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Zafirlukast, a Cysteinyl Leukotriene Receptor 1 Antagonist, Reduces the Effect of Advanced Glycation End-Products in Rat Renal Mesangial Cells *In Vitro*

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

E 1 Liping Yan
B 2 Ani Sun
C 3 Xinwei Xu

1 Administration Division, Weifang People's Hospital, Weifang, Shandong, P.R. China
2 Infection Control Office, Weifang People's Hospital, Weifang, Shandong, P.R. China
3 Nephrology Department, Weifang People's Hospital, Weifang, Shandong, P.R. China

Corresponding Author: Xinwei Xu, e-mail: xinweixuwf@163.com
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Background: Zafirlukast is an antagonist of cysteinyl leukotriene receptor 1 (CysLTR1). Advanced glycation end-products (AGEs) are formed by the glycation of lipids and proteins in hyperglycemia, including diabetes mellitus. Zafirlukast has not previously been studied in diabetic nephropathy. This study aimed to investigate the effects of zafirlukast on rat renal mesangial cells cultured with AGEs *in vitro*.

Material/Methods: Mesangial cells were cultured in AGEs (0, 20, 50, 100 μg/ml), and with AGEs (100 μg/ml) and zafirlukast (2.5 μm, 5 μm, and 100 μm). An enzyme-linked immunoassay (ELISA) was used to measure the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and monocyte chemoattractant protein-1 (MCP-1). Reactive oxygen species (ROS) were assessed by intracellular fluorescence measurement of 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and detection kits were used to measure malondialdehyde (MDA), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD). Cell apoptosis was assessed by flow cytometry, and Western blot was used to measure protein levels.

Results: In mesangial cells cultured with AGEs, markers of inflammation, oxidative stress, and apoptosis and levels of CysLTR1 increased, and these effects were reduced by zafirlukast in a dose-dependent manner. The effects of zafirlukast as a CysLTR1 antagonist protected mesangial cells from the effects of AGE *in vitro*.

Conclusions: Zafirlukast, a CysLTR1 antagonist, reduced the levels of inflammatory cytokines, markers of oxidative stress, and cell apoptosis induced by AGE in mesangial cells in a dose-dependent way. Future *in vivo* studies are needed to investigate the potential role for zafirlukast in models of diabetic nephropathy.

MeSH Keywords: Diabetic Nephropathies • Mesangial Cells • Oxidative Stress

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Background

Worldwide, diabetic nephropathy is a severe complication of diabetes mellitus that occurs in between 20–40% of patients with type 1 and type 2 diabetes, and is a major cause of chronic kidney disease and end-stage renal disease [1]. Diabetic nephropathy is classified into four grades of severity, from mild to severe, which are associated with increased thickening of the glomerular basement membrane and increased mesangial cellularity associated with increased inflammation [2]. Therefore, early diagnosis and timely treatment is important to prevent the progression of early diabetic nephropathy to end-stage renal disease and renal failure. Treatment requires the control of blood glucose and blood pressure [2]. Diabetic nephropathy is also associated with renal fibrosis, reduced glomerular filtration rate, and proteinuria, and is a complex disease. The pathogenesis of diabetic nephropathy has also been reported to involve inflammation and cell apoptosis induced by oxidative stress [3].

Previously published studies on the pathogenesis of diabetic nephropathy have investigated the role of inflammation and the expression of pro-inflammatory cytokines [4–6]. Studies have shown that inhibition of inflammation can reduce the progression of diabetic nephropathy. For example, in a rat model of streptozotocin-induced diabetic nephropathy, *Allium tuberosum* inhibited the oxidative stress and inflammation induced by hyperglycemia, resulting in reduced renal damage [7]. Also, the omega-3 fatty acid-derived bioactive lipid, resolvin, was reported to have reduced inflammation in diabetic nephropathy [8].

Oxidative stress can induce inflammation by activating the production of inflammatory cytokines, and also activates apoptosis signaling pathways [3]. Oxidative stress also induces the advanced glycation end-product (AGE) pathway, the protein kinase C pathway, the JAK/STAT pathway, the MAPK pathway, the mTOR pathway, the transforming growth factor β (TGF-β) and SMAD pathway, and the NADPH oxidase (NOX) pathway [3]. Therefore, targeting inflammation and relevant signaling pathways might be an effective therapeutic approach for diabetic nephropathy. However, the identification of the most relevant potential therapeutic targets remains to be investigated.

Non-enzymatic glycation is an important component of the pathogenesis of diabetic nephropathy that generates advanced glycation end-products (AGEs) via a series of chemical reactions [9]. AGEs as pathogenic factors in diabetic nephropathy result from non-enzymatic glycation and constantly accumulate, leading to renal injury by increasing renal lipid accumulation, changing the autophagy and lysosome pathways, inflammation, and cell cycle arrest of podocytes [10–13]. Currently, studies that use AGEs-induced cell models of diabetic nephropathy have benefit for preliminary or early studies that involve drug testing, including for inhibitors of inflammation. For example, recently, Lee et al. investigated the inhibitory effects of chrysin *in vivo* in a mouse model of glomerular fibrosis and in AGEs-treated murine renal mesangial cells *in vitro* [14]. AGEs-induced inflammatory responses in macrophages *in vitro* have been used to study the role of TGF-β-activated kinase 1 [15].

Zafirlukast is an antagonist of cysteinyl leukotriene receptor 1 (CysLTR1) that is currently prescribed for chronic asthma. Zafirlukast has a dual role in stimulating insulin secretion, and it also has anti-inflammatory effects [16,17]. AGEs that are formed by the glycation of lipids and proteins in conditions of hyperglycemia, including diabetes mellitus, are important inflammatory mediators in diabetes that increase the expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, and induce cell apoptosis [13,18,19]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that is synthesized in mesangial cells and is associated with the progression of diabetic nephropathy [20–22]. CysLTR1 is expressed in renal mesangial cells [23]. Also, nuclear factor-κB (NF-κB) is a conserved nuclear transcription factor that is expressed in inflammation and shows cross-talk with reactive oxygen species (ROS) during inflammation [24].

Inflammation is involved in the pathogenesis of diabetic nephropathy, and as well as promoting insulin secretion, zafirlukast has previously been shown to have anti-inflammatory effects [25,26]. However, zafirlukast has not previously been studied in diabetic nephropathy. Therefore, this study aimed to investigate the effects of zafirlukast on rat renal mesangial cells cultured *in vitro* with AGEs.

Material and Methods

Cell culture and treatment

Renal mesangial cells derived from Sprague-Dawley rats (CRL-2573) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Thermofisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2. The cells were treated with increasing concentrations of advanced glycation end-products (AGEs) (0, 20, 50, 100 μg/ml). Mesangial cells were cultured with AGEs (100 μg/ml) and zafirlukast at doses of 2.5 μm, 5 μm, and 100 μg/ml. The cells were incubated for 24 h.

Enzyme-linked immunoassay (ELISA) detection of inflammatory cytokines

ELISA was used to measure the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and monocyte

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chemoattractant protein-1 (MCP-1) in the culture supernatant after the mesangial cells were treated with zafirlukast and AGEs. The ELISA kits were used according to the manufacturer's instructions.

Detection of reactive oxygen species (ROS)

The redox-sensitive fluorescent probe for reactive oxygen species (ROS), 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA) was used to detect the intracellular ROS. The cells were incubated with carboxy-H_2-DCFH-DA at a concentration of 1 μm in the dark for 30 min. Then, carboxy-H2-DCFDA was removed, and the cells were washed with phosphate-buffered saline (PBS). AGEs at different concentrations (0, 20, 50, 100 μg/ml), the mixture of AGE (100 μg/ml) and zafirlukast (2.5 μm) and a mixture of AGE (100 μg/ml) and zafirlukast (5 μm), respectively were added to the cells that were treated with carboxy-H2-DCFDA. The method was performed in the dark.

Flow cytometry for cell apoptosis

The cells after treatment were digested by 0.25% trypsin and collected. Then the cells were washed with PBS for three times and resuspended in binding buffer. Then, 5 μL Annexin Annexin V-fluorescein isothiocyanate (FITC) and 10 μL of propidiμm iodide (PI) staining solution were added. Cell apoptosis was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot

After treatment, the cultured mesangial cells were lysed and collected to obtain the total proteins. The protein samples underwent separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked in Tris-buffered saline in Tween-20 with 5% dried skimmed milk powder for 1 h followed by overnight incubation at room temperature with the primary antibody and the secondary antibody. The membranes were washed three times before undergoing enhanced chemiluminescence (ECL) analysis of the protein expression levels using Quantity One software (Bio-Rad, Hercules, CA, USA).

Measurement of malondialdehyde (MDA), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)

MDA was analyzed using the MDA lipid peroxidation kit, based on the thiobarbituric acid (TBA) method (Biovision Inc., Milpitas, CA, USA). LDH was measured using a colorimetric assay kit (K726-500) (Biovision Inc., Milpitas, CA, USA). GSH-Px and SOD were detected using commercial ELISA kits (Biovision Inc., Milpitas, CA, USA). All assays were performed according to the manufacturer's instructions.

Statistical analysis

Numerical data were expressed as the mean±standard deviation (SD). Data were analyzed using SPSS version 11.03 software (IBM, Chicago, IL, USA) and GraphPad Prism version 7.03 (GraphPad Software, La Jolla, CA, USA). The difference between groups was analyzed by one-way analysis of variance (ANOVA) with Dunnett's test. A P-value <0.05 was considered to be statistically significant.

Results

Mesangial cells cultured in advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm, 5 μm, and 100 μm) and the expression of inflammatory cytokines, reactive oxygen species (ROS), and cell apoptosis

The expression of inflammatory cytokines, ROS, and cell apoptosis in mesangial cells increased with increasing concentration of AGEs (Figures 1, 2). Monocyte chemoattractant protein-1 (MCP-1) expression was significantly increased with increasing concentrations of AGEs at 0, 20, 50, and 100 μg/ml.

AGEs enhanced the expression of extracellular matrix (ECM) components

Figure 3 shows that the expression of fibronectin, collagen I, and collagen IV were significantly increased in the AGEs-induced study groups compared with the control group, and this result was dose-dependent.

AGEs promoted the expression of cysteinyl leukotriene receptor 1 (CysLTR1)

Figure 3 shows that increasing concentrations of AGEs at 0, 20, 50, 100 μg/ml were significantly associated with expression levels of CysLTR1. This finding supported a potential role for CysLTR1 in the effects of AGE in renal mesangial cells.

Zafirlukast reduced the expression of inflammatory cytokines and MCP-1 in AGEs-induced renal mesangial cells

Compared with the cells incubated with AGEs alone, the cells incubated with AGEs and zafirlukast (2.5 μm and 5 μm) showed reduced expression of inflammatory cytokines (Figure 4). MCP-1 expression was significantly correlated with

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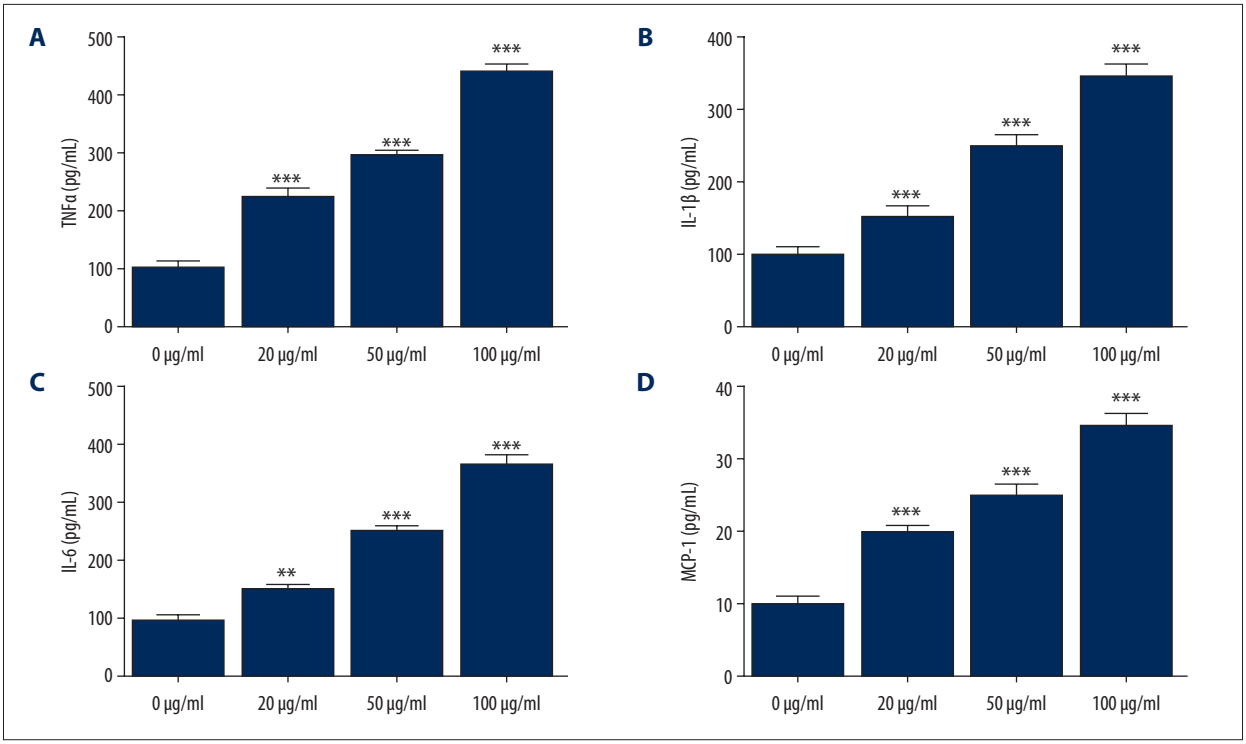

Figure 1. Levels of tumor necrosis factor-α (TNF-α) (**A**), interleukin-1β (IL-1β) (**B**), IL-6 (**C**), and monocyte chemoattractant protein-1 (MCP-1) (**D**) in mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm, 5 μm, and 100 μm). ** P<0.01 and *** P<0.001 *vs.* the control group.

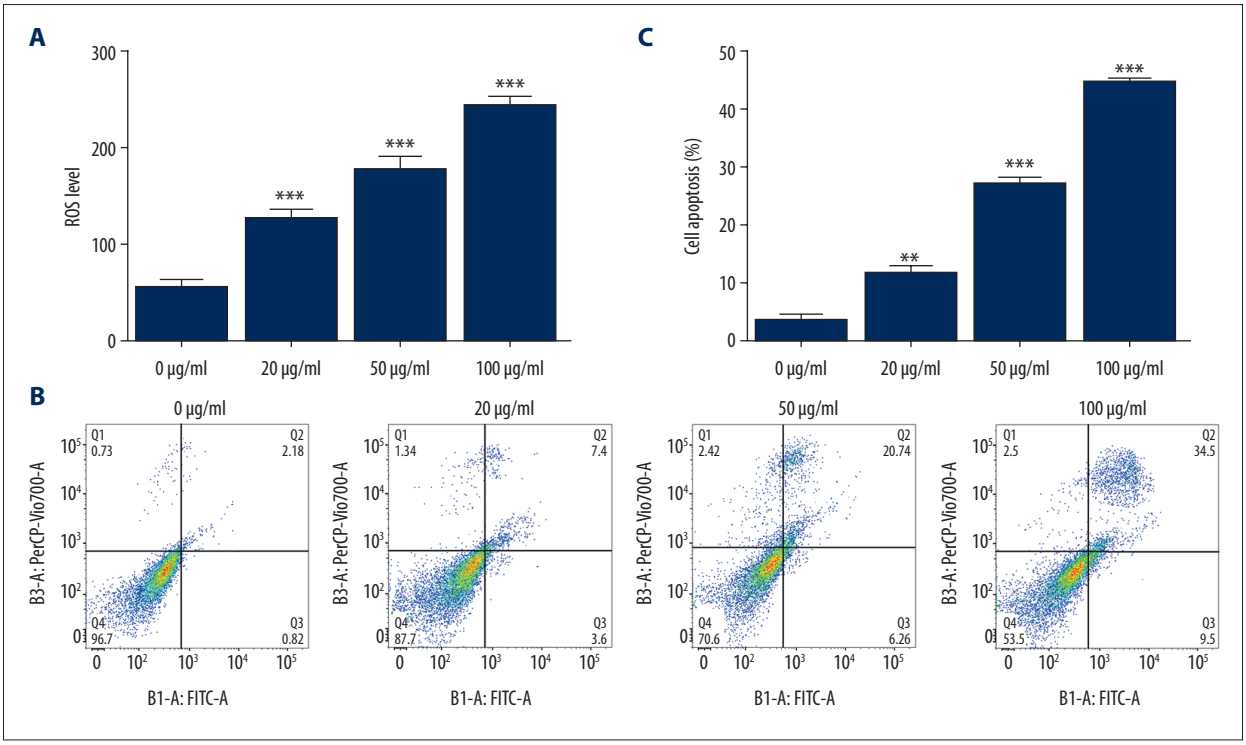

Figure 2. The level of reactive oxygen species (ROS) in mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm, 5 μm, and 100 μm). (**A**) The cell apoptosis evaluated by flow cytometry in the study groups (**B, C**). ** P<0.01 and *** P<0.001 *vs.* the control group.

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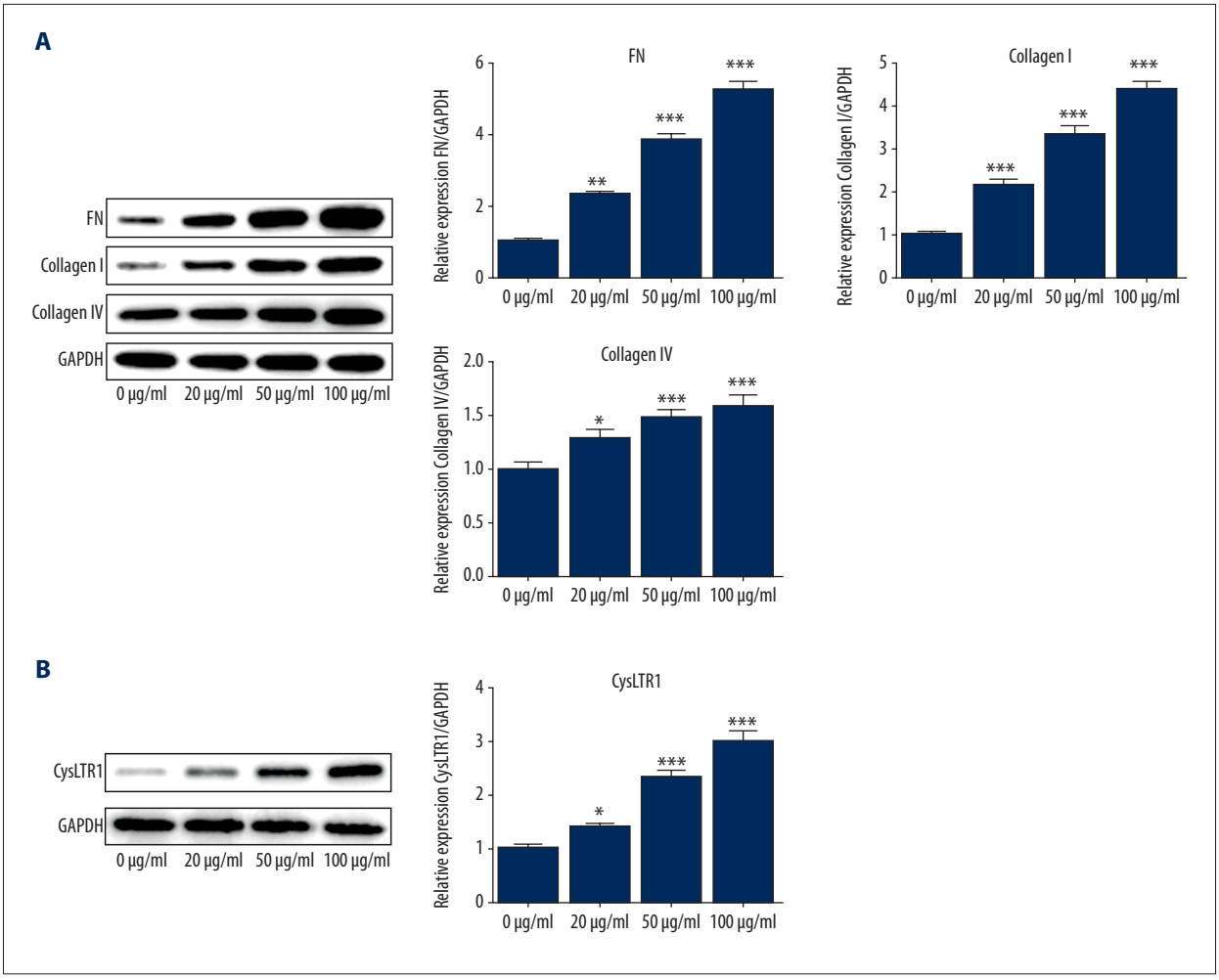

Figure 3. The expression of fibronectin, collagen I, and collagen IV in mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm, 5 μm, and 100 μm). (**A**) Dose-dependent expression of fibronectin, collagen I, and collagen IV in the AGEs-induced groups compared with the control group. (**B**) Cysteinyl leukotriene receptor 1 (CysLTR1) expression in the different study groups. * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group.

the concentration of AGEs. In comparison with the group treated with AGEs alone, the association between MCP-1 and AGE was reversed in the presence of AGE and zafirlukast (2.5 μm). The expression of MCP-1 was further reduced in the presence of AGE and zafirlukast (5 μm).

Zafirlukast reduced the activation of nuclear factor-κB (NF-κB) in AGEs-induced renal mesangial cells

Compared with the control group, the expression of p-NF-κB was increased in the presence of AGEs, which indicated that NF-κB could be activated by AGEs. Compared with the mesangial cells cultured in AGEs alone, p-NF-κB expression was reduced in the presence of AGEs with zafirlukast (2.5 μm or 5 μm) indicating that the activation of NF-κB induced by AGEs was inhibited by zafirlukast in a dose-dependent manner.

Zafirlukast reduced the expression of oxidative stress factors in AGEs-induced renal mesangial cells

As shown in Figure 5, in comparison with the control group, the levels of reactive oxygen species (ROS) were increased in the AGEs-induced study group. The ROS level was reduced in the group treated with AGEs and zafirlukast indicating that oxidative stress induced by AGEs was inhibited by zafirlukast. Expression of malondialdehyde (MDA), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were investigated. As shown in Figure 5, the expression levels of MDA, LDH, GSH-Px, and SOD induced by AGEs were inhibited by zafirlukast, which supported the inhibitory effect of zafirlukast on oxidative stress induced by AGEs.

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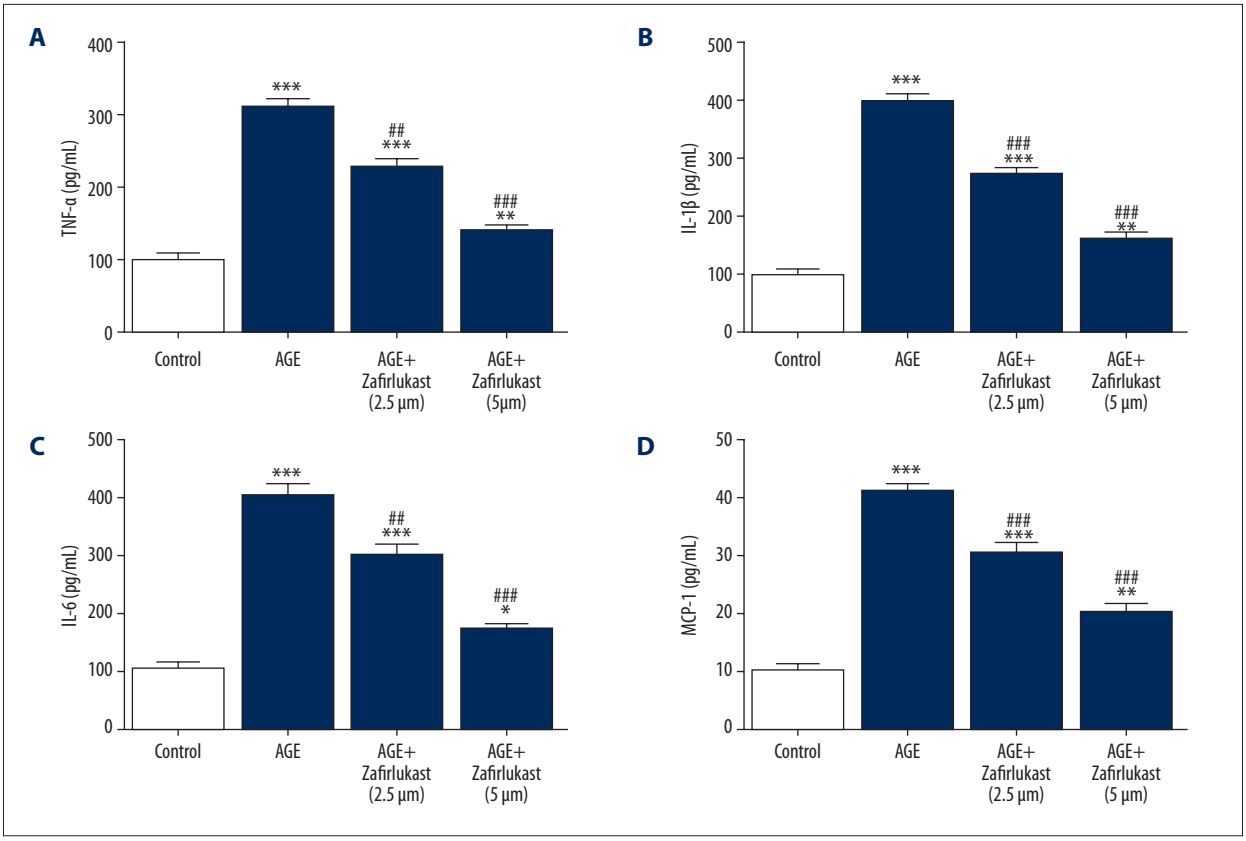

Figure 4. (**A–D**) Levels of monocyte chemoattractant protein-1 (MCP-1), IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) in mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm and 5 μm). Compared with the cells incubated with AGEs alone, the cells incubated with AGE and zafirlukast (2.5 μm and 5 μm) showed reduced expression of inflammatory cytokines. MCP-1 expression was significantly correlated with the concentration of AGE. * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group. ## P<0.01 and ### P<0.001 *vs.* the advanced glycation end-product (AGE) group.

Zafirlukast reduced apoptosis of renal mesangial cells induced by AGEs

Apoptosis of mesangial cells was significantly increased in the presence of AGEs compared with the control group (Figure 6). Mesangial cell apoptosis was reduced in the presence of AGEs and zafirlukast (2.5 μm). In the group treated with AGEs and zafirlukast (5 μm), the cell viability reached >80%, which indicated that zafirlukast inhibited apoptosis of renal mesangial cells induced by AGEs. Apoptosis-associated proteins were also studied. As shown in Figure 7, the expression level of Bcl-2, an anti-apoptosis protein, was highest in the control group compared with the other study groups, and was lowest in AGEs-induced group. The expression of Bcl-2 in the group treated with AGEs and zafirlukast was higher than that in the group treated with AGEs alone, indicating that zafirlukast reduced apoptosis of renal mesangial cells by inhibiting the reduction of Bcl-2 induced by AGEs. Bax, cleaved caspase-3 and cleaved caspase-9, pro-apoptotic proteins, showed a reverse trend to that of Bcl-2 in all the study groups, further confirming that zafirlukast reduced apoptosis of renal mesangial cells by inhibiting the increase of pro-apoptotic proteins induced by AGEs.

Zafirlukast inhibited the expression of extracellular matrix (ECM)-associated factors by AGEs

As shown in Figure 7, compared with the control group, the expression of fibronectin, collagen I, and collagen IV were significantly increased in AGEs-induced cells compared with the other study groups. The expression of fibronectin, type I collagen, and type IV collagen were reduced in the study groups treated with AGEs and zafirlukast (2.5 μm or 5 μm) compared with the AGEs-treated group, indicating that the expression ECM-associated factors induced by AGEs was inhibited by zafirlukast.

Discussion

Zafirlukast, an antagonist of cysteinyl leukotriene receptor 1 (CysLTR1), is approved for the treatment of chronic asthma

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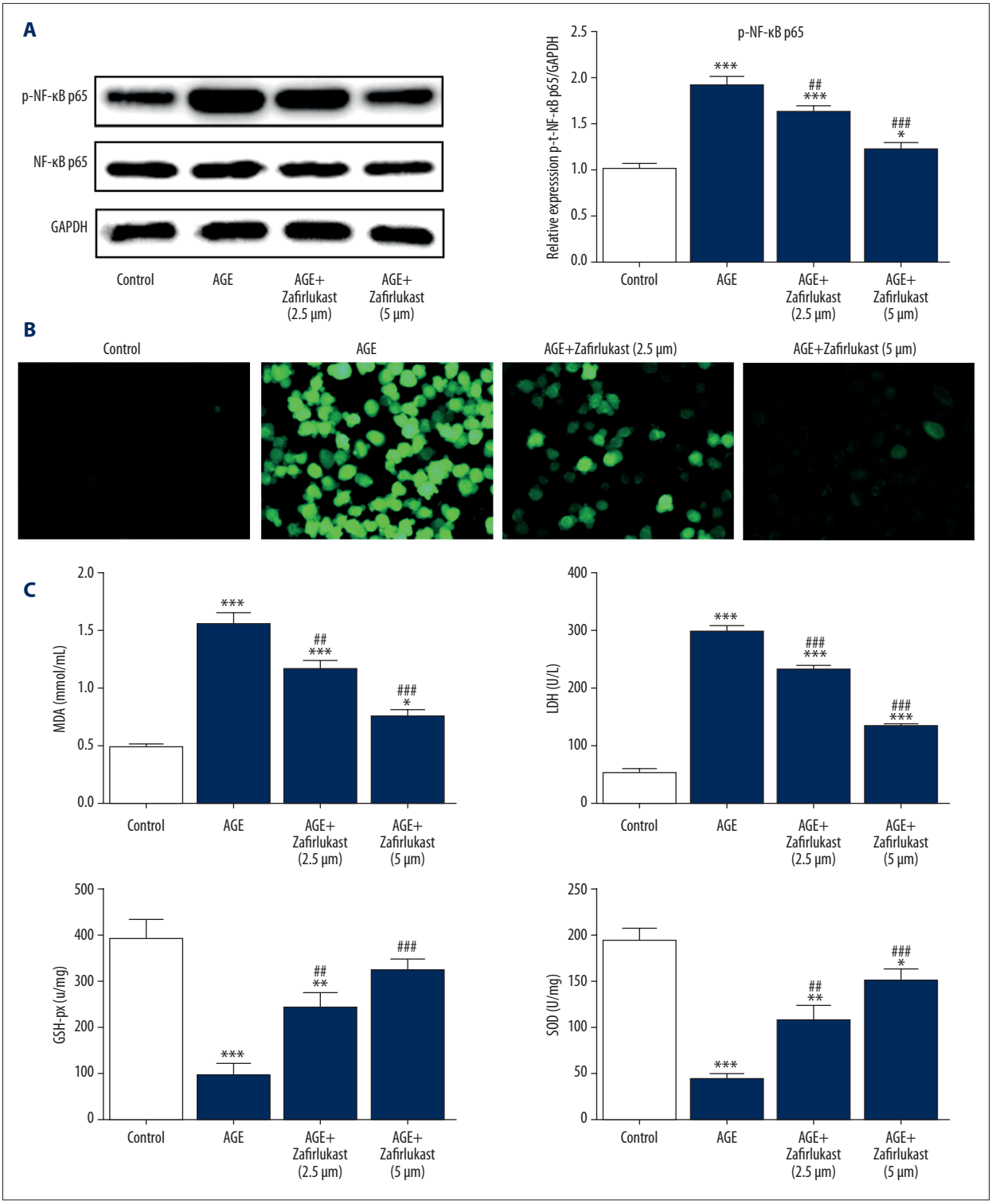

Figure 5. The level of reactive oxygen species (ROS) in the mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm and 5 μm). (**A**) The level of reactive oxygen species (ROS), or cell redox status, assessed by fluorescence intensity of intracellular 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the different study groups. (**B, C**) The level of malonaldehyde (MDA), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in the different study groups. * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group. ## P<0.01 and ### P<0.001 *vs.* the advanced glycation end-product (AGE) group.

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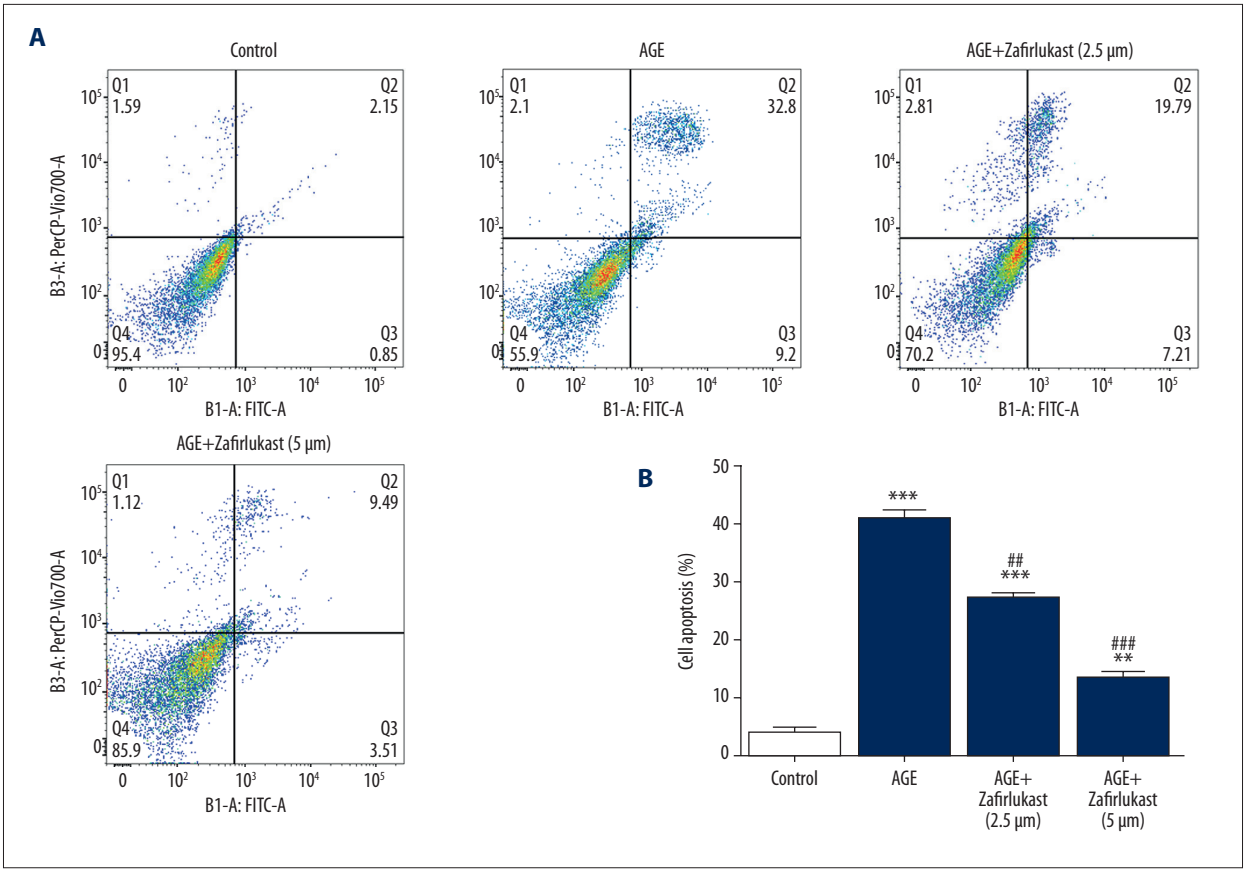

Figure 6. (**A, B**) Cell apoptosis evaluated by flow cytometry in the different groups of mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm and 5 μm). ** P<0.01 and *** P<0.001 *vs.* the control group. ## P<0.01 and ### P<0.001 *vs.* the advanced glycation end-product (AGE) group.

as an anti-inflammatory agent and has also been reported to promote insulin secretion [25]. Zafirlukast is a potential hypoglycemic agent that was investigated in this study for its *in vitro* effects on renal mesangial cells that were cultured with increasing doses of advanced glycation end-products (AGEs). AGEs play an important role in the cellular and molecular events associated with the pathogenesis of diabetic nephropathy [9]. The results of this study showed that the expression of inflammatory cytokines by mesangial cells were increased with increasing concentration of AGEs. The expression of extracellular matrix (ECM)-associated proteins, ROS levels, and apoptosis by mesangial cells also increased with increasing concentration of AGEs. The findings from this *in vitro* study were consistent with the findings from previous studies on the expression of inflammatory cytokines and ECM induced by AGEs in the progression of diabetic nephropathy [26,27].

In the present study, the expression of CysLTR1 by mesangial cells cultured in AGEs was investigated. AGEs increased the expression of CysLTR1 by mesangial cells in a dose-dependent manner, which supported the role of CysLTR1 in the effects of AGEs in renal mesangial cells. Also, in this study, increased expression of inflammatory cytokines induced by the culture of renal mesangial cells with AGEs was reduced by zafirlukast. Inflammation has a central role in progression of diabetic nephropathy, and the production of pro-inflammatory cytokines further recruits inflammatory cells that result in the progression of renal injury [6,28]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine involved in the recruitment of inflammatory cells that contributes to the initiation and the progression of diabetic nephropathy, which was investigated in the present study [22]. The expression of MCP-1 by renal mesangial cells cultured with AGEs *in vitro* increased in a dose-dependent way and was reduced when the cells were treated with zafirlukast, a finding that supported the anti-inflammatory effect of zafirlukast in this study. Also, NF-κB, which is activated through phosphorylation, has been reported to be an important inflammatory mediator [29]. In this study, increased expression of p-NF-κB was induced by AGEs and was inhibited by zafirlukast. Approaches to reduce inflammation might delay the progression of diabetic nephropathy and, as this study has shown, zafirlukast had an anti-inflammatory effect in AGEs-treated renal mesangial cells.

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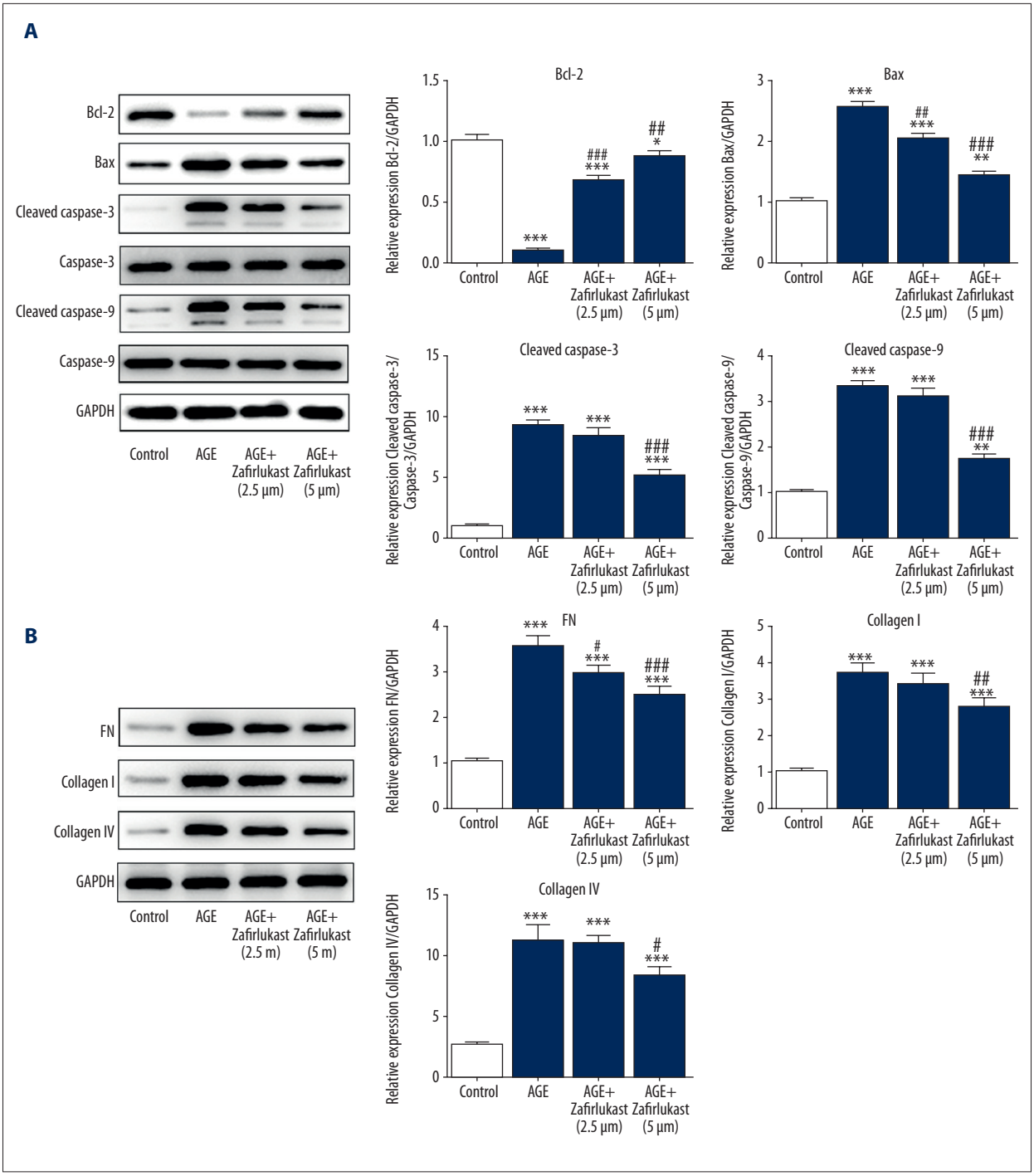

Figure 7. The expression of apoptosis-associated proteins and extracellular matrix (ECM)-associated proteins from mesangial cells cultured in increasing concentrations of advanced glycation end-products (AGEs) and treated with zafirlukast (2.5 μm and 5 μm). (**A**) The expression of Bcl-2, Bax, cleaved caspase-3, and cleaved caspase-9 in the different groups. (**B**) The expressions of fibronectin, collagen I, and collagen IV in the different groups. * P<0.05, ** P<0.01 and *** P<0.001. # P<0.05, ## P<0.01 and ### P<0.001 *vs.* the advanced glycation end-product (AGE) group.

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Oxidative stress has a role in the pathogenesis of diabetic nephropathy [30]. Excess free radicals contribute to insulin resistance, cell injury, inflammation, and fibrogenesis, which exacerbate diabetic nephropathy [31]. Reactive oxygen species (ROS) generated by AGEs have been reported to promote chronic inflammation [32]. Therefore, oxidative stress and inflammation are two main areas for the identification of potential therapeutic targets in diabetic nephropathy, but they remain to be investigated. In the present study, ROS that included malondialdehyde (MDA), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) associated with cultured mesangial cells were measured. As shown in Figure 5, the expression of ROS induced by treatment with AGEs was reversed by zafirlukast.

Deposition of components of extracellular matrix (ECM) is also a feature of diabetic nephropathy, and studies have been conducted to investigate approaches to reduce ECM. For example, the findings from a recent study by Ma et al. [33] showed that protocatechuic acid, a phenolic anthocyanin compound, reduced ECM deposition by human mesangial cells *in vitro* that were cultured in conditions of high glucose [33]. In the present study, zafirlukast was shown to reduce AGEs-induced renal mesangial cell ECM components, fibronectin, type 1 collagen, and type IV collagen.

Mesangial cell apoptosis was investigated in the present study by measurement of the pro-apoptotic factors, Bax, cleaved caspase-3 and cleaved caspase-9, and the anti-apoptotic factor, Bcl-2. Also, apoptosis was directly investigated in renal mesangial cells cultured in AGEs and treated with zafirlukast using fluorescence flow cytometry. The expression of Bcl-2 in renal mesangial cells cultured in AGEs and treated with zafirlukast was greater than that in the group treated with AGEs alone, indicating that zafirlukast reduced apoptosis of renal mesangial cells. These findings are supported by previous studies that have shown that renal mesangial cell injury can be induced by AGEs, which have attempted to identify anti-apoptosis agents [34–37].

This preliminary *in vitro* study has attempted to investigate the effects of zafirlukast on AGEs-induced renal mesangial cells in culture and has included an investigation of inflammatory cytokines, oxidative stress, apoptosis, and ECM factors. The findings have shown that the effect of AGEs on mesangial cells *in vitro* could be reversed by zafirlukast. Given that the anti-inflammatory role of zafirlukast is established, and its effects on the reduction of hyperglycemia have also been reported, further studies are recommended to determine whether zafirlukast might have potential therapeutic applications in diabetic nephropathy.

Conclusions

Currently, the cysteinyl leukotriene receptor 1 (CysLTR1) antagonist, zafirlukast, is an approved drug for the treatment of chronic asthma. In this *in vitro* study, using cultured mesangial cells treated with advanced glycation end-products (AGEs), zafirlukast reduced the levels of inflammatory cytokines, markers of oxidative stress, apoptosis, and factors associated with extracellular matrix (ECM) formation. Previous studies have shown the role of zafirlukast in increasing insulin production. The findings from this preliminary *in vitro* study support the need for future *in vivo* studies to investigate the potential role for zafirlukast in diabetic nephropathy.

Conflict of interest

None.

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