Preventive Effects of Grape Extract on Ischemia/Reperfusion-Induced Acute Kidney Injury in Mice

Mamoru Ohkita,* Haruna Hayashi, Kohei Ito, Natsuko Shigematsu, Ryosuke Tanaka, Hidenobu Tsutsui, and Yasuo Matsumura

Laboratory of Pathological and Molecular Pharmacology, Osaka University of Pharmaceutical Sciences; 4–20–1 Nasahara, Takatsuki, Osaka 569–1094, Japan.

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Since grape extract (GE) contains oligomeric proanthocyanidins and numerous polyphenols, dietary GE supplements may exert protective effects against various diseases. The present study investigated the pharmacological effects of GE derived from Chardonnay in vitro and in vivo. GE (100 μg/mL) completely inhibited tumor necrosis factor-α-induced endothelin-1, monocyte chemoattractant protein-1, interleukin-1β, and intercellular adhesion molecule-1 gene expression in cultured endothelial cells. GE also strongly stimulated the phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) pathway. In the in vivo study, the effects of GE on ischemic acute kidney injury (AKI) were examined using male C57bl/6J wild-type and eNOS−/− mice. Right nephrectomized mice were exposed to 45 min of ischemia in the left kidney and this was followed by reperfusion. Although renal functional parameters in AKI mice significantly increased 48 h after reperfusion, the administration of GE (0.1 and 1 mg/kg, intravenous (i.v.)) 5 min before ischemia dose-dependently improved post-ischemic renal dysfunction in wild-type mice. Renal histopathological studies on AKI mice revealed tubular necrosis, proteinaceous casts in tubuli, and medullary congestion. The administration of GE ameliorated this damage in wild-type mice, but not in eNOS−/− mice. Furthermore, GE significantly restored decreases in the renal nitric oxide metabolite content due to ischemia in wild-type mice, but not in eNOS−/− mice. Thus, eNOS is closely involved in the renoprotective effects of GE, strongly suggesting that GE supplements are useful as a prophylactic treatment for the development of ischemic AKI.

Key words grape extract; acute kidney injury; ischemia; reperfusion; endothelial nitric oxide synthase (eNOS)

INTRODUCTION

Ischemia often occurs in several organs, and is followed by the reperfusion phenomenon with blood flow recovery. This phenomenon itself is a very important process in terms of blood flow recovery, but induces severe organ dysfunction and tissue injury in reperfused organs.1) In acute kidney injury (AKI), the glomerular filtration rate markedly decreases, which results in the accumulation of urea nitrogen and creatinine, and, thus, a state exists in which the homeostasis of body fluids and electrolytes cannot be maintained.2) Ischemia/reperfusion (I/R) also causes decreases in renal blood flow and the glomerular filtration rate as well as renal tubular injury.2–4) Damaged renal tubule cells then become detached and occlude the renal tubular lumen, thereby increasing the tubular inner pressure and causing the backleak of the glomerular filtrate from the renal tubule lumen to the interstitium, which reduces the glomerular filtration rate. On the other hand, previous studies reported that the main causal factors in I/R-induced AKI are based on transient increases in reactive oxygen species (ROS), cytokines, and adhesion molecules in cells such as neutrophils and vascular endothelial cells.2,4) Thus, the suppression of the above factors may contribute to preventing the development of AKI.

Nitric oxide (NO) is a major substance of endothelial cell-derived vasorelaxation factors, and its synthesis in vivo is regulated by NO synthase (NOS).5) There are three isozymes of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Endothelial cells constitutively express eNOS, and this eNOS-derived NO plays an important role in the regulation of blood flow. When shear stress caused by blood flow, or physiologically active substances, such as acetylcholine, bradykinin, and endothelin, act on endothelial cells, eNOS is activated.5) NO produced by endothelial cells expands blood vessel diameters and blood flow is properly regulated. Studies conducted to date elucidated the physiological and pathophysiological roles of NO and revealed that NO itself plays an important role in maintaining homeostasis in various organs and tissues.6)

Grape extract (GE) mainly consists of oligomeric proanthocyanidin and includes numerous polyphenols, such as catechin and taxifolin. Dietary GE supplements, particularly grape seed extract, have been reported to exert diverse protective effects, including anti-inflammatory and antioxidant effects, against cardiovascular diseases,8,9) diabetes,10,12) and cancer.13) Therefore, GE supplements may exert prophylactic and beneficial effects against the development of these diseases.

The present study investigated whether the administration of GE exerts protective effects against I/R-induced renal damage in mice. We also examined the involvement of NO in the renoprotective effects of GE.

MATERIALS AND METHODS

Materials All reagents were commercial products of the highest grade available. Proanthocyanidin-rich extract was

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prepared from commercially available grape seeds and skin (Chardonay), and the contents of proanthocyanidins and monomeric flavanols were measured as described previously.14-16 Briefly, grape seeds and skin were extracted with 10% aqueous ethanol at 80°C for 1 h. After filtration with a nylon mesh, the 10% aqueous ethanol extract was chromatographed on DIAION™ HP20. The 70% aqueous ethanol elute was concentrated and then freeze-dried to obtain a proanthocyanidin-rich extract. The extract was composed of 73% proanthocyanidins, 12% catechins, and 7% epicatechin.

**Cell Culture**

EA.hy926 endothelial cells (kindly gifted by Prof. Cora-Jean S. Edgell, Department of Pathology and Laboratory Medicine, University of North Carolina, U.S.A.), a human umbilical vein cell line, were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with high glucose (4.5 g/L), 10% fetal calf serum, 100 µg/mL streptomycin, and 100 U/mL of penicillin, at 37°C in a 5% CO₂ atmosphere. Prior to the initiation of experiments, EA.hy926 cells were incubated with serum-free DMEM overnight.

**Measurement of Gene Expression**

RNA extraction from cultured cells and its purification were performed using RNAiso Plus (TaKaRa Bio Inc., Shiga, Japan) and the TURBO DNA-free™ Kit (Thermo Fisher Scientific, Tokyo, Japan). Quantitative RT-PCR was performed using the PrimeScript™ RT reagent Kit (TaKaRa Bio Inc.) and CFX96™ Real-Time System (Bio-Rad Laboratories Inc., Tokyo, Japan). Human endothelial (ET)-1, monocyte chemotactic protein (MCP)-1, interleukin (IL)-beta, intercellular adhesion molecule (ICAM)-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sequences were as follows: forward primer, 5’-GCC ACC TTG ACA TCA TTT TG-3’ (ET-1), 5’-GAT CCT AGT GCA GAG GCT CG-3’ (MCP-1), 5’-CAG CTA CGA ATC CCC GAC CAC C-3’ (IL-1β), 5’-TGA CCG TGA ATG TGCT CCT CC-3’ (ICAM-1), 5’-CAC CCA CTC CTC CAC CTT TG-3’ (GAPDH); reverse primer, 5’-ACG GCT GTG TGC CTT TGT GG-3’ (ET-1), 5’-TGG TCT TCC AGG TGT GC AT-3’ (MCP-1), 5’-GCC AGG GGA ACC GCC GAT TTT C-3’ (IL-1β), 5’-AGG CTT GCT GCT TTT GCT GTT-3’ (ICAM-1), 5’-CCA CCA CCC TGT GTG TCAG-3’ (GAPDH). Relative mRNA expression levels were normalized to that of GAPDH.

**Western Blot Analysis**

Extracted protein (50 µg) was fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gels (7.5%) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, U.K.). After blocking with 5% skim milk or Blocking One-P (Nacalai Tesque, Inc., Kyoto, Japan), membranes were incubated overnight with antibodies against eNOS, phosphorylated-eNOS (serine (Ser)-1177), Akt, phosphorylated-Akt (Ser-473), and actin at 4°C, and then incubated with secondary antibodies. Blots were developed with Pierce™ ECL Western blotting Substrate (Thermo Fisher Scientific) and quantified using the Quantity One software (Bio-Rad Laboratories).

**Animals and Experimental Design**

Male C57bl/6J wild-type and eNOS−/− mice (The Jackson Laboratory, Bar Harbor, ME, U.S.A.) weighing 15–25 g were used in experiments. Mice were housed with free access to food and water. Animal care methods and experimental protocols were conducted according to the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences. The right kidney was excised two weeks before the experiment (from 8-week-old mice). These mice (at 10 weeks old) were then divided into four groups: (1) sham-operated control without ischemia, (2) vehicle-treated ischemic AKI, (3) ischemic AKI pretreated with GE (0.1 mg/kg, intravenous (i.v.)), and (4) ischemic AKI pretreated with GE (1 mg/kg, i.v.). The left kidney was exposed via a small flank incision under anesthesia to occlude the renal artery and vein with a Schwartz micro serefine. After the ischemic period for 45 min, the micro serefine was removed to restore blood flow. GE or vehicle (saline) was injected 5 min before ischemia in a volume of 1 mL/kg. The kidney of sham-operated control mice was treated identically, except for occlusion. Mice subjected to ischemic AKI were housed in cages for 24 h and subsequently in metabolic cages to collect urine for 24 h. Blood sampling from the thoracic aorta and excision of the left kidney were then performed under anesthesia. Urine and plasma samples as well as the kidneys were used to assess renal function and in histological studies.

**Analytical Procedures**

Plasma creatinine (Pcr) and blood urea nitrogen (BUN) levels were measured using the blood urea nitrogen-test-Wako and creatinine-test-Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively.

**Histological Studies**

Ablated left kidneys were fixed in phosphate-buffered 10% formalin. Paraffin-embedded renal tissues were sliced for staining with hematoxylin and eosin. Histopathological changes were assessed in accordance with the method described by Caramelo et al. The grading of histological changes was performed in a blinded manner.

**NO Metabolite Measurements**

NO metabolite (NOx: NO2− + NO3−) concentrations in renal tissue were measured using the microdialysis method. Briefly, a left side abdominal incision was made under anesthesia approximately 1 h before the ischemia treatment, and a dialysis probe was then inserted into the exposed kidney. The probe was connected to an ENO-20 NOx Analyzer (Eicom, Kyoto, Japan), the dialysate was intermittently passed through at a rate of 2.0 µL/min, and the NOx content in the dialysate (22 µL) obtained every 12 min was measured in real time. In the case of culture medium, the medium diluted with a mobile phase was injected into the ENO-20 NOx Analyzer. The analysis was performed by combining a diazo coupling method with HPLC. Nitrite and nitrate levels were measured by comparisons with the results obtained using standard solutions (NO-STD, Eicom).

**Statistical Analysis**

Data represent the mean ± standard error of the mean (S.E.M.) and were analyzed by an ANOVA followed by Dunnett’s or Bonferroni’s test for multiple comparisons. Histological changes were analyzed by the Kruskal–Wallis non-parametric test combined with a Steel-type multiple comparison test. Statistical analyses on differences in NOx levels between the vehicle and GE treatments in wild-type and eNOS−/− mice were performed using a two-way repeated ANOVA. p-Values of less than 0.05 were considered to be significant.

**RESULTS**

**Inhibitory Effects of GE on Gene Expression in Cultured Endothelial Cells**

Various causal factors, such as cytokines, adhesion molecules, and vasoactive hormones, are responsible for I/R-induced renal injury. Therefore, we examined the effects of GE on ET-1, MCP-1, IL-1β, and ICAM-1 gene expression. As shown in Fig. 1A, GE significantly inhibited tumor necrosis factor (TNF)-α-induced ET-1 mRNA expression...
expression in cultured endothelial cells. The same suppressive effects were observed in TNF-α-induced MCP-1, IL-1β, and ICAM-1 mRNA expression (Figs. 1B–1D).

Effects of GE on the eNOS Protein in Cultured Endothelial Cells We examined the effects of GE on the PI3K/Akt/eNOS pathway. The addition of GE dose-dependently increased NOx content in the culture medium (Fig. 2A). GE had no effect on Akt or eNOS protein expression, but significantly increased phosphorylated Akt (p-Akt) and eNOS (p-eNOS) protein expression (Figs. 2B–F). The PI3K inhibitor LY 294002 (10 µM) completely abolished increases in GE-induced p-Akt and p-eNOS protein expression. Thus, these results suggest that GE induces NO production in endothelial cells by activating the PI3K/Akt/eNOS pathway.

Effects of GE Administration on I/R-Induced Renal Dysfunction in Mice Renal function 48 h after reperfusion showed a marked deterioration in wild-type mice (Fig. 3). BUN and Pcr values were significantly higher in vehicle-treated AKI mice (150.3 ± 4.5 and 2.04 ± 0.34 mg/dL, respectively) than in the sham-operated control without ischemia (35.9 ± 1.6 and 0.44 ± 0.02 mg/dL, respectively). Intravenous bolus injection of GE 5 min before ischemia reduced renal dysfunction in a dose-dependent manner. On the other hand, these renoprotective effects by GE were not observed in eNOS−/− mice, even at a dose of 1 mg/kg (Fig. 4).

Effects of GE Administration on I/R-Induced Renal Tissue Injuries in Mice Histopathological studies showed severe renal tissue injuries in vehicle-treated AKI wild-type mice. This renal damage was defined as tubular necrosis (Fig. 5B; scores, 3.67 ± 0.21), medullary congestion and hemorrhage (Fig. 5F; scores, 3.67 ± 0.21), and proteinaceous casts (Fig. 5J; scores, 4). The administration of GE (1 mg/kg, i.v.) significantly reduced the development of renal tissue injuries (Figs. 5D, 5H, 5L). On the other hand, GE had no effects on renal damage in eNOS−/− mice (Fig. 6). No histopathological changes were observed in the renal tissue of sham-operated control without ischemia (A, E, and I in Figs. 5 and 6).

Changes in NO Metabolite Levels in Renal Tissue during I/R In all groups, the ischemia treatment markedly reduced the NO metabolite content in renal tissue (Fig. 7). Although the NOx content gradually increased after reperfusion, the extent of recovery was more pronounced when 1 mg/kg of GE was administered. Furthermore, when GE was administered to eNOS−/− mice, the increase observed in the NOx content in renal tissues after reperfusion was very small and similar to that in the vehicle-treated AKI group.

DISCUSSION

The present study showed that the administration of GE suppressed post-ischemic renal dysfunction in wild-type, but not eNOS−/− mice. Histological studies on vehicle-treated AKI wild-type mice revealed renal tissue injuries, such as tubular necrosis, medullary congestion and hemorrhage, and proteinaceous casts in tubuli, and this damage was significantly prevented by the pre-ischemic administration of GE. These findings confirmed that GE improved reductions in renal function and tissue damage caused by I/R.

eNOS is distributed in vascular endothelial cells in the kidney, such as glomerular capillaries. eNOS-derived NO exerts organ protective effects through vasodilation, blood flow retention, the removal of ROS, the suppression of platelet...
aggregation and leukocyte adhesion, and other processes.\(^5,7\)

Thus, NO is regarded as a renoprotective substance against I/R in various cell types and animal models.\(^5,6\) The present study showed that the ameliorating effects of GE on ischemic AKI almost completely disappeared in eNOS\(^{-/-}\) mice. This result suggests that eNOS is closely involved in the renal protective effects of GE. Shao et al. demonstrated that grape seed proanthocyanidin extracts (GSPE) protected cardiomyocytes from I/R-induced injury and these cardioprotective effects were associated with the Akt/eNOS signaling pathway.\(^18\)

Furthermore, the results obtained using cultured vascular endothelial cells showed that GE dose-dependently promoted the phosphorylation of Akt and the eNOS protein. These increments were completely suppressed under the PI3K inhibitor LY-294002-treated condition. Moreover, in the case of excised blood vessels, the vasorelaxant effects of GE were dependent on I/R-reperfusion, as demonstrated by the inhibition of NO production and Akt/eNOS phosphorylation. These findings highlight the potential of GE as a therapeutic agent for renal protection against I/R injury.\(^5,6,7\)

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**Fig. 2. Effects of GE on NO Production and Its Mechanisms in EA.hy926 Cells**

A: quiescent EA.hy926 cells were treated with the indicated concentrations of GE for 2h, and the concentration of NO\(_x\) (NO\(_2^-\) + NO\(_3^-\)) in the culture medium was then measured (n = 4). B to F: quiescent EA.hy926 cells were pretreated with LY294,002 (10 µM) for 1 h and then incubated with or without the indicated concentrations of GE for 10min. The expression levels of each protein were quantified by a densitometric analysis. Results represent the mean ± S.E.M. (n = 5). *p < 0.05, **p < 0.01 significantly different from no addition. ‡p < 0.01 significantly different from each concentration of GE alone.

**Fig. 3. Effects of GE on Renal Function 48 h after Ischemia/Reperfusion in Wild-Type Mice**

Effects of GE administered before ischemia (0.1 or 1 mg/kg, i.v.) on blood urea nitrogen (BUN) and plasma creatinine (Pcr) concentrations 48 h after ischemia/reperfusion. GE was given 5 min before ischemia. Results represent the mean ± S.E.M. (n = 6). *p < 0.05, **p < 0.01, significantly different from vehicle-treated AKI mice. AKI: acute kidney injury.
on eNOS and markedly abolished by the PI3K inhibitor wortmannin (Sato and Ohkita et al., unpublished observations). Therefore, GE appears to enhance NO production mainly via the PI3K/Akt/eNOS pathway. These findings strongly support the present results showing that GE had no effect on I/R-induced renal dysfunction or tissue injuries in eNOS\(^{-/-}\) mice.

In the present study, to examine the effects of GE on NO production during I/R, the renal NOx content was intermittently measured as an indicator of NO production. The results obtained showed that the NOx content significantly decreased during ischemia and then gradually improved to approximately 60% of the basal level in the vehicle-treated AKI group after reperfusion. No significant difference was observed in the changes in NOx content between wild-type and eNOS\(^{-/-}\) mice for 84 min after ischemia/reperfusion. In the present study, we did not investigate the changes of NOx content after that, but there is a possibility that the recovery of NOx in eNOS\(^{-/-}\) mice may have required more time than that of wild type mice. In fact, renal tissue damage 48 h after I/R in eNOS\(^{-/-}\) mice was slightly exacerbated in comparison with wild-type mice. On the other hand, the NOx content in the GE-treated group started to rise early after reperfusion, and then improved the decreased NOx content to the basal level. In contrast, the NOx content in GE-treated eNOS\(^{-/-}\) mice did not show these changes and remained at the same level as vehicle-treated AKI mice. Therefore, in the model of ischemic AKI, GE appears to promote NO production early in reperfusion, and the renal vasodilator effects of NO may be involved...
in subsequent renal functional and histological improvement.

Although the precise mechanisms underlying post-ischemic renal dysfunction and damage remain unclear, many factors (including vasoactive substances, neutrophil invasion, cytokines, and ATP depletion) contribute to renal injury. The present study showed that GE inhibited ET-1, MCP-1, IL-1β, and ICAM-1 gene expression, which were the most important and deleterious factors in I/R-induced renal damage. Thus, improvements in renal function and renal tissue in wild-type mice may be attributed to these suppressive effects of GE. However, our results are confined to the effects of GE on ET-1, MCP-1, IL-1β, and ICAM-1 mRNA expression in cultured endothelial cells and do not extend to interpret these gene and protein levels in vivo, which means the necessity to examine the changes of these factors in the kidney after ischemia/reperfusion in future studies. On the other hand, ROS are also regarded as a causative factor clearly related to the onset of ischemic AKI. We previously demonstrated that
I/R-induced AKI was attenuated by various agents that exhibit antioxidant activity, such as α-lipoic acid and t-carnosine. Furthermore, an extract of French maritima pine bark, such as flavangenol and pycnogenol, which contains oligomeric proanthocyanidins (OPCs), similar to GE, is a radical scavenger. This extract may also potentiate defenses against intracellular oxidative stress by inducing the activities of several antioxidant enzymes. Moreover, we previously reported that flavangenol improved I/R-induced AKI in rats. Therefore, the amelioration of post-ischemic renal dysfunction and tissue injuries by GE appears to be closely related to its antioxidant effects; however, further studies are needed to elucidate the relationship between the renoprotective effects of GE observed in the present study and direct and/or indirect antioxidant activity.

I/R enhances the expression of various genes, such as cytokines, vasoactive peptides, and adhesion molecules, in many tissues. Nuclear factor-kappa B (NF-κB), a transcriptional factor, plays an important role in the control of gene expression. Thus, the suppression of NF-κB may be a therapeutic strategy for I/R-induced tissue damage. There is accumulating evidence to show that GE exerts suppressive effects on NF-κB activation in macrophages and vascular endothelial cells. Lu et al. reported that GSPE exerted protective effects on the cerebral cortex of streptozotocin-induced diabetic rats by down-regulating the advanced glycation end products (AGEs)/receptor for AGEs/NF-κB pathway. Wei et al. also found that the beneficial effects of GSPE in renal I/R-induced injuries were based on increases in antioxidant activity and decreases in NF-κB activation. On the other hand, we previously demonstrated that endogenous and exogenous NO reduced NF-κB activation by suppressing inhibitor of κB (I-κB) degradation in cultured endothelial cells. Therefore, GE-induced NO production is at least partly associated with NF-κB inhibition, followed by improvements in post-ischemic renal lesions; however, the molecular mechanisms underlying the favorable effects of GE need to be examined in more detail.

Flavonoids, such as catechin, epigallocatechin gallate, taxifolin, and proanthocyanidin, exert protective effects against I/R injury in the heart, brain, kidney, and liver. On the other hand, the main constituents of GE used in the present study are OPCs, which represent approximately 70% of all components. OPCs contained in GE are mainly composed of dimer and oligomer chains of catechin and/or epicatechin. GE also includes several polyphenols, such as monomer catechin and taxifolin, and other condensed tannins; however, the amounts of these components are very small. Although our results clearly demonstrate the beneficial effects of GE at the in vivo level, further studies are needed to identify the most important components of GE, evaluate the effects of dietary supplementation with GE on AKI, and clarify its pharmacokinetics. In conclusion, the administration of GE exerted renoprotective effects against I/R-induced AKI by increasing eNOS-derived NO production. GE supplements may be beneficial as a prophylactic treatment against the development of ischemic AKI.

Conflict of Interest The authors declare no conflict of interest.
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