NF-*κ*B- and STAT3-associated genes such as ICAM1, VCAM1, TGFβ, IL1β and MCP1 and expression of TGFβ-associated extracellular matrix proteins such as fibronectin and collagen IV. These effects can be attributed to type A γ-aminobutyric acid receptor ($GABA_AR$) which interacts with baicalin and, subsequently, regulates intracellular concentration of Ca^{2+}. Based on these results, baicalin, which interacts with $GABA_AR$, can mediate anti-inflammatory and anti-fibrotic effect via both Ca^{2+} induced and oxidative stress induced NF-*κ*B and JAK2/STAT3 pathway in DN conditioned PTEC.

Key words: baicalin, diabetic nephropathy, human proximal tubular cell, NF-κB, JAK/STAT, $GABA_AR$

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I. INTRODUCTION

Diabetes mellitus (DM) is defined as a metabolic disorder, including lack of insulin production and insulin resistance with high concentration of blood sugar. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are called as intermediate hyperglycemia or prediabetes[1]. According to the recent research in 2017, DM is prevalence in 425 million of patients and is now considered to global epidemic[1]. Also, it is predicted to increase up to 629 million in 2045, according to the results by International Diabetes Federation (IDF)[1]. DM is chronic disease and associated with metabolic diseases such as atherosclerosis, end-stage renal disease (ESRD), and blindness[2]. The vascular complications of DM is the main reason for impaired life expectancy in patients with DM[2].

To prevent and improve diabetic vascular diseases induced by DM, glucose concentration in blood is considered as an efficacious target of remedy[2,3]. Regular

exercises and proper diet improve metabolic signaling pathways via blood glucose level on the aspect of Type 2 diabetes mellitus (T2DM)[3]. In addition, treatment of medications is necessary when needed to manage acute and chronic complications[3]. The commonly used medications for DM are metformin, sulphonylureas, glucagon-like-peptide-1 (GLP-1) agonists, dipeptidylpeptidase4 (DPP4) inhibitors and sodium-glucose cotransporter 2 (SGLT2) inhibitors including insulin to enhance the body's natural response on control of blood glucose concentration[1]. In Korea, prescription of combination therapies of anti-diabetic medications including DPP4 inhibitors have been increased and exceeded single treatment in 2016[3]. Recently, many anti-diabetic medications are studied to confirm mechanism of other effects more than lowering blood sugar level due to much attention focused on the management of microvascular and macrovascular complications[4-6].

Hyperglycemia, if left not well managed over the long term, can lead to the development of disabling health complications, affecting the eyes, nerves, kidneys, and the heart[3]. Excessive glucose supply can accelerate alteration in microvascular function including capillary basement membrane, such as arterioles in the glomeruli, retina, myocardium, skin, and muscle, by increasing their thickness, leading to the development of diabetic microangiopathy[6]. This thickening eventually induces multiple clinical problems such as hypertension, delayed wound healing, and tissue hypoxia, as a result, failure of various organ systems[6]. People with diabetic complications have an increased risk of serious life-threatening health problems which demand high medical care costs and lower quality of life[1].

Diabetic nephropathy (DN), one of diabetic complications, is a main cause of ESRD[7], thereby, high mortality and morbidity among T2DM patients[8,9] and 30.3% of patients with DM have it in Korea[3]. While histological changes in the glomerulus

has been traditionally focused in DN, the changes in tubulointerstitium has become widely acknowledged as an initiator of pathogenesis of DN, particularly in tubulointerstitial fibrosis, which correlate with dysfunction of kidney[4,10]. There is a growing body of evidence that the renal proximal tubular epithelial cell (PTEC) plays an important role in the pathogenesis of DN[9]. Markers of tubular dysfunction have a good correlation with urinary albumin excretion[11,12]. In the 'retrieval hypothesis', high levels of albumin can appear in the urine in nephrotic amounts when glomeruli is normal but tubular reabsorption does not occur[11,13]. In addition to normal glomerulus, leaking protein due to impaired glomerular permeability can be prevented from proteinuria by tubular reabsorption[11] and protein overload can cause intrinsic tubular toxicity[14] which may provide a pathogenic link to DN[11]. This means that tubules are primarily origin of albuminuria[11,13]. Also, there are direct indicators of injury of the proximal tubule, N-acetyl-β-D-glucosaminidase, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM1), which are kinds of tubular biomarkers and reflect demise of proximal tubular reabsorption of filtered proteins[11]. Taken together, a lot of evidence has been suggested that direct effects of high glucose mediate inflammation and fibrosis in PTEC[4,15].

DN is well-characterized with the accumulation of extracellular matrix (ECM)[7]. Hyperglycemia, which is the major phenomena of DM, is the major contributor of the progression to the ESRD and downstream of this condition is connected to inflammation and subsequent ECM expansion, as a result, DN[7]. Hyperglycemia can induce inflammation through three pathways; 1) intracellular signaling and metabolism, 2) glycation and oxidative stress, 3) glomerular hyper filtration and hypertension[7]. Advanced glycation end-products (AGEs) are results of non-

enzymatic reaction between reducing sugars and amine residues on proteins, lipid, or nucleic acids[16] and can be produced in hyperglycemia *in vivo*[17]. According to activated inflammation pathways, nuclear factor κB (NF-κB) and janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, especially JAK2 and STAT3[18], are major and the most important transcription factors in the pathogenesis of DN[7]. NF-κB and STAT3 can translocate to nucleus via phosphorylation of IκB and JAK2, *respectively*, followed by activating gene transcription[18,19]. NF-κB and STAT3 bind to the promoter sites of several genes that are significantly involved in the pathogenesis of DN, such as pro-inflammatory cytokines; tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), interleukin (IL) 1β and IL6, chemokines; CC chemokine ligand 2 [CCL2, also known as monocyte chemoattractant protein1 (MCP1)], and adhesion molecules; intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1)[7,20-24]. Specifically, TGFβ is known as a key molecule for the accumulation of ECM, such as fibronectin (FN) and collagen type IV (COL4)[25,26], via smad2/3 or AP-1 transcription factor pathway[17,26,27], as wound healing mechanism, which, when it is chronic, can lead to renal fibrosis and deterioration of kidney function. These suggest that attenuation of diabetes-associated molecules, including oxidative stress and inflammatory response, is critical in the prevention of DN progress.

Baicalin is a flavonoid glycoside substance extracted from *Scutellaria baicalensis*, which has been suggested to have a role on glucose metabolism[28,29]. Especially, baicalin can activate AMPK pathway and AKT pathway and suppress hepatic gluconeogenesis, including expression of gluconeogenic genes, such as glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and glucose

transporter 2 (GLUT2) in HepG-2 cells[30]. In addition, it has been reported that it plays a role as the anti-inflammatory and anti-fibrosis in several kinds of cell[25,31]. For example, the anti-fibrotic effect of baicalin is reported in bleomycin induced pulmonary fibrosis of mice model via TGFβ induced ERK1/2 signaling pathway[32]. As the pathogenesis of DN can be characterized as the process of fibrosis via inflammation, a therapeutic effect of baicalin on DN is expected. However, there is a lack of evidence in human proximal tubular cells with diabetic *milieu* induced by high glucose levels.

Recently, the multiple physiological effects of baicalin have been demonstrated, however, the receptors and cellular cascades mediating its effects are still not clear. Type A γ-aminobutyric acid receptor ($GABA_AR$) is reported that it has ligand-receptor interaction with baicalin[33]. $GABA_AR$ is a heteropentameric GABA-gated chloride channel[34,35] and involved in most of the inhibitory synaptic transmissions in the central nervous system[33,36]. It has benzodiazepine binding site which is used for psychoactive drugs[35] and there were previous studies that baicalin may bind to this region[33,35] with less side effects. Benzodiazepines are reported that they modulate the chloride flux through the ion channel complex of $GABA_AR$ after binding[35]. Although $GABA_AR$ subunits was identified in rat PTEC[37], there is limited information concerning the expression of $GABA_AR$ subunits and what their physiological role in the human kidney.

Considering that TNFα can activate inflammatory process and AGE can stimulate oxidative stress-associated pathogenesis, it is assumed that hyperglycemia with TNFα and AGE may have synergetic effect on inducing DN condition *in vitro*. Given that baicalin inhibited inflammatory signaling pathway in other cells, it is anticipated that baicalin has anti-fibrotic effect in many kinds of cells, such as

endothelial cells. However, baicalin has lack of evidence concerning its protective effect in PTEC, which plays an important role on initiation of DN.

In addition, there is no investigation on receptor in mediating the roles of baicalin in human kidney cells. Considering that $GABA_AR$ is suggested as receptor for baicalin as anxiolytic ligand in neuron cells[35], it is anticipated that baicalin can also interact with $GABA_AR$ in kidney cells and activate pathway. Therefore, in this study, the protective effect and mechanism of the effect of baicalin in human PTEC, HK-2 cells, was examined.

II. MATERIALS AND METHODS

1. Cell culture and treatment

Primary human kidney cortex proximal tubule cell line (adult male), HK-2 cell, was purchased from American Type Culture Collection (ATCC, CRL 2190, Gaithersburg, Maryland**,** USA). For culture, Keratinocyte Serum Free Media (KSFM, 10724011, Gibco, Grand Island, NY, USA) supplemented with 30μg/ml bovine pituitary extract (13028-014, gibco, Grand Island, NY, USA) and 0.1ng/ml epidermal growth factor (10450-013, gibco, Grand Island, NY, USA) was used. Cell culture media was changed every 48hrs. Cells were grown in humidified chamber conditioned by 37 ℃, 5% CO_2 atmosphere and subcultured at 70-80% confluence using 0.25% trypsin-EDTA solution (SH30042.01, Hyclone, Logan, Utah, USA).

Cells at 80-90% confluence were used in all experiments. Cells were treated with 5 or 10μM baicalin (572667, Sigma-Aldrich, Saint Louis, MO, USA), TNFα (SRP3177, Sigma-Aldrich, Saint Louis, MO, USA) at 100ng/ml, AGE at 100μg/ml for 72hrs or 96hrs to induce DN condition.

2. Preparation of AGEs

AGE was prepared as previously described[38-40]. Briefly, 100mg/ml bovine serum albumin (BSA; A0100050, GenDEPOT, Korea) was incubated with 0.5M D-glucose (G7021, Sigma-Aldrich, Saint Louis, MO, USA) in DPBS (SH30028.02, HyClone Logan, Utah, USA) in the 37 ℃ dehumidifier chamber (J-100M, JISICO, Gyeonggi-do, Korea) for 10wks in dark. After the incubation, protein concentrations were determined using the bicinchoninic acid (BCA) assay (23225, Pierce, Rockford, IL, USA) according to the supplier's protocol.

3. Immunocytochemistry and confocal microscopy imaging

Fibronectin and collagen IV, kinds of protein of extracellular matrix, were analyzed by immunocytochemistry (ICC) followed by imaging laser confocal microscopy. For ICC, cells were cultured on 22x22mm coverslip (0101050, Marienfeld, Lauda-Konigshofen, Germany). Before seeding, the cell culture slide was coated with Poly-D-lysine (P6407, Sigma Aldrich, St. Louis, MS, USA) at concentration of 50μg/ml for overnight at 4 ℃. Prior to ICC, cells were treated with or without 30mM glucose, 100μg/ml AGE, 100ng/ml TNFα and 5μM baicalin for 96hrs in 37 ℃ humidified incubator.

For ICC, cells were fixed in containing 4% (*v/v*) paraformaldehyde (PC2031-100-00, Biosesang, Gyeonggi-do, Korea) for 10mins at room temperature, and were washed three times with PBS containing 0.1% Tween 20 (0777, VWR life science, Solon, Ohio, USA) (PBST). To block unspecific binding of the antibodies, cells were incubated in PBST containing 10% (*v/v*) goat serum (S26, EMD Millipore, Billerica, MA, USA) at room temperature for 1hr. Then, cells were incubated in primary antibody for fibronectin (diluted at 1:500, ab6328, abcam, Cambridge, UK) and collagen IV (diluted at 1:150, NB1206586, Novus Biologicals, Centennial, CO, USA) diluted in blocking solution (10% goat serum in PBST) overnight at 4 ℃. After three times of washing for 5mins with PBST, cells were incubated in blocking solution containing Alexa Fluor 488 (ab150077, abcam, Carlsbad, USA) to detect COL4 and Alexa Fluor 647 (ab150115, abcam, Carlsbad, CA, USA) to detect FN with confocal microscopy. For counter staining, cells were incubated with 4`,6-Diamidino-2-Pheylindole, Dilactate (DAPI) solution (D3571, Invitrogen,

Carlsbad, CA, USA) diluted blocking solution at 5ug/ml for 10mins. After washing, the cells were mounted with coverslip with fluorescent mounting medium (S3023, Dako, Carpinteria, CA, USA). The cells were imaged at room temperature using confocal microscopy (LSM780, Carl Zeiss, Stuttgart, Germany) with a 40X water objective lens; and the images were processed by Zen Blue Edition for Windows (Carl Zeiss, Stuttgart, Germany).

4. Quantitative real time PCR (qRT-PCR)

For mRNA extraction to examine transcriptional levels, cells were collected in Tri-RNA Reagent (FATRR 001, Favorgen, Kaohsiung, Taiwan) and extraction protocol, which includes chloroform (UN1888, DUKSAN, Gyeonggi-do, Korea), and iso-propyl alcohol (UN1219, DUKSAN, Gyeonggi-do, Korea) was used. After extraction, cDNA was synthesized from RNA samples of HK-2 cells by reverse transcriptase PCR using M-MLV Reverse Transcriptase kit (M1705, Promega, MA, USA). The PCR protocol followed the kit.

The gene expression levels of inflammatory and fibrotic markers were tested by quantitative real time PCR(qRT-PCR) using TOPreal^{TM} qPCR2X PreMIX (SYBR Green with low ROX) (RT500M, enzynomics, Daejeon, Korea) on LightCycler 480 System (Roche, Basel, Switzerland). All reactions were performed in triplicate. RNA levels were normalized to the level of GAPDH and calculated as delta-delta threshold cycle (ΔΔCT). Primers used for qRT-PCR are listed in Table1.

The expression level of $GABA_AR$ was tested by semi quantitative real time PCR ($_{semi}$PCR) using TopTaq DNA Polymerase kit (200205, QIAGEN,

Hilden, Germany). PCR primers designed for the receptor subunits (α_{1-6}, β_{1-3}, and γ_{1-3}) are listed in the Table2. The PCR protocol was as follows: pre-denaturation at 94 ℃ for 7mins, denaturation at 94 ℃ for 30secs, annealing at 60 ℃ for 30secs, and amplification at 72 ℃ for 30secs.

Table 1. List of primers

Name	Primer	Product size	T_m (℃)
FN: forward	AAGAGGCAGGCTCAGCAAAT	176	60
FN: reverse	TCGCAGTTAAAACCTCGGCT		
$COL4A_1$: forward	CTCCACGAGGAGCACAGC	414	60
$COL4A_1$: reverse	CATCCCTGGTAAGCCTGGTG		
$COL4A_2$: forward	CTGCCACTACTACGCCAACA	153	60
$COL4A_2$: reverse	CCGGCTCACAGGTTCTTCAT		
TGFβ: forward	GCGTGCTAATGGTGGAAACC	122	60
TGFβ: reverse	GAGCAACACGGGTTCAGGTA		
ICAM1: forward	GGTAGCAGCCGCAGTCATAA	122	60
ICAM1: reverse	TGTTCGGTTTCATGGGGGTC		
VCAM1: forward	TTGACCGGCTGGAGATTGAA	239	60
VCAM1: reverse	TCTGGGGGCAACATTGACAT		
IL1β: forward	AGTACCTGAGCTCGCCAGT	171	60
ILβ: reverse	CCCTTGCTGTAGTGGTGGTC		
IL6: forward	CAATAACCACCCCTGACCCAA	101	60
IL6: reverse	GCGCAGAATGAGATGAGTTGTC		
MCP1: forward	AGCAGCAAGTGTCCCAAAGA	131	60
MCP1: reverse	GTCTTCGGAGTTTGGGTTTGC		

FN; fibronectin; $COL4A_1$, collagen type IV alpha chain 1; $COL4A_2$, collagen type IV alpha chain 2; TGFβ, transforming growth factor beta; ICAM1, intracellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1; IL, interleukin; MCP1, monocyte chemoattractant protein1.

Table 2. List of primers

$GABA_AR$ subunit	Primer	Product size	T_m (℃)
α1: forward	GGACCCGTTTCAGACCATGA	236	60
α1: reverse	TACAGCAAGGTGCCATCCTC		
α2: forward	GCTCGGAATTCTCTCCCCAAA	139	60
α2: reverse	CATCCCAAGCCCATCCTCTTT		
α3: forward	TCTGGACGGCTATGACAACC	190	60
α3: reverse	TGGAAGGATCTTCATGGGGC		
α4: forward	GGCACACCTCGGTCTTACTT	227	60
α4: reverse	TTCCCAGTAGCCCCTATGGT		
α5: forward	TATGCCTTCGTCTTCTCGGC	191	60
α5: reverse	TCCTTCGGAATGTTTGGGGG		
α6: forward	CGAGAGCGCCCATCTTACAA	197	60
α6: reverse	GGCCGCAAGCTATTCAACAC		
β1: forward	ATCGAGAGAGTCTGGGGCTT	200	60
β1: reverse	TATGCTGGCGACATCGATCC		
β2: forward	TCTGGACAACAGAGTGGCAG	138	60
β2: reverse	AGTCCATAAAGGACGGTGCC		
β3: forward	AGGTGCCTATCCTCGACTGT	161	60
β3: reverse	TGATCCCGAGGGCAACTCTA		
γ1: forward	AGGGGTGAGGTTGGTCTTCT	210	60
γ1: reverse	ACTGTGGGCCTCACTCCTAT		
γ2: forward	TGACTCCAAAAGTTCCTGAGGG	205	60
γ2: reverse	CGTCTGTCATACCACGTTTGC		
γ3: forward	AATCCCAAGACACCGACGTG	214	60
γ3: reverse	GTGCTGTTGAATCGAAGGCG		

5. Western Blots

Fully cultured and conditioned HK-2 cells were extracted by RIPA Lysis Extraction Buffer (89900, Thermo Scientific, Waltham, MA, USA) containing protease/phosphatase inhibitor cocktail (5872s, cell signaling technology, Danvers, MA, USA). Suspensions were centrifuged at 13,000 rpm at 4 ℃ for 15mins. To determine protein concentration, the BSA assay (23225, Pierce, Rockford, IL, USA) was performed; 20μg of protein was subjected to 7~10% SDS-PAGE gels and transferred to the nitrocellulose membranes (10401196, Capitol Scientific, Austin, TX, USA). Transferred membranes were incubated in the blocking solution (TBS buffer with 0.1% Tween 20) containing 5% BSA (*w/v* ratio) for 1hr at room temperature. All antibodies used in this study are listed in Table 3 below. Primary antibodies were reacted overnight at 4 ℃; secondary antibodies were reacted for 1hr 30mins at room temperature. Pierce ECL Substrate (32106, Thermo Scientific, Waltham, MA, USA) was used to amplify the detectable signals for film development. The intensities of the protein bands developed on films were quantified by using ImageJ (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, Madison, WI, USA). The band intensities were quantified and represented in bar graphs.

Table 3. List of primary and secondary antibodies

Primary Antibodies				
Name		**Manufacturer**	**Dilution**	**Gel %**
1.	GAPDH	2118,Cell Signaling	1:2000	10, 8, 14
2.	YY1	SC7341, Santa Cruz	1:1000	10
3.	TGFβ	3711,Cell Signaling	1:1000	14
4.	ICAM1	ab2213, abcam	1:500	8
5.	VCAM1	ab134047, abcam	1:2000	8
6.	NF-κB	8242,Cell Signaling	1:1000	10
7.	pIκB$^{Ser32/36}$	9246, Cell Signaling	1:1000	10
8.	pIκB^{Tyr42}	ab24783, abcam	1:1000	10
9.	Total-IκB	ab32518, abcam	1:1000	10
10.	pSTAT3	9145, Cell Signaling	1:2000	9
11.	STAT3	4904, Cell Signaling	1:2500	9
12.	pJAK2	3230, Cell Signaling	1:500	9
13.	JAK2	8082, Cell Signaling	1:500	9
14.	IL1β	NBP1-19775, Novus Biologicals	1:1000	13
15.	IL6	ab6672, abcam	1:500	13
16.	MCP1	MAB2791, R&D Systems	1:500	13
Secondary Antibodies				
1.	Anti-rabbit IgG, HRP-linked antibody	7074,Cell Signaling	1:3000	All
2.	Anti-mouse IgG, HRP-linked antibody	7076,Cell Signaling	1:3000	All
3.	Goat anti-Rabbit IgG(H+L)-HRP	SA002-500, GenDEPOT	1:5000	All
4.	Goat anti-Mouse IgG(H+L)-HRP	SA001-500, GenDEPOT	1:5000	All

The numbers in manufacturer are catalog number of products. Optimized antibody concentrations are presented at dilution. Percentage of gels which were used in SDS-PAGE is presented. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; YY1, Yin Yang 1; p, phosphorylation, HRP, horseradish peroxidase.

6. cAMP and cGMP production measurement

To examine the cellular responses in HK-2 cells stimulated by baicalin, cAMP and cGMP production were measured by Cyclic AMP ELISA KIT (581001, Cayman Chemical Technology, Ann Arbor, MI, USA) and Cyclic GMP ELISA KIT (581021, Cayman Chemical Technology, Ann Arbor, MI, USA). For this assay, cells were treated with 5 and 10μM baicalin for 1hr. Then, cells were incubated 0.1M HCl for 20mins and supernatants were assayed directly. cAMP or cGMP in the lysates was acetylated to increase sensitivity using potassium hydroxide and acetic anhydride. Assay was performed according to instructions. cAMP or cGMP productions were calculated by reading HRP activity at OD_{405nm} with plate reader (VersaMax, Mplecular Device, Sunnyvale, CA, USA).

7. Ca^{2+} influx measurement

Fully cultured HK-2 cells were seeded into black well and clear bottom 96-well plates at 4×10^4 cells per well. Alterations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration by baicalin and bicuculine in HBSS (LB003-04, WELGENE, Gyeongbuk, Korea) containing 2mM $CaCl_2$ (2506-1405, DAE JUNG, Gyeonggi-do Korea) were assessed by the Fluo-8 No Wash Calcium Assay kit (ab112129, abcam, Cambridge, UK) according to the manufacturer's instructions. Fluorescence intensities were measured in a FLUOstar OPTIMA microplate reader (413-101, BMG Labtech Inc. Ortenberg, Germany). Then, 5μM baicalin with and without 100μM bicuculine were injected by the equipped syringe on-board reagent injectors. Samples were excited at 485nm and Ca^{2+}-bound Fluo-8 fluorescence was recorded at 525nm. The fluorescence intensity was recorded 40 times.

8. Statistical analysis

All data was analyzed using SPSS software Version 18.0 for windows (IBM, Armonk, NY, USA). All data was represented as mean±SD and the mean differences between the conditions were assessed by independent *t-test*. $P<0.05$ was considered statistically significant.

III. RESULTS

1. The effects of baicalin on fibrosis process in HK-2 cells exposed to diabetic *milieu*

To examine the anti-fibrotic effects of baicalin on PTECs, the expressions of fibrotic markers, such as fibronectin (FN) and collagen IV (COL4) associated with DN in response to long-term exposition to diabetic *milieu*, were measured at protein and gene level. In response to exposure of hyperglycemia (30mM), protein expression of COL4 (Figure 1) and gene expressions of FN, collagen IV α chain 1 ($COL4A_1$) and collagen IV α chain 2 ($COL4A_2$) (Figure 2) were significantly increased, and baicalin attenuated these increases. The co-treatment of AGEs and TNFα, mediating oxidative stress and inflammation, *respectively*, with hyperglycemia for 96hrs demonstrated synergetic fibrotic effects, and baicalin attenuated the increase in both FN and COL4 in the same condition at protein level (Figure 1). Transcription levels of FN were considerably increased in response to 96hrs co-treatments of AGEs or/and TNFα in hyperglycemic conditions, compared to control, and baicalin significantly attenuated these increases (Figure 2). Baicalin considerably attenuated the increased $COL4A_1$ transcription level, induced by exposure of AGEs and AGEs with TNFα in hyperglycemic conditions for 96hrs, while it attenuated the increased $COL4A_2$ transcription level, induced by 96hrs exposure of TNFα and AGEs with TNFα in hyperglycemic conditions (Figure 2).

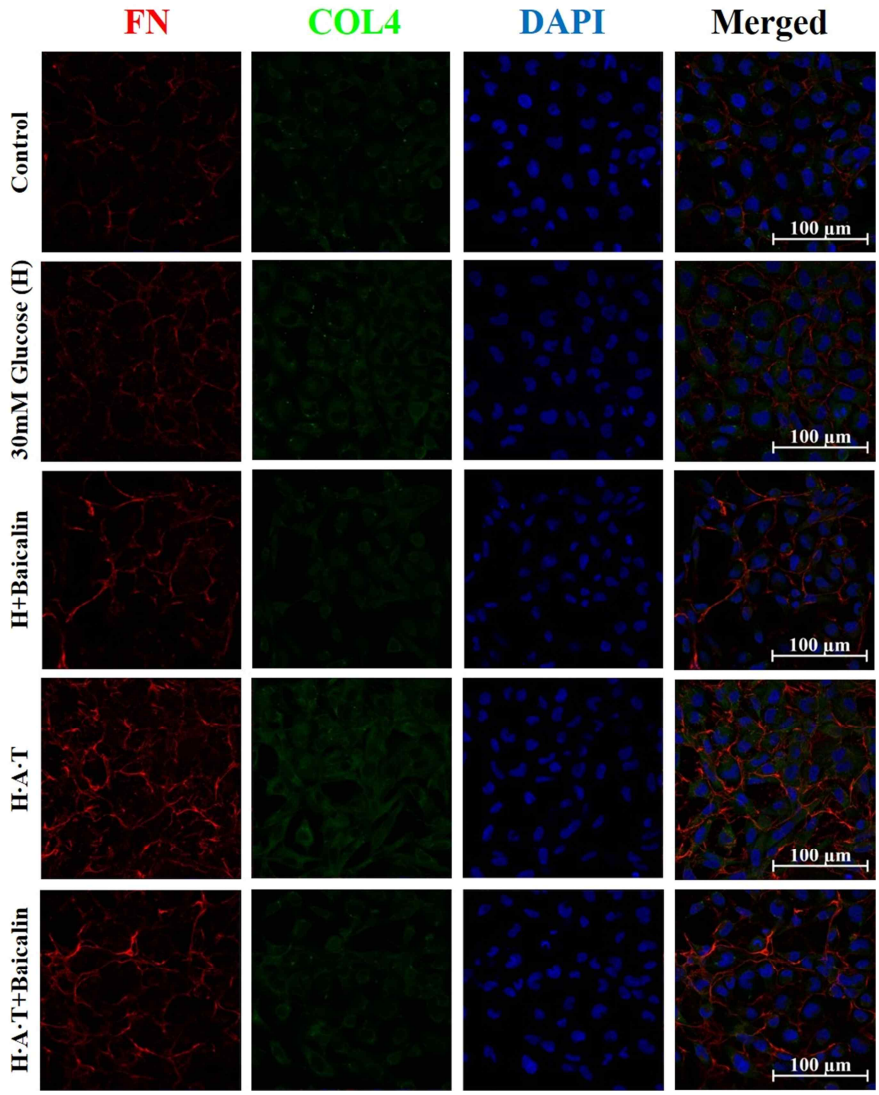

Figure 1. The effects of baicalin on protein expression levels of fibrosis markers. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 96hrs. Normal control represents culture condition in the normal glucose growth medium only. Expression of extracellular matrix proteins (FN and COL4) in HK-2 cells was detected by confocal laser scanning microscopy following ICC. A; advanced glycated end-products, COL4; collagen type IV, FN; fibronectin, T; tumor necrosis factor α.

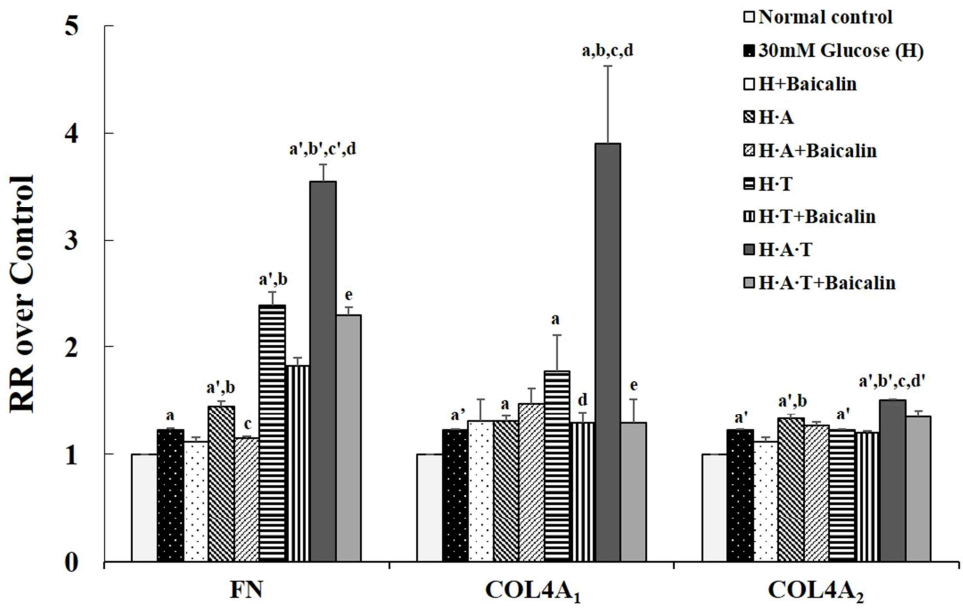

Figure 2. The effects of baicalin on gene expression levels of fibrosis markers. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 96hrs. Expression levels of FN, $COL4A_1$ and $COL4A_2$ in HK-2 cells were analyzed by qRT-PCR. Data is represented by relative ratio to normal control. $^{a}P<0.05$ and $^{a'}P<0.001$ compared with normal control, $^{b}P<0.05$ and $^{b'}P<0.001$ compared with H, $^{c}P<0.05$ and $^{c'}P<0.001$ compared with H·A, $^{d}P<0.05$ and $^{d'}P<0.001$ compared with H·T, $^{e}P<0.05$ compared with H·A·T. A; advanced glycated end-products, $COL4A_1$; collagen type IV α chain 1, $COL4A_2$; collagen type IV α chain 2, FN; fibronectin, T; tumor necrosis factor α.

2. The effects of baicalin on activation of transcription factors in HK-2 cells exposed to diabetic *milieu*

To investigate anti-inflammatory effect of baicalin, activation levels of NF-κB and STAT3, which can translocate to nucleus in response to oxidative stress and/or inflammation[7], were examined by western blot. According to the results, baicalin significantly inhibited the nuclear translocation of NF-κB in response to hyperglycemic condition and the condition with AGEs and TNFα for 1hr without affecting total NF-κB level (Figure 3). In addition, considering that phosphorylated STAT3 by JAK2 can be induced during pathogenesis of DN[18], co-treatment of AGEs and TNFα with hyperglycemia for 1hr increased phosphorylation levels of JAK2 and STAT3 and baicalin decreased the increases without significant change of total JAK2 and STAT3 levels (Figure 4).

Given that nuclear translocation of NF-κB and STAT3 transactivates inflammation-inducing molecules[41], our results suggest that baicalin can attenuate inflammation via regulating transcription factors, NF-κB and STAT3 in HK-2 cells.

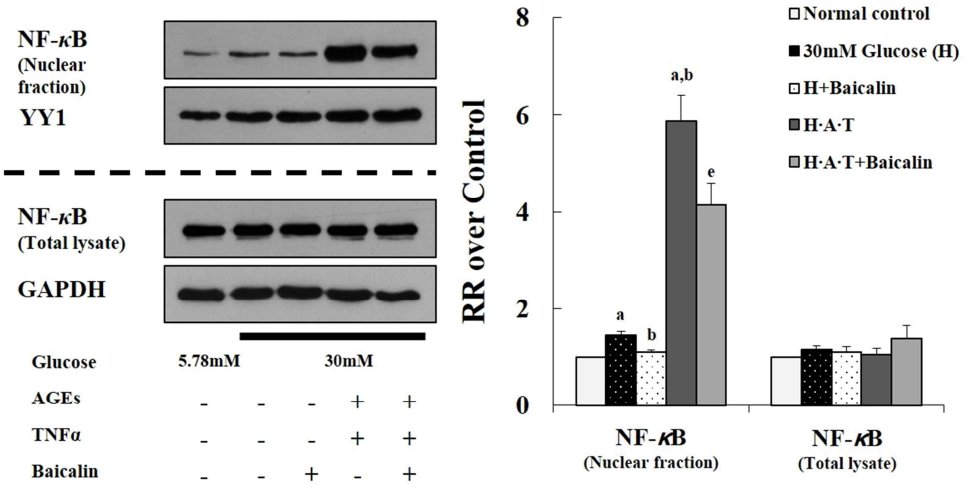

Figure 3. The effects of baicalin on translocation of NF-κB during inflammation. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 1hr. Baicalin (5μM) decreased the levels of nuclear translocation of NF-κB. Bar-graph represents relative ratio to control. NF-κB of nuclear fraction and YY1 were each cropped from the same gel. High-contrast was not used. $^{a}P<0.05$ compared with control, $^{b}P<0.05$ compared with H. $^{e}P<0.05$ compared with H·A·T. A; advanced glycated end-products, T; tumor necrosis factor α.

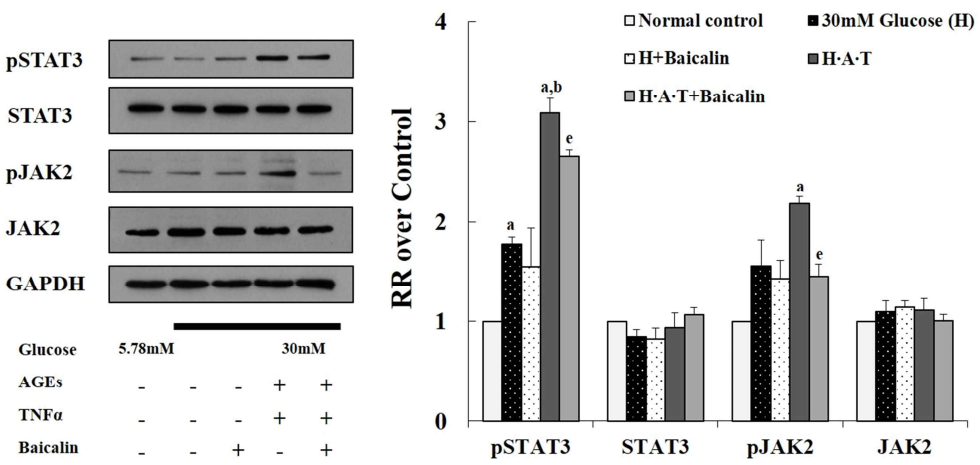

Figure 4. The effects of baicalin on phosphorylations of JAK2/STAT3 pathway during inflammation. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 1hr. Baicalin (5μM) decreased the levels of phosphorylation on STAT3 and JAK2 compared with H·A·T. Bar-graph represents relative ratio to control. All membranes were each cropped from the same gel. High-contrast was not used. $^{a}P<0.05$ compared with control, $^{b}P<0.05$ compared with H. $^{e}P<0.05$ compared with H·A·T. A; advanced glycated end-products, T; tumor necrosis factor α.

3. The effects of baicalin on expression of inflammatory molecules in HK-2 cells exposed to diabetic *milieu*

To examine anti-inflammatory effects of baicalin, expression levels of inflammatory markers, transactivated by NF-κB and STAT3 in response to oxidative stress and/or inflammation and mediating expression of fibrosis markers[7], were measured at protein and gene level. According to the results, increased gene expressions of TGFβ, ICAM1, VCAM1, IL1β, IL6 and MCP1 in response to sole or co-treatments of AGEs and TNFα in hyperglycemic conditions for 72hrs were attenuated by baicalin, except IL6 (Figure 5). Consequently, although the effect of baicalin on IL6 was not considerable, protein expressions of TGFβ, ICAM1, VCAM1, IL1β and MCP1 in hyperglycemia with AGEs and TNFα were substantially decreased by baicalin (Figure 6).

Considering that transactivation of NF-κB and STAT3 can induce IL1β, activating inflammatory pathways[7], ICAM1, VCAM1 and MCP1 which can accelerate inflammatory process by inducing firm adhesion of immune cells[41] and TGFβ which can mediate fibrogenic process by increasing expression of FN and COL4[42], our results suggest that baicalin can attenuate inflammation and inflammation-induced fibrosis in HK-2 cells.

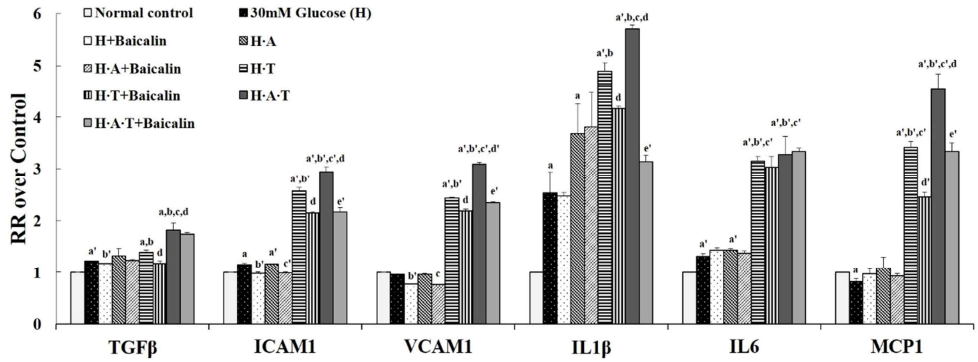

Figure 5. The effects of baicalin on transcriptional levels of inflammatory markers during inflammation. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 72hrs. Baicalin (5μM) decreased gene expressions of TGFβ, ICAM1, VCAM1, IL1β, and MCP1. Bar-graph represents relative ratio to control. $^{a}P<0.05$ and $^{a'}P<0.001$ compared with control, $^{b}P<0.05$ and $^{b'}P<0.001$ compared with H, $^{c}P<0.05$ and $^{c'}P<0.001$ compared with H·A, $^{d}P<0.05$ and $^{d'}P<0.001$ compared with H·T, $^{e}P<0.05$ and $^{e'}P<0.001$ compared with H·A·T. A; advanced glycated end-products, T; tumor necrosis factor α.

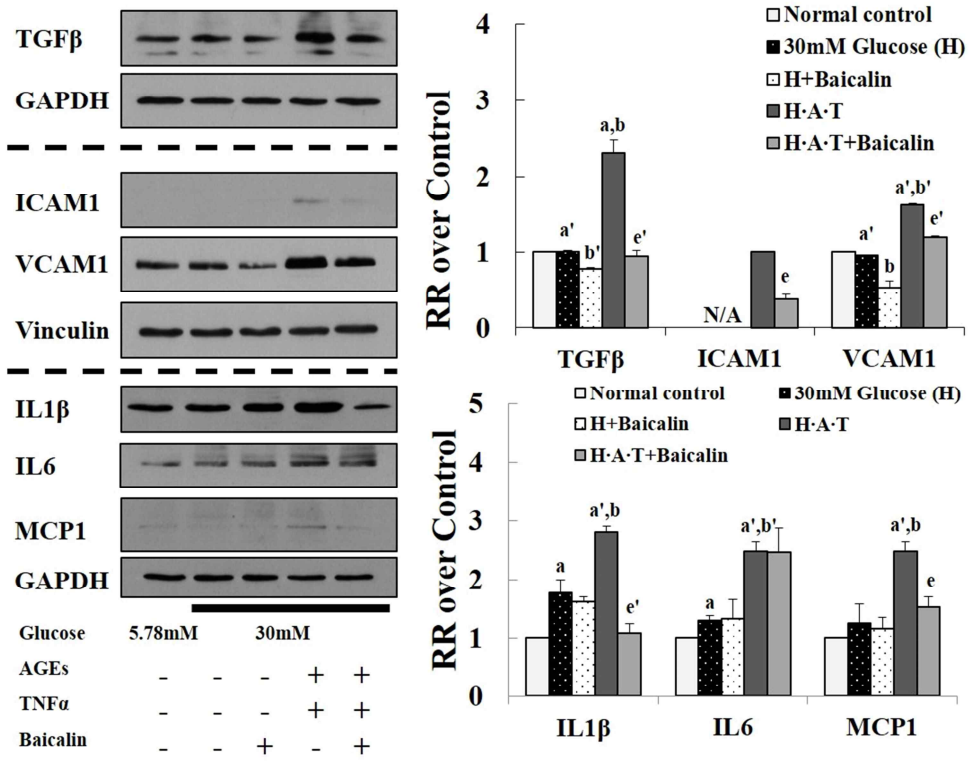

Figure 6. The effects of baicalin on protein expression levels of inflammatory markers during inflammation. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 72hrs. Baicalin (5μM) decreased protein expression levels of TGFβ, ICAM1, VCAM1, IL1β, and MCP1, except IL6. Bar-graph represents relative ratio to control. TGFβ and GAPDH were from different parts of the same gel and ICAM1, VCAM1 and vinculin were from same gel. IL1β, IL6, MCP1 and GAPDH were from same gel. High-contrast was not used. $^{a}P<0.05$ and $^{a'}P<0.001$ compared with control, $^{b}P<0.05$ and $^{b'}P<0.001$ compared with H, $^{e}P<0.05$ and $^{e'}P<0.001$ compared with H·A·T. A; advanced glycated end-products, T; tumor necrosis factor α.

4. Regulation of NF-κB activation via IκB phosphorylations by baicalin

To further investigate the suppressive mechanism of baicalin regarding NF-κB activation, phosphorylation levels of Ser32/36 and Tyr42 sites on IκB[43] were examined. Baicalin significantly attenuated the phosphorylation levels of both Ser32/36 and Tyr42 on IκB induced by co-treatment of hyperglycemia, AGEs and TNFα without affecting total IκB level (Figure 7(a)). As in the case of NF-κB activation, inhibition of phosphorylations on Ser32/36 and Tyr42 by BAY 11-7082 and PDTC, *respectively*, also attenuated nuclear translocation of NF-κB (Figure 7(b)). Our results together indicate that baicalin can inhibit NF-κB translocation via attenuating oxidative stress- and inflammation-induced IκB phosphorylations, thereby, it exerts protective effects on renal fibrosis in human renal proximal tubular cells.

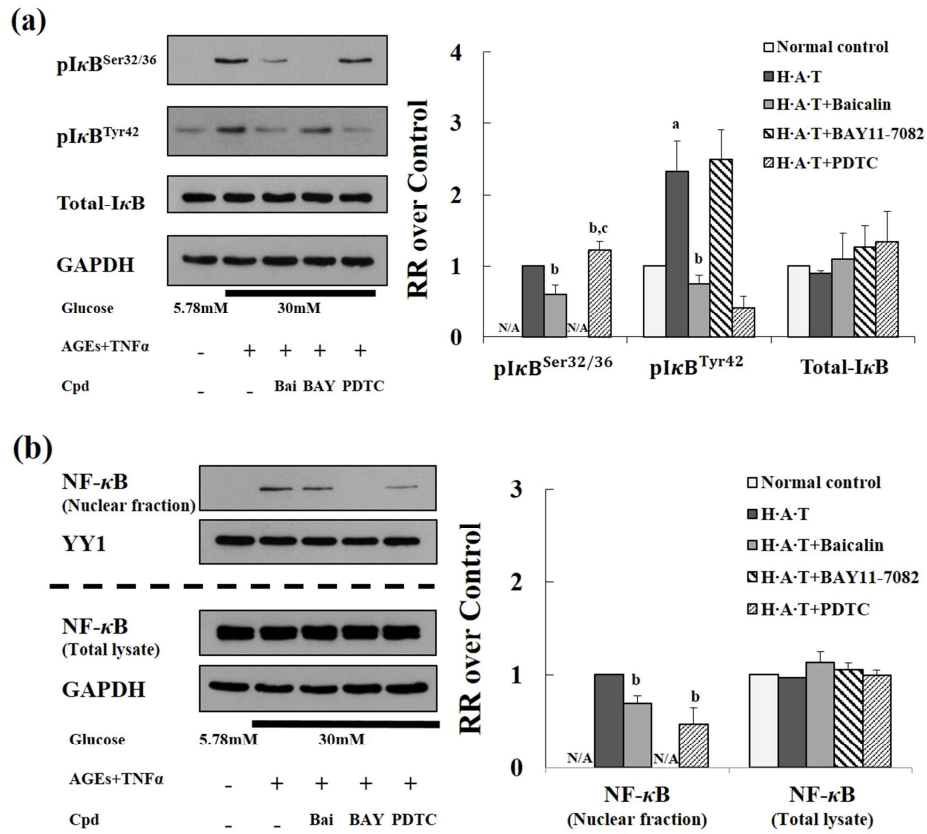

Figure 7. The effects of baicalin on upstream of NF-κB activation. HK-2 cells were cultured in the normal glucose (5.78mM) or high glucose (30mM) medium with or without TNFα (100ng/ml) and AGEs (100μg/ml) for 1hr. Baicalin (5μM) decreased (a) phosphorylation level on Ser32/36 and Tyr42 of IκB in HK-2 cells that were treated by A and/or T in high glucose medium. As a result, in (b), baicalin decreased nuclear translocation of NF-κB. Bar-graph represents relative ratio to control. All bands of (a) were from same gel. NF-κB of nuclear fraction and YY1 in (b) were each cropped from the same gel. High-contrast was not used. $^{a}P<0.05$ and $^{a'}P<0.001$ compared with control, $^{b}P<0.05$ and $^{b'}P<0.001$ compared with H·A·T, $^{c}P<0.05$ and $^{c'}P<0.001$ compared with H·A·T+Baicalin. A; advanced glycated end-products, T; tumor necrosis factor α, BAY; BAY11-7082; PDTC: Pyrrolidine dithiocarbamate.

5. cAMP and cGMP production in response to baicalin

To determine the transduction pathways of baicalin contributing the suppressive effects on renal fibrosis, cellular productions of cAMP and cGMP in response to baicalin were measured by ELISA kit. In the previous study, baicalin can activate large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) via production of cAMP and cGMP and activated BK_{Ca} can regulate Ca^{2+} influx which is one of secondary messengers mediating NF-κB activation[44,45]. Epinephrine and atrial natriuretic peptide (ANP) were used as positive control for cAMP and cGMP production, *respectively*. Although 10μM baicalin stimulation statistically increased production of both cAMP and cGMP, these increases were neglectable compared to the positive controls (Table 4). In addition, the increases of cAMP and cGMP in response to 10μM baicalin in mesenteric artery smooth muscle cells of rats were much larger[44]. These results suggest that the effect of baicalin on increasing production of cAMP and cGMP were not considerable enough to activate downstream pathway, including BK_{Ca}.

Condition	Control	Baicalin		Epi 10μM	ANP 100nM
		5μM	10μM		
cAMP (nM)	0.556±0.060	0.585±0.095 (1.05)	0.661±0.050* (1.18)	106.698 ±17.266**	-
cGMP (nM)	0.025±0.006	0.038±0.006 (1.52)	0.045±0.006* (1.80)	-	19.792 ±11.252*

Table 4. The effects of baicalin on cAMP and cGMP production

Numbers in parentheses represent fold-increase compared to control. Cells were treated with baicalin (5μM and 10μM), epinephrine and ANP for 1hr. cAMP and cGMP production were measured by ELISA kit. Normal control means that cells were cultured in normal growth media. Data (collected from three independent experiments) represent means±SD relative to normal control. *$P<0.05$ and **$P<0.001$ compared with control. ANP; atrial natriuretic peptide, cAMP; cyclic AMP, cGMP; cyclic GMP, Epi; epinephrine.

6. The kinds of $GABA_AR$ subunits expressed in human PTEC

To confirm the modulating role of $GABA_AR$, the kinds of subunits of it in HK-2 cells were examined by $_{semi}$PCR. Among various isoforms, dominant $GABA_AR$ subunits in human PTEC were α_3, α_4, β_3, γ_1, and γ_3 (Figure 8). Given that baicalin can interact with $GABA_AR$ at benzodiazepine binding site between α and γ subunit, the role of $GABA_AR$ on baicalin-induced pathway in human PTEC is expected.

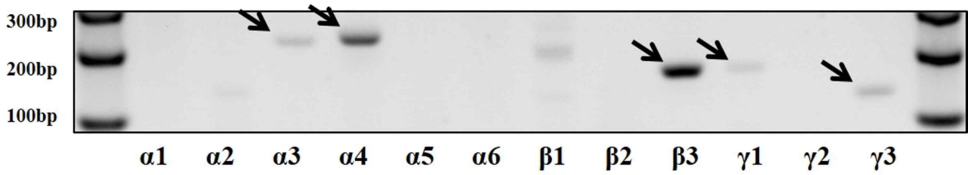

Figure 8. The kinds of $GABA_AR$ subunits expressed in HK-2 cells. Cells with normal growth condition are subjected to $_{semi}$PCR amplification using primers of $GABA_AR$ subunits.

7. The effects of baicalin on regulating intracellular Ca^{2+} concentration via $GABA_AR$

To further investigate pathway of baicalin, inhibiting NF-κB and STAT3 transactivation, changes of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in Fluo-8 loaded HK-2 cells were measured by fluorescence, considering that Ca^{2+} in cytosol can induce IKK complex and Src kinase to activate NF-κB and STAT3, *respectively*[45,46], and the expected receptor for baicalin, $GABA_AR$, can interact with voltage-dependent Ca^{2+} channel (VDCC) which modulates Ca^{2+} influx[47]. As a result, 5μM baicalin and 100μM bicuculine, a selective inhibitor of $GABA_AR$[48], was injected at cycle 1. According to the results, baicalin decreased $[Ca^{2+}]_i$ during the cycles and pre-treatment with bicuculine abated decrease in Ca^{2+} influx induced by baicalin (Figure 9). Considering reduction of Ca^{2+} influx to cell inside by baicalin, the effect of bicuculine on baicalin can present the role of $GABA_AR$ mediating between baicalin pathway and change of Ca^{2+} influx.

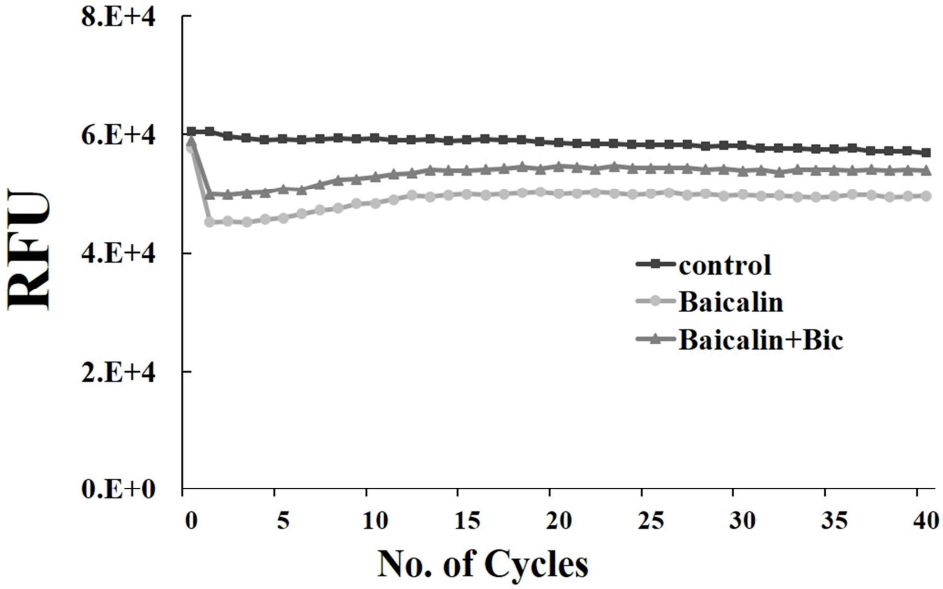

Figure 9. The effect of baicalin on Ca^{2+} influx to cytosol. HK-2 cells loaded with Fluo-8 dye in HBSS containing 2mM $CaCl_2$ were treated with baicalin (5μM) with or without bicuculine (100μM). Intensity of fluorescence of Fluo-8 was measured 40 times. Baicalin attenuated intracellular concentration of Ca^{2+} and this attenuation was reduced by $GABA_AR$ inhibition by bicuculine. Bic: bicuculine.

8. Roles of $GABA_AR$ in regulating activation of inflammation-associated transcription factors inhibited by baicalin

To identify the significance of $GABA_AR$ during baicalin treatment, HK-2cells, treated with $GABA_AR$ inhibitor, bicuculine, were subjected to western blot analysis and NF-κB activation level and JAK2/STAT3 phosphorylation levels were measured. Bicuculine diminished the anti-inflammatory effect of baicalin regarding phosphorylations of IκB on both Ser32/36 and Tyr42, without considerable change of total-IκB protein level (Figure 10(a)). Along with the results, addition of bicuculine increased NF-κB translocation which was attenuated by baicalin (Figure 10(b)), while NF-κB in total lysate had no substantial change. Besides, inhibition of JAK2 and STAT3 phosphorylations by baicalin was attenuated by bicuculine without significant change of total JAK2 and STAT3 (Figure 11). In consequence, baicalin can inhibit IκB and JAK2 phosphorylations and, subsequent, NF-κB and STAT3 transactivations by reducing $[Ca^{2+}]_i$ via $GABA_AR$

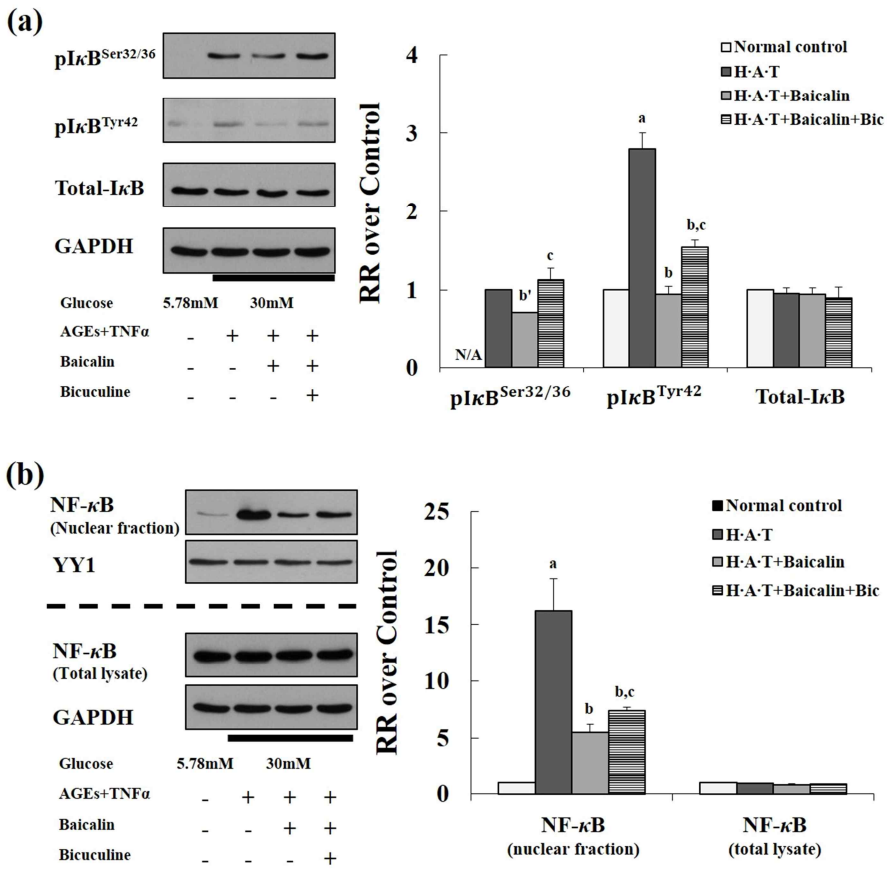

Figure 10. The effect of bicuculine on inhibitory effect of NF-κB activation by baicalin. (a) Attenuated phosphorylation levels on Ser32/36 and Tyr42 of IκB and (b) decreased nuclear translocation of NF-κB by baicalin (5μM) in HK-2 cells treated by TNFα (100ng/ml) and AGEs (100μg/ml) in hyperglycemic medium were inhibited by $GABA_AR$ inhibition by bicuculine. Bar-graph represents relative ratio to control. N/A; non-available, indicating not detectable band on western blot. All bands of (a) were from same gel. NF-κB of nuclear fraction and YY1 in (b) were cropped from the same gel. High-contrast was not used. $^{a}P<0.05$ compared with normal control, $^{b}P<0.05$ and $^{b'}P<0.001$ compared with H·A·T, $^{c}P<0.05$ compared with H·A·T+Baicalin. A; advanced glycated end-products, T; tumor necrosis factor α, Bic: bicuculine.

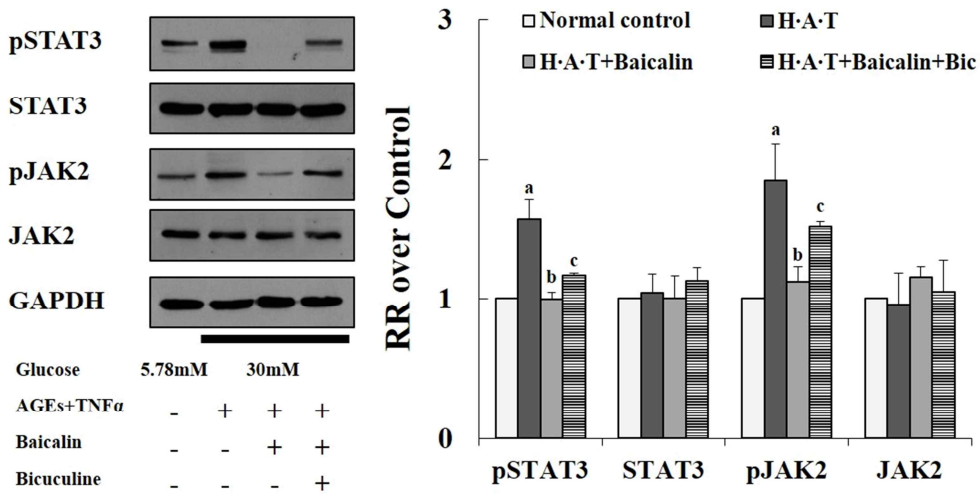

Figure 11. The effect of bicuculine on inhibition of JAK2/STAT3 phosphorylation by baicalin. Attenuated phosphorylation levels on JAK2 and STAT3 by baicalin (5μM) in HK-2 cells treated by TNFα (100ng/ml) and AGEs (100μg/ml) in hyperglycemic medium was reduced by $GABA_AR$ inhibition by bicuculine. Bar-graph represents relative ratio to control. All bands were from same gel. High-contrast was not used. $^{a}P<0.05$ compared with normal control, $^{b}P<0.05$ and $^{b'}P<0.001$ compared with H·A·T, $^{c}P<0.05$ compared with H·A·T+Baicalin. A; advanced glycated end-products, T; tumor necrosis factor α, Bic: bicuculine.

IV. DISCUSSION

The present study showed that baicalin has inhibitory effect on fibrosis process induced by long term diabetic *milieu* in human PTEC, HK-2 cells. Baicalin attenuated IκB and JAK2 phosphorylations and NF-κB and STAT3 transcativation, thereby, it is suggested that baicalin has anti-inflammatory and anti-fibrotic effects via regulating expression levels of inflammation and fibrosis markers. In addition, it was shown that baicalin exerts the effects through $GABA_AR$, which has benzodiazepine-binding site. To the best our knowledge, this was the first study reporting a specific pathway of baicalin on anti-inflammatory and anti-fibrotic effects via $GABA_AR$ in human PTEC.

Baicalin is expected as a therapeutic substance for DM, based on the several studies that baicalin can regulate glucose metabolism including glucose production, glucose uptake via Akt signaling and insulin secretion in various animal models and *in vitro* systems[28,29]. In addition to its anti-hyperglycemic properties, therapeutic potential of baicalin has been reported, such as anti-inflammation, anti-fibrosis, anti-cancer, anti-anxiety, anti-oxidant and anti-microbial effects in many kinds of condition, including *in vivo* systems[31,32,49,50]. In the case of DN and renal fibrosis, activated by chronic oxidative stress and inflammation which can occur with chronic hyperglycemia[7] and characterized as accumulation of ECM, the protective effect of baicalin is expected .To the best of our knowledge, this is the first study examining the anti-fibrogenic effect of baicalin in human PTECs exposed to diabetic *milieu* including hyperglycemic condition. Although baicalein, a aglycone form of baicalin, was reported to abate the renal fibrosis in unilateral ureteric obstruction (UUO) mice models via inactivating NF-κB[51], UUO represents models for obstructive nephropathy rather than DN[52]. In addition, it is shown that 90% of baicalein can be metabolized to baicalin[50], thereby, the effect of baicalin and

baicalein in vitro system is necessary to investigate the role, independently, in any particular pathologic conditions. Therefore, the present study showed the mechanism that baicalin attenuates inflammation and fibrosis process on DN conditioned human PTEC, HK-2 cell.

The effects of baicalin on fibrosis in DN-induced HK-2 cells were examined by measuring expression levels of ECM-associated proteins, in this study, FN and COL4. Given that FN and COL4 are accumulated within glomerular basement membranes and their accumulation can lead to renal dysfunction in human and rodent[53,54], baicalin attenuated the increased protein and gene expression levels of the proteins activated by AGEs and TNFα with hyperglycemia. The increase of transcription of FN and COL4 can be induced upon the transactivation of Smad2/3-Smad4 transcription regulatory complexes which are regulated by TGFβ signaling pathway[26,42].

Production of TGFβ, one of inflammatory cytokines, is stimulated by NF-κB and STAT3 transactivation via IκB and JAK2 phosphorylation, *respectively*[26,27,42,55]. In NF-κB pathway, IκB proteins, kinds of inhibitory protein of NF-κB, have various phosphorylation sites, activated by different stimulation and, thereby, dissociate from NF-κB which can translocate to nucleus and transactivate the target genes. Phosphorylation sites of serines 32 and 36 (Ser32/36) on IκB are typically activated by canonical pathway; activated IKK phosphorylates IκB and leads to ubiquitination and degradation of IκB[43,56] upon activation by stimulus, such as pro-inflammatory cytokines, TNFα[43]. On the other hand, phosphorylation of IκB on tyrosine 42 (Tyr42) is mainly affected by reactive oxygen stress (ROS) during oxidative stress and may or may not be degraded as part of the process mediated by casein kinase II, possibly downstream of Syk[43]. In JAK/STAT pathway, JAK coupled receptors, including cytokine receptors for interleukins and interferons, can phosphorylate

JAK and receptor itself in response to ligand binding and subsequently, activate STAT family of nuclear transcription factors[55]. In kidney cells, phosphorylated STAT3 is mediated by JAK2-dependent pathways and inhibition of JAK2 can regulate STAT3 activation and expression of target genes transactivated by STAT3, including TGFβ[18].

In addition to TGFβ, nuclear-translocated NF-κB and STAT3 transactivate inflammatory mediators such as pro-inflammatory cytokines; TNFα, IL1β and IL6 which can activate inflammation associated transcription factors as positive feedback, chemokines; MCP1 and adhesion molecules; ICAM1 and VCAM1 which facilitate firm adhesion of leukocytes, subsequently, maintaining chronic inflammation[7,41]. In this study, we showed that baicalin abated nuclear translocation of NF-κB and phosphorylation of STAT3 through inhibiting phosphorylations of $I\kappa B^{Ser32/36}$, $I\kappa B^{Tyr42}$ and JAK2 and, subsequently, transcription levels of TGFβ, ICAM1, VCAM1, IL1β and MCP1 which were increased in response to AGEs and TNFα with hyperglycemic condition, demonstrating anti-fibrotic effects of baicalin via its antioxidant and anti-inflammatory properties.

To determine the upstream of IκB and JAK2 phosphorylations mediated by baicalin, considering that Ca^{2+}, which acts as a secondary messenger, can induce nuclear translocation of NF-κB via calmodulin kinase II (CaMKII) and IKK complex, which phosphorylates IκB[45] and activation of STAT3 through Src kinase and JAK2 phosphorylation[46], change of $[Ca^{2+}]_i$ in HK-2 cells was measured and baicalin reduced influx of Ca^{2+} to cytosol upon injection. According to the previous study[45], increase of the influx of Ca^{2+} to cytosol via voltage sensitive Ca^{2+} channel or from endoplasmic reticulum can activate Ca^{2+} dependent proteins, such as calmodulin and PKC[45]. Calmodulin phosphorylates IKK complex and NF-κB via calcineurin and CaMKII, *respectively*, and activated PKC can activate Akt, which

has ability to phosphorylate directly NF-κB and IKK complex. In addition, it is suggested that increase of Ca^{2+} influx in rat hippocampal neurons can trigger activation of Src family kinases, required for phosphorylation of STAT3 at Tyr705 and Ser727 via JAK-dependent and Akt-dependent pathway, *respectively*[46]. Taken together, the effect of baicalin attenuating activation of NF-κB and STAT3 is mediated by Ca^{2+} influx.

Given that IκB and JAK2 can be phosphorylated by influx of Ca^{2+} to cell cytosol[45], large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) is suggested to be able to inhibit Ca^{2+} influx by baicalin[44]. In this previous study, baicalin increased cAMP and cGMP production levels and activated BK_{Ca} via the cAMP/PKA and cGMP/PKG pathways in mesenteric artery[44]. Activated BK_{Ca} could export K^+ to extracellular and induce membrane hyperpolarization of the cells, thereby closing voltage-dependent Ca^{2+} channel (VDCC). According to the results, although cAMP and cGMP were increased by baicalin treatment, it seems that the increased amount may not be sufficient to induce, through BK_{Ca}, alteration of Ca^{2+} flux mediating nuclear translocation of NF-κB and STAT3 in comparison to the increased amount in rat mesenteric artery smooth muscle cells[44]. In this study, considering that baicalin decreased Ca^{2+} influx, it can exert anti-inflammatory and anti-fibrotic effect via other protein, which can affect to change of Ca^{2+} flux, rather than BK_{Ca}.

$GABA_AR$ has been suggested as a receptor for baicalin in benzodiazepine (BZD) binding sites of neurons and the receptor is a GABA-gated, ionotropic chloride channel, composed of heteropentameric subunits[57]. The activation of $GABA_AR$, interacting with GABA can lead to various effect on pharmaceutical properties; including neuroprotective, anti-hypertensive, anti-diabetic, anti-cancer, anti-oxidant, anti-inflammatory in human colon cells and mouse macrophage cells, and reno-protective effect[58]. Considering that $GABA_AR$ has the eight subclasses based on

sequence homology (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-3})[35] and $GABA_AR$ is reciprocal to Cl^- influx, the composition of subunits is expected to be important for specific function. BZD binding sites exist between the α and γ subunit of $GABA_AR$; specifically, $\alpha_{2,3}$ and $_5$ subunits are more region specific than α_1 containing $GABA_AR$ which is abundant in brain[59], and baicalin exhibits substantial preference for α_3-containing BZD site of $GABA_AR$[33,60]. Given that BZD is a kind of therapeutics used for numerous indications, including anxiety and muscle relaxation[60] and α_3-containing $GABA_AR$ has considerable contribution of anxiolysis and minor effect of addiction, myorelaxation and anticonvulsive[59], $GABA_AR$ with α_3 subunit contributes moderately toward clinical effect. In terms of specific function of $GABA_AR$, Cl^- influx by it can induce anxiolysis in neurons via membrane hyperpolarization[57] and additionally, reduction of intracellular Ca^{2+} concentration affected by ion channel, which is sensitive to voltage, such as VDCC, is expected[61]. To identify the physiological roles of $GABA_AR$ on anti-inflammatory and anti-fibrosis effect of baicalin in human PTECs, we confirmed kinds of $GABA_AR$ subunits expressed in the cells; α_3, α_4, β_3, γ_1, and γ_3. Accordingly, it was anticipated that baicalin with $GABA_AR$ induces increase of intracellular Cl^- influx, thereby, reduction of $[Ca^{2+}]_i$ in HK-2 cells. As expected, bicuculine, one of $GABA_AR$ inhibitors, attenuated decrease of $[Ca^{2+}]_i$ and reduced inhibitory effect of baicalin on nucleus translocation of NF-κB via both Ser32/36 and Tyr42 of IκB phosphorylations and phosphorylation of STAT3 and JAK2. As a result, the binding of baicalin with the site between α_3 and γ subunit of $GABA_AR$ in HK-2 cells can play an important role of inhibiting Ca^{2+} influx and downstream of the process.

Taken together, the results of this study indicate baicalin as a candidate therapeutic agent for DN since baicalin attenuated increase of inflammatory and fibrotic markers via NF-κB and JAK2/STAT3 pathways, activated by oxidative stress and

inflammation. In addition, we suggest that $GABA_AR$ is a receptor for baicalin mediating the protective effect of baicalin through regulating Cl^- influx and VDCC.

Although it was reported that baicalein has anti-fibrosis effect via NF-κB pathway, baicalin is metabolically distinct from baicalein and this study is regarding DN condition rather than UUO. Furthermore, even though $GABA_AR$ subunits in renal proximal tubular cells in rat and rabbit kidney cortex were studied[37], there was lack of evidence concerning human proximal tubular cells. Thereby, this is the first study to identify an inhibitory effect of baicalin on DN induced fibrogenic process and suggest the attribution of the effect to its regulatory roles of Ca^{2+} influx via $GABA_AR$ in HK-2 cells.

Schematic representation of the study is shown in Figure 12.

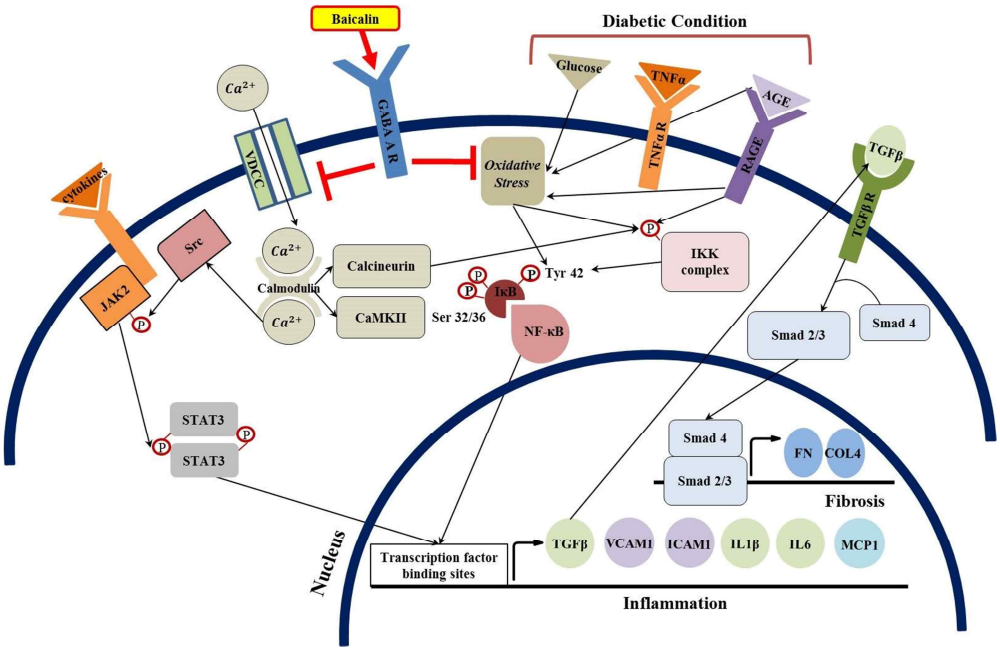

Figure 12. schematic illustration of this study. Baicalin may interact with $GABA_AR$ in HK-2 cells, in turn; it induces anti-inflammatory and anti-fibrotic effect. After interaction with $GABA_AR$, baicalin inhibits translocation of NF-*κ*B and STAT3 to nucleus by attenuating phosphorylation of I*κ*B on both Ser32/36 and Tyr42 and JAK2, *respectively*. As a result, baicalin abates expression of inflammatory cytokines, such as TGFβ, ICAM1, VCAM1, IL1β and MCP1 and this downregulates production of fibrosis markers, such as FN and COL4. CAMKII; calmodulin kinase II, COL4; collagen type IV, FN; fibronectin, $GABA_AR$; type A γ-aminobutyric acid receptor, ICAM1; intracellular adhesion molecule 1, IL; interleukin, JAK2; Janus kinase 2, RAGE; receptor for advanced glycation end products, MCP1; CC chemokine ligand 2, STAT3; signal transducers and activators of transcription 3, TGFβ R; transforming growth factor β receptor, TNFα; tumor-necrosis factor α receptor, VCAM1; vascular cell adhesion molecule 1, VDCC; voltage dependent calcium channel.

V. CONCLUSION

Baicalin has various pharmaceutical functions including anti-inflammatory effects, although its molecular mechanism is unclear. In this study, we observed that, according to the stimulation, co-treatment of AGEs and TNFα with hyperglycemia, accelerating pathogenesis of DN, translocation of NF-κB and STAT3 activated expressions of inflammatory and fibrosis markers, which is reduced by addition of baicalin. Given that $GABA_AR$ is expressed in human PTECs, baicalin, interacting with $GABA_AR$, can attenuate the fibrogenic process via inhibiting both Ca^{2+}- and oxidative stress-induced NF-κB and STAT3 activation. As a result, we suggest baicalin as a therapeutic for diabetic nephropathy.

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ABSTRACT (IN KOREAN)

고혈당에 의해 유도되는 인간 신장 근위세뇨관의 섬유화 과정의 Baicalin의 치료적 역할

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남정은

바이칼린은 *Scutellaria baicalensis* 에서 추출된 flavonoid glycoside 의 일종이며, 당 대사에 대한 효과뿐만 아니라 여러 치료적 특성 때문에 항 염증 효과가 있을 것으로 제안되었다. 당뇨병은 염증 반응과 산화 스트레스를 유도하고 그것에 대하여 신장 섬유증과 신부전으로 이어질 수 있는 만성적인 고혈당 상태가 특징이다. 바이칼린은 폐와 간에서 항 섬유화 효과가 보고되었지만 신장에서의 역할과 그 작용에 대한 연구는 부족하다. 본 연구에서는 당뇨병 환경이 유도된 인간 신장 근위세뇨관 (HK-2) 세포에서 바이칼린이 갖는 보호 효과를 확인했고 그 효과의 기전을 조사하였다.

바이칼린은 산화스트레스와 염증반응으로 인해 나타나는 IκB 인산화를 통한 NF-κB 활성화와 JAK2에 의한 STAT3의 인산화 수준을 약화시켰다. 그 결과 바이칼린은 NF-κB 와 STAT3와 연관된 ICAM1와 VCAM1, TGFβ, IL1β, MCP1의 발현량을 상당히 줄였고 TGFβ와 연관된 세포 외 매트릭스 단백질의 일종인 fibronectin 과 collagen IV 의 발현량 또한 감소시켰다. 이러한 효과는 바이칼린과 결합하여 세포 내 칼슘 이온 농도를 조절할 수 있는 $GABA_AR$가 기여한다고 볼 수 있다.

이러한 결과들을 기반으로 당뇨병성 신증 상태가 유도된 인간 신장 근위세뇨관에서, $GABA_AR$ 와 결합한 바이칼린은 칼슘이온과 산화스트레스로 인해 유도된 NF-κB pathway 를 통해 항 염증과 항 섬유화 효과를 가진다고 제시한다.

핵심되는 말: 바이칼린, 당뇨성 신증, 인간 신장 근위세뇨관, NF-κB, JAK/STAT, $GABA_AR$

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