Research Article

Cudrania tricuspidata Root Extract Prevents Methylglyoxal-Induced Inflammation and Oxidative Stress via Regulation of the PKC-NOX4 Pathway in Human Kidney Cells

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Diabetic nephropathy is a microvascular complication induced by diabetes, and methylglyoxal (MGO) is a reactive carbonyl species causing oxidative stress that contributes to the induction of inflammatory response in kidney cells. Cudrania tricuspidata (CT), cultivated in Northeast Asia, has been used as traditional medicine for treating various diseases, including neuritis, liver damage, and cancer. In this study, we determined whether a CT root extract (CTRE) can prevent MGO-induced reactive oxygen species (ROS) production and inflammation and assessed underlying mechanisms using a kidney epithelial cell line, HK-2. We observed that CTRE inhibited MGO-induced ROS production. Additionally, CTRE ameliorated the activation of MGO-induced inflammatory signaling pathways such as p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and c-JUN N-terminal kinase (JNK). Consistent with these results, expressions of p-nuclear factor-kappa B (NFκB) and inflammatory cytokines, tumor necrosis factor-α, interleukin-1 (IL-1), and IL-6, were decreased when compared with MGO-only exposed HK-2 cells. CTRE alleviated the MGO-induced decrease in nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and antioxidant enzyme mRNA expressions. MGO induced the expression of NADPH oxidase 4 (NOX4); CTRE pretreatment inhibited this induction. Further studies revealed that the NOX4 expression was inhibited owing to the suppression of MGO-induced protein kinase C (PKC) activation following CTRE treatment. Collectively, our data suggest that CTRE attenuates MGO-induced inflammation and oxidative stress via inhibition of PKC activation and NOX4 expression, as well as upregulating the Nrf2-antioxidant enzyme pathway in HK-2 cells.

1. Introduction

Diabetic nephropathy (DN) is one of the most important and common complications of diabetes [1]. Glomerular hypertrophy, matrix protein accumulation, and tubular injury are major pathological features, and eventually, the loss of kidney function. [2, 3]. Chronic hyperglycemia is known to cause and accelerate kidney damage. Hyperglycemia promotes the formation of advanced glycation end products (AGEs), which are known to play an important role in the development of DN.

Methylglyoxal (MGO) is the main precursor of AGEs and can induce intracellular damage by increasing reactive oxygen species (ROS), mitochondrial damage, and inflammation. Therefore, MGO accumulation has been implicated in vascular complications of diabetes and chronic inflammatory diseases, including cardiovascular disease, cancer, cognitive dysfunction, and bone loss [4–10].

Cudrania tricuspidata Bureau (CT), which belongs to the Moraceae family, is widely distributed throughout Northeast Asia, including Korea, China, and Japan. It has been used as a traditional medicine for tumors, liver damage, jaundice, contusions, chronic gastritis, rheumatism, neuritis, and inflammation in East Asia [11, 12]. The roots, stems, leaves, and fruits of CT reportedly contain large amounts of phenolic compounds, including flavonoids, xanthones, diterpenoids,
alkaloids, and terpenoids [12–14]. In particular, the roots and stems of CT possess the highest total phenol and flavonoid content when compared with that of leaves and fruits, as well as a strong antioxidant and reducing capacity [13]. Cudratricusxanthone A and cudraflavanone A are reported as active components of CT [15, 16], and cudratricusxanthone A reportedly attenuates renal injury in septic mice [17]. In this study, we investigated the effect of the CT root extract (CTRE) on MGO-induced ROS production and expression of inflammatory cytokines in HK-2 cells, a human tubular epithelial cell line, and assessed the underlying molecular mechanisms.

2. Materials and Methods

2.1. Preparation of Extracts from CT Root (CTRE). In this study, we used 70% (v/v) ethanolic extracts of dried CT root (CTRE). Dried CT root (CTR) was purchased from Miryang Kkujippong Farm Cooperative (Gyeongsangnam-do, Korea) and powdered using a mechanical pulverizer. The dried CTR powder (1.0 kg) was subjected to extraction in 70% ethanol for 3 h using a reflux extraction system by KOC Biotech (Daejeon, Republic of Korea). CTRE was dissolved in dimethyl sulfoxide (DMSO; Duchefa Biochemie B.V., Haarlem, Netherlands) at a concentration of 100 mg/mL and then further diluted with a culture medium for the experiment to reach the required concentration.

2.2. Cell Culture. The human renal proximal tubular cell line, HK-2, was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). HK-2 cells were maintained in RPMI 1640 (Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Welgene) at 37 °C in an atmosphere of 5% CO₂ in 95% air.

2.3. Cell Viability Assay. Cell viability was estimated using a D-Plus™ CCK cell viability assay kit (Dongin LS, Seoul, Korea) in accordance with the manufacturer’s protocol. HK-2 cells (3 × 10⁵ cells/well) were seeded in 96-well plates. After 24 h of culture, the cells were treated with CTRE or MGO. To assess toxicity following CTRE treatment, the cells were treated with different concentrations of CTRE (5–40 μg/mL) for different times, ranging between 3 and 24 h. To measure cell viability following MGO treatment, the cells were treated with different concentrations of MGO (0.125–1.0 mM) for 24 h or 0.5 mM MGO for different periods, ranging between 3 and 24 h. Following incubation, the CTR- or MGO-treated medium was replaced with 100 μL of CCK working solution, and the cells were further incubated for 4 h. The optical density (OD) was measured at 450 nm using a VersaMax Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The OD value of control cells was considered to represent 100% viability [18]. For use in subsequent studies, we selected an MGO concentration of 0.5 mM, which showed 50% cell viability, and a CTRE dose of 20 μg/mL, which demonstrated the highest inhibitory effects.

2.4. Measurement of Intracellular Reactive Oxygen Species (ROS) Levels. To examine time- and dose-dependent effects of MGO on ROS production, HK-2 cells (1.5 × 10⁵ cells/well) were seeded in 6-well plates and cultured for 24 h. Then, cells were treated with 0.5 mM MGO for different periods (0.5–12 h) or with different concentrations of MGO (0.125–1.0 mM) for 2 h. To determine the inhibitory effect of CTRE on MGO-induced ROS production, cells were incubated with vehicle (DMSO) or 20 μg/mL CTRE for 1 h and then further incubated for 2 h, with or without 0.5 mM MGO. Intracellular ROS levels were measured through flow cytometry using 2,7-dichlorodihydrofluorescin diacetate (DCHF-DA, Invitrogen, San Diego, CA, USA), as described in our previous report [19].

For fluorescence images, HK-2 cells (2 × 10⁴ cells/well) were incubated overnight in the Nunc™ Lab-Tek™ II Chamber Slide™ system (Thermo Scientific, Rochester, NY, USA). The cells were pretreated with vehicle or 20 μg/mL CTRE for 1 h and then further treated with or without 0.5 mM MGO for 2 h. The cells were treated with 10 μM DCFH-DA for 30 min at 37 °C. The cells were fixed in 5% neutral buffered formalin (NBF, Sigma, St. Louis, MO, USA) for 20 min at 4 °C. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) diluted in DPBS (1:1000) at 25°C for 5 min. Then, cells were mounted with fluorescence mounting medium (Dako North America, Inc.) and observed under a confocal microscope (LSM700; Carl Zeiss Inc., Oberkochen, Germany).

2.5. Western Blotting. HK-2 cells (5 × 10⁵ cells) were incubated in 90 mm dishes for 24 h. Then, the cells were pretreated with vehicle or 20 μg/mL CTRE for 1 h and then further treated with 0.5 mM MGO for 1 h (for evaluation of p-PKCβ) or 2 h. For inhibition of p-PKCβ signaling, cells were treated with 1 μM GF 109203X for 2 h before CTRE treatment. Total cellular protein extractions and western blotting were performed as described in our previous report [20]. Primary antibodies used in this study are listed in Supplementary Table 1. Chemiluminescent signals were developed by treating the blot with ECL reagents, Immobilon-Western chemiluminescent HRP substrate (Millipore Corp., Billerica, MA, USA), and visualized using the LAS4000 imaging system (Fujifilm Corp., Tokyo, Japan). Protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. RNA Isolation and Quantitative Real-Time PCR (RT-qPCR). HK-2 cells (2 × 10⁵ cells) were incubated in 60 mm dishes for 24 h. The cells were pretreated with vehicle or 20 μg/mL CTRE for 1 h and then treated with or without 0.5 mM MGO for 2 h. Total RNA isolation and RT-qPCR were performed as described in our previous report [21]. In brief, total RNA was extracted using RNAiso Plus Reagent (TaKaRa Bio Inc., Shiga, Japan) and 2 μg of total RNA was reverse-transcribed using a PrimeScript™ 1st-strand cDNA synthesis kit (TaKaRa Bio Inc.). RT-qPCR was performed using a reaction mixture comprising SYBR Green Master Mix (TaKaRa Bio Inc.). Results were calculated using the 2⁻ΔΔCΤ relative quantification method and normalized to
the expression level of cyclophilin B. Primer pair sequences are shown in Supplementary Table 2.

2.7. Statistical Analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± standard error of the mean (SEM) of three independent experiments. (b) Cell morphology of the control and CTRE groups (5, 10, 20, and 40 μg/mL) treated for 24 h was examined by light microscopy (original magnification, ×40). CTRE: Cudrania tricuspidata root extract.

3. Results

3.1. CTRE Does Not Affect HK-2 Cell Viability and Morphology. To determine whether CTRE has cytotoxic effects on HK-2 cells, we examined the viability of HK-2 cells after treatment with different concentrations of CTRE for different incubation periods using the CCK8 assay. On treating HK-2 cells with 5–40 μg/mL CTRE for 3–24 h, no differences in cell viability were observed between the control and CTRE-treated cells at any conditions investigated (Figure 1(a)). As shown in Figure 1(b), the cellular morphology did not differ between the control and 5–40 μg/mL CTRE-treated cells (Figure 1(b)). These results indicate that the CTRE concentrations used in this study did not demonstrate any cellular toxicity in HK-2 cells.

3.2. CTRE Inhibits ROS Production in MGO-Treated HK-2 Cells. To determine cell damage induced by MGO, HK-2 cells were treated with 0.125–1.0 mM MGO for 24 h, followed by measurement of cell viability. Cell viability decreased in a dose-dependent manner from 0.5 mM MGO when compared with control cells (Figure 2(a)). Next, HK-2 cells were treated with 0.5 mM MGO, which showed 50% cell viability; when assessed for 3–24 h, cell viability was significantly decreased after 12 h of treatment (Figure 2(b)). As MGO induces cytotoxicity by ROS overproduction in various cell lines [22–25], we measured intracellular ROS levels by flow cytometry in MGO-treated HK-2 cells. As shown in Figure 2(c), ROS production was significantly increased in HK-2 cells treated with 0.5 mM MGO for 2 h compared with that in control cells. On treating HK-2 cells with different concentrations of MGO (0.125–1.0 mM) for 2 h, ROS production was significantly increased when treated with 0.25 and 0.5 mM MGO when compared with that of control cells (Figure 2(d)). We then investigated the effect of CTRE on MGO-induced ROS production and found that pretreatment with CTRE significantly reduced ROS production compared with cells treated with 0.5 mM MGO for 2 h (Figure 2(e)). Confocal image analyses confirmed that MGO greatly increased ROS production, which was strongly attenuated by CTRE pretreatment (Figure 2(f)).

3.3. CTRE Decreases the Activation of MAPK and NFκB and the Expression of Inflammatory Cytokines in MGO-Treated HK-2 Cells. As MGO-induced oxidative stress activates mitogen-activated protein kinase (MAPK)/nuclear factor-kappa B (NFκB) signaling and induces an inflammatory response [26–28], we examined the expression of inflammatory cytokines in HK-2 cells treated with 0.5 mM MGO in the presence or absence of CTRE. MGO significantly increased the expression of interleukin-1 (IL-1) β, IL-6, and tumor necrosis factor (TNF) α when compared with that in control cells; however, CTRE pretreatment significantly reduced the expression of these genes when compared with that observed.
Figure 2: Continued.
in MGO-only exposed cells (Figure 3(a)). We investigated whether the inhibition of MGO-induced cytokine expression by CTRE was mediated via the regulation of MAPK/NFκB activation. Accordingly, we examined the phosphorylation of MAPKs and NFκB in HK-2 cells treated with 0.5 mM MGO, with or without CTRE pretreatment. As shown in Figure 3(b), MGO significantly increased the phosphorylation of P38, extracellular signal-regulated kinase (ERK), and c-JUN N-terminal kinase (JNK) when compared with that in control cells (Figure 3(b)). However, CTRE pretreatment significantly reduced the phosphorylation of these proteins when compared with that in MGO-treated cells (Figure 3(b)). Furthermore, MGO-induced phosphorylation of NFκB was significantly reduced by CTRE pretreatment (Figure 3(c)). These results suggest that CTRE may inhibit the inflammatory cytokine expression by downregulating MAPK/NFκB activation.

3.4. CTRE Recovers MGO-Induced Decrease in Nrf2 and mRNA Expressions of Antioxidant Enzymes. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) acts as a master regulator of cytoprotective responses, including antioxidant, anti-inflammatory, and detoxification [29, 30]. To investigate whether CTRE affects the expression of Nrf2 and antioxidant enzymes, we examined the mRNA expression of Nrf2 and Nrf2 target antioxidant enzymes in MGO-treated HK-2 cells, with and without CTRE pretreatment. The mRNA expression levels of antioxidant genes such as glyoxalase 1 (GLO1), catalase (CAT), glutamate-cysteine ligase catalytic subunit (GCLC), heme oxygenase-1 (HO-1), glutathione peroxidase (GPX), and NAD(P)H quinone dehydrogenase (NQO1) were significantly reduced following MGO treatment, and all, except CAT, were significantly increased by CTRE pretreatment when compared with that in the MGO-only exposed cells (Figure 4(a)). Consistent with these results, Nrf2 mRNA expression was also significantly increased when compared with that in the MGO-only exposed cells (Figure 4(b)). These results suggest that CTRE may ameliorate the MGO-induced decrease in the antioxidant enzyme gene expression, contributing to a decrease in ROS production.

3.5. CTRE Suppresses NOX4 Expression by Downregulating PKCβ Phosphorylation in MGO-Treated HK-2 Cells. In renal proximal tubular epithelial cells, ROS production is primarily regulated by NOX4 [31–34]. To investigate whether the inhibition of MGO-induced ROS production by CTRE can be attributed to the regulation of NOX4 expression, we measured the protein expression level of NOX4 by western blotting. NOX4 protein levels were significantly increased 2 h after MGO treatment when compared with that in control cells and were significantly reduced by CTRE pretreatment when compared with that in MGO-treated cells (Figure 5(a)). As PKC, particularly PKCβ, and AMPK pathways are involved in the induction of NOX4 in tubular cells [35], we measured the phosphorylation of PKCβ or AMPKα

![Figure 2: CTRE inhibits ROS production in MGO-treated HK-2 cells.](image)
in the absence or presence of CTRE in MGO-treated HK-2 cells. The phosphorylation of PKCβ was significantly increased in MGO-treated cells when compared with that in control cells and was significantly reduced following CTRE pretreatment when compared with that in MGO-treated cells (Figure 5(b)). Conversely, MGO reduced the phosphorylation of AMPKα when compared with that in control cells; however, CTRE pretreatment did not inhibit the MGO-induced decrease of phosphorylated-AMPKα in HK-2 cells (Figure 5(b)). To examine whether p-PKCβ regulates NOX4 expression, HK-2 cells were treated with GF 109203X (PKC inhibitor) and/or CTRE and then treated with MGO. We observed that pretreatment with GF 109203X significantly inhibited PKCβ phosphorylation and blocked the expression of NOX4 induced by MGO treatment (Figures 5(c) and 5(d)). Furthermore, we observed additive effects on the inhibition of NOX4 expression following cotreatment with CTRE and GF 109203X (Figure 5(d)). These results suggest that the antioxidant effect of CTRE is caused by the downregulation of NOX4 and ROS production through the inhibition of PKCβ phosphorylation.

4. Discussion

DN is a major complication of diabetes and a common cause of end-stage renal disease. Although DN contributes to the overall morbidity and mortality in diabetes patients, the pathophysiological mechanisms of DN remain unclear [36,
A multidisciplinary therapeutic approach, including renin-angiotensin system (RAS) inhibitors, has long been used to treat DN. The efficacy of sodium-glucose cotransporter 2 (SGLT2) inhibitors has been recently proven and thus added as a new treatment option; however, current therapeutic approaches still do not completely inhibit DN progression [38].

During the progression of DN, glomerular changes such as mesangial expansion, glomerular basement membrane thickening, and podocyte loss are considered clinical signs; however, tubular interstitial inflammation and tubular damage are also critical features [2, 39, 40]. In particular, early changes in tubular epithelial cells are considered an important factor in DN progression [40, 41]. Inflammation from tubular epithelial cells can lead to hyperfiltration, matrix expansion, and apoptosis by causing damage to glomerular cells through inflammatory mediators such as cytokines and chemokines [42–44]. Mohamed et al. reported that the suppression of local inflammation through overexpression of anti-inflammatory factors in tubular epithelial cells improved albuminuria and DN in streptozotocin-induced diabetic mice [44]. Therefore, suppression of inflammation in tubular epithelial cells may be an effective prevention strategy against the development of DN.

Medicinal plants and bioactive natural compounds have been used as alternative treatments for kidney diseases, including DN [45–49]. Previous studies have shown that the fruit, leaves, and roots of CT also have various biological activities, including antioxidant [50, 51], anti-inflammatory [52], antiobesity [53], hepatoprotective [54], and neuroprotective [16, 55] effects. It has recently been reported that CT displays antisenescence effects on endothelial cells exposed to high glucose, a major diabetic factor [56]. In addition, renal protective effects have been reported for cudratricus-xanthone A, an active component of CT, in cecal ligation and puncture-induced septic mice [17]; however, the effects of CT on DN have not been studied. In this study, we investigated the effects of the ethanolic extract of CT root on

![Figure 4](image-url)
Figure 5: Continued.
MGO-induced oxidative stress and inflammation in HK-2 cells, a human kidney epithelial cell line. MGO, a highly reactive dicarbonyl compound, is known as the major precursor for advanced glycation end products (AGEs) and is generated as a side-product derived from glycolysis [57]. MGO- and MGO-mediated AGEs are increased in DN patients, and they are important factors in DN progression [58–60]. It is well established that MGO increases oxidative stress and cell damage/death in endothelial cells [61] and several kidney cells including mesangial cells [62, 63], podocytes [64], and tubular epithelial cells [26, 27, 65]. According to several studies [26–28, 35],...
including the present study, MGO-induced oxidative stress activates MAPK/NFκB signaling and induces an inflammatory response in renal tubular epithelial cells. Our results show that CTRE pretreatment inhibits MGO-induced ROS overproduction, as well as MAPK and NFκB activation and production of inflammatory cytokines, illustrating the antioxidant and anti-inflammatory effects of CTRE. Previous studies have identified cudraxanthone B, cudratricusxanthone A, cudratricusxanthone O, cudrallavanone A, cudrallavanone B, isogentisin, and 8-prenylxanthone as active ingredients that have anti-inflammatory effects in the CT root [12, 16, 66–68]. Therefore, these active ingredients might decrease the MGO-induced inflammatory response in HK-2 cells.

An increase in antioxidant enzymes through the Nrf2-Keap1 pathway is a major antioxidant response [69]. Cudrallavanone A, isolated from CT root bark, has shown anti-inflammatory effects by Nrf2 activation in microglial cells [16], and a CT leaf extract reportedly exerts antioxidant effects through Nrf2 activation in hepatocytes [70]. Cudratricusxanthone A isolated from the CT root has shown renal protective effects by inducing antioxidant enzymes [17]. Similar to these previous reports [16, 17, 70], CTRE also increased expression of Nrf2 and antioxidant enzymes, suggesting that MGO-induced ROS overproduction may be attenuated by CTRE through increased Nrf2-mediated antioxidant enzyme expression.

NADPH oxidases (NOXs) are a major source of ROS in diabetic kidneys and are important mediators of redox signaling in glomerular and tubulointerstitial cells in diabetic conditions [35]. The NOX family has seven isoforms, and the renal system predominantly expresses NOX1, NOX2, and NOX4 isoforms, of which NOX4 is the most abundant isoform [35, 71, 72]. NOX1 and NOX2 are located in the plasma membrane and require activation by the isoform-specific assembly of different subunits; however, NOX4 is located in the ER, has no regulatory subunit, and is constitutively expressed [71, 73]. Thus, NOX4 has been referred to as a "constitutively active" enzyme, and overall ROS production by NOX4 under various stimulation conditions may be directly regulated by its expression level [35]. NOX4 is highly expressed in the renal cortex, which is mainly composed of tubular epithelial cells [74]. Furthermore, the expression of NOX4 was increased in renal proximal tubular epithelial cells under high glucose conditions; however, NOX2 or NOX1 expression was not changed [31]. Therefore, downregulation of NOX4 expression under diabetic conditions can be employed as a strategy to suppress tubular inflammation. In this study, CTRE pretreatment significantly reduced MGO-induced NOX4 expression in HK-2 cells, suggesting that NOX4 downregulation by CTRE contributed to decreased ROS production.

It is known that the PKCβ/NOX4 and AMPKα/NOX4 pathways are major regulatory pathways for ROS production in renal tubular cells under diabetic conditions [31, 35, 75]. According to a previous report [76], inhibition of PKCβ signaling attenuates diabetes-induced NOX4 expression and oxidative stress, consistent with our results showing that CTRE inhibits MGO-induced PKCβ activation, subsequently inhibiting NOX4 expression in HK-2 cells. Meanwhile, inactivation of AMPK by diabetic stimuli increases NOX4 expression and subsequent ROS production in mesangial cells [35, 77]. AMPK activation using AMPK activators, such as metformin or AICAR (5-aminooimidazole-4-carboxamide-1-beta-d-riboruranoside), attenuates high glucose-induced NOX4 expression in tubular epithelial cells [78, 79]. Similar to previous study [77], the expression of phosphorylated-AMPK was significantly reduced following MGO treatment; however, no change was observed following CTRE pretreatment. These results indicate that the inhibition of MGO-induced NOX4 expression by CTRE in HK-2 cells can be attributed to the downregulation of PKCβ activation, but not by the upregulation of AMPK activation.

In summary, our study revealed that CTRE prevents MGO-induced inflammation via attenuation of oxidative stress through inhibition of the PKC-NOX4 pathway, as well as by increasing the Nrf2-antioxidant enzyme pathway in HK-2 cells (Figure 6). Considering the multifactorial and systemic properties of DN and the effects of CTRE on HK-2 cells, we suggest that CTRE or its components might be candidate agents in combination therapy for attenuating chronic inflammation and oxidative stress underlying DN progression.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Donghee Kim and Jayeon Cheon contributed equally to this work.

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Supplementary Materials

Table S1: antibodies used in the present study. Table S2: primer sequences used for RT-qPCR. (Supplementary Materials)

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