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Effect of *Scutellaria Baicalensis* Georgi Extract on Oxidant-Induced Apoptosis in Renal Epithelial Cells

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Renal epithelial cells에서 oxidant에 의한 apoptosis에 미치는 黃芩의 영향

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목적 : 黃芩과 黃芩의 주요 flavonoid 성분인 baicalein이 신장세뇨관 상피세포에서 산화제에 의한 apoptosis에 미치는 효과를 살펴보고자 한다.

방법 : 신장세뇨관 상피세포주인 opossum kidney (OK) 세포를 유기산화제인 *t*-butylhydroperoxide (*t*BHP)에 노출시켜 apoptosis를 일으킨 후 관련된 변화를 관찰하였다.

결과 : *t*BHP는 농도에 의존하여 apoptosis를 유발시켰는데, 이러한 효과는 黃芩과 baicalein에 의해 농도 의존적으로 방지되었다. *t*BHP에 의한 OK 세포사는 항산화제인 Trolox와 N-acetylcysteine에 의해 방지되었다. *t*BHP는 mitogen-activated protein kinase의 subfamily인 extracellular signal-regulated kinase (ERK)를 활성화시켰다. ERK 억제제인 PD98059와 U0126은 *t*BHP에 의한 세포 사망을 방지하였다. *t*BHP에 의한 ERK 활성화는 U0126에 의해 억제되었으나 黃芩과 baicalein에 의해서는 영향을 받지 않았다. 철착염제인 deferoxamine은 *t*BHP에 의한 세포 사망과 ERK 활성화를 방지하였다. *t*BHP에 의한 세포 사망은 casopase 억제제인 BOD-D-FMK와 zDEVD-FMK에 의해 방지되었다.

결론 : 黃芩은 산화제에 의한 세포 사망을 방지하는데, 이는 kinase 억제, 항산화제 역할 및 철착염제의 작용에 기인하지 않았다. 黃芩의 이러한 효과는 산화제에 의한 신부전 예방 및 치료제로 개발하는데 이용될 수 있는 가능성을 보였다.

Key Words: *Scutellaria Baicalensis* Georgi, apoptosis, oxidants

Ⅰ. Introduction

Many environmental toxicants and chemicals including metabolic poisons and chemotherapeutic drugs cause cell injury by mechanisms involving reactive oxygen species (ROS)[1]. Oxygen metabolites involved in cytotoxicity are superoxide, hydrogen peroxide (H_2O_2), hydroxyl radical, and organic hydroperoxide. ROS have been implicated in the pathogenesis of a number of renal diseases including ischemia/reperfusion injury, glomerulonephritis, and nephrotoxicant-induced acute renal failure[2-4]. Several *in vivo* and *in vitro* studies have demonstrated that renal tubular cells have the potential to produce oxygen free radicals in response to various stimuli[5,6]. Therefore, agents that can scavenge ROS may serve as a possible preventive intervention for ROS-mediated renal diseases. In this context, the search for natural antioxidants and other preparations of plant origin to achieve this objective has been intensified.

Flavonoids are found in almost every plant and act as pharmacological active constituents in many

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herbal medicines. They have multiple biological activities including vasodilatory[7], anticancer, anticarcinogenesis, antiinflammatory, antibacterial, antiallergic, and antiviral effects[8,9]. Such effects of flavonoids are associated with their abilities to inhibit lipid peroxidation[10]. *Scutellaria balicalensis* Georgi (SbG) has been widely employed for the purpose of clearing away heat and toxic material in traditional Chinese medicine for centuries[11]. SbG has been known to contain numerous flavonoids including baicalein as its major constituent[12] and have antioxidant activity[13].

Members of the mitogen-activated protein kinase (MAPK) family are serine/threonine kinases involved in the regulation of a wide range of cellular responses, including cell proliferation, differentiation, and survival[14,15]. The MAPK family has been classified into three major subfamilies: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK/SAPK), and the p38 kinase. The ERK pathway is mainly induced in response to mitogens and growth factors and plays a major role in regulating cell growth, survival, and differentiation[16]. In contrast, JNK and p38 pathways are activated in response to chemical and environmental stress. Their activation is most frequently associated with induction of apoptosis[16,17].

Flavonoids have been demonstrated to suppress hydrogen peroxide-induced apoptosis by modulating activation of MAPK signaling pathways which have been known to involve in oxidant-induced apoptosis[18,19]. However, it is unclear whether SbG affects oxidant-induced apoptosis through modulation of MAPK in renal epithelial cells.

Thus, the present study was carried out to (1) examine effects of SbG on oxidant-induced apoptosis, (2) determine whether MAPK signal pathways are involved in anti-apoptotic effect of these agents in renal epithelial cells, and (3) compare its effects with those of baicalein (a major flavonoid constituent of SbG). Organic hydroperoxide, *t*-butylhydroperoxide (*t*BHP), has been employed as an *in vitro* drug model for the study of mechanisms underlying of oxidant-induced cell injury and for the screening of drugs that may protect cells from oxidative stress in various cell types[20,21].

II. Materials and Methods

1. SbG preparation

SbG was purchased from Oriental Medicine Hospital in Kyung-Ju, Oriental Medicine College of Dongguk University. The drugs were crushed and aqueous extract was prepared as following : 300 g of crushed crude drug was heated at 100℃ for 8 hr in 2,000 ml distilled water and was filtered with gauze. The extract thus obtained was concentrated under reduced pressure to give 46 g. The extractwas dissolved in the incubation medium. The concentration used in the experiment was based on the dry weight of the extract.

2. *Chemicals*

Trolox, deferoxamine (DFO), propidium iodide, *N*-acetylcysteine (NAC), Hoechst 33258, Boc-Asp (Ome)-CH_2F (BOC-D-FMK) and Z-Asp (OCH3)-Glu (OCH3)-Val-Asp (OCH3)-FMK (z-DEVD-FMK) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Tween 20, PD98059, and U0126 were purchased from Calbiochem (California, USA). Antibodies of MAPK subfamilies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals were of the highest commercial grade available.

3. Culture of opossum kidney OK cells

Opossum kidney (OK) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Sigma Chemical Co.) containing 10% fetal bovine serum at 37℃ in 95% air / 5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates in DMEM/F12 medium containing 10% fetal bovine serum. All experiments started 3-4 days after plating when a confluent monolayer culture was achieved.

4. Measurement of cell viability

Cell viability was determined by a trypan blue exclusion assay. The cells were harvested using 0.025% trypsin, incubated with 4% trypan blue solution, and were counted using a hemacytometer under light microscopy. Cells failing to exclude the dye were considered nonviable, and the data were expressed as a percentage of control cells.

5. Measurement of apoptotic cells

Apoptosis was estimated by a TUNEL and fluorescent dye (Hoechst 33258) staining assay. Cells were grown on 22-mm glass coverslips in 6-well plates. After treatment with oxidnats, the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 hr at 4℃. The fixed cells were stained by the TUNEL method using an in situ Apoptosis Detection Kit and horse-radish peroxidase (POD) (Boehringer Mannheim, Germany). After TUNEL staining, the cells were stained again with hematoxylin and analyzed under a light microscope (BX50, Olympus, Japan). In other experiments, the fixed cells were stained with 10 M Hoechst 33258 for 15 min at 37 ℃. Then cells were washed twice with PBS and examined by confocal microscopy (LSM510, ZEISS, Germany). Apoptotic cells were identified by condensation and fragmentation of nuclei.

6. Western blot analysis and immunoblotting

Cells were harvest at various times after *t*BHP treatement and disrupted in lysis buffer [1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4)]. Cell debris was removed by centrifugation at 10,000 g for 10 min at 4℃. The resulting supernatants were resolved on a 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with rabbit monoclonal antibodies against phosphorylated form of ERK, JNK, and p38. The Membranes were washed and incubated with the respective secondary antibodies conjugated with 5% non-fat fried milk. The signals were visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

7. Statistical analysis

The data are expressed as mean±SE and the difference between two groups was evaluated using Student's *t*-test. Multiple group comparison was done using one-way analysis of variance followed by the Dunnett's test. A probability level of 0.05 was used to establish significance.

III. Results

1. Effects of SbG and baicalein on tBHP-induced apoptosis in OK cells

Cells were exposed to 0.05 mM *t*BHP for 24 hr

and apoptosis was assessed by fluorescent dye staining (Fig. 1A) and TUNEL assay (Fig. 1B). The results indicated that cells treated with *t*BHP exhibited DNA fragmentation, typical morphological features of apoptosis. Such changes were markedly prevented by SbG (0.1mg/ml). Quantitative analysis of apoptosis by Hoechst staining demonstrated that *t*BHP caused approximately 37% of cells underwent apoptotic cell death and its effect was markedly prevented by SbG and baicalein (2 μM) (Fig. 1C).

We examined the effect of SbG and baicalein on cell viability in cells exposed to various concentrations of *t*BHP. As shown in Fig. 2, *t*BHP caused the loss of cell viability over concentration range of 0.005-0.1 mM in a dose-dependent manner. Exposure of cells to *t*BHP in the presence of 0.1 mg/ml SbG or 2 μM baicalein produced a significant increase in cell viability.

In order to determine the potency of the protective effect of SbG and baicalein, the cells were exposed to *t*BHP in the presence of various concentrations of SbG and baicalein. As shown in Fig. 3, SbG prevented *t*BHP-induced cell death over concentration range of 0.08-0.2 mg/ml. Similarly,

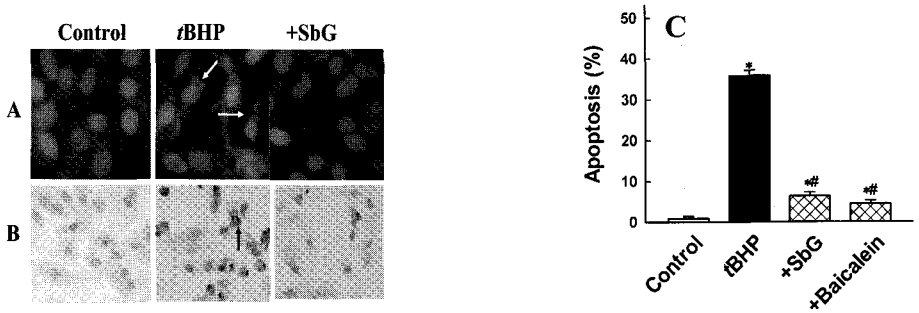

Fig. 1. Hoechst 33258 Staining (*A*) and TUNEL Assay (*B*) in Ospossum Kidney Cells treated with *t*-butylhydroperoxide (*t*BHP). Cells were treated with 50 M *t*BHP at 37℃ for 24 hr in the presence or absence of 0.1 mg/ml *Scutellaria baicalensis* Georgi extract (SbG) and 2 μM baicalein. *C*, Quatitation of apoptotic cells was estimated by Hoechst staining. Data are mean±SE of three experiments.

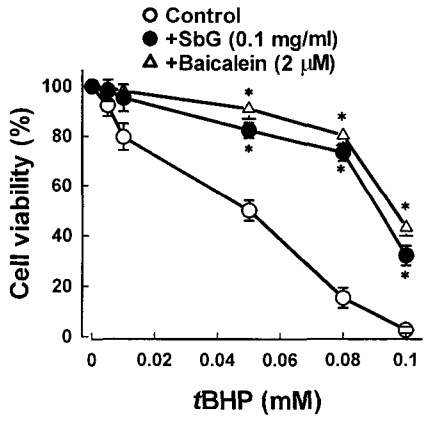

Fig. 2. Effects of *Scutellaria baicalensis* Georgi Extract (SbG) and Baicalein on *t*BHP- induced Cell Death in Ospossum Kidney Cells. Cells were treated with various concentrations of *t*BHP at 37℃ for 24 hr in the presence or absence of 0.1 mg/ml *Scutellaria baicalensis* Georgi extract (SbG) and 2 μM baicalein. Data are mean±SE of four experiments. *p<0.05 vs. *t*BHP alone.

baicalein also blocked the cell death in a dose-dependent manner over concentration range of 1-5 μM. However, SbG at 0.5 and 1 mg/ml and baicalein at 10 μM decreased cell viability, indicating that these agents resulted in cell death at higher concentrations.

2. Role of lipid peroxidation in tBHP-induced apoptosis

In order to clarify if lipid peroxidation is involved in *t*BHP-induced apoptosis, the effect of well-known antioxidants on *t*BHP-induced apoptotic cell death was examined. *t*BHP-induced cell death was significantly prevented by Trolox and NAC (Fig. 4),

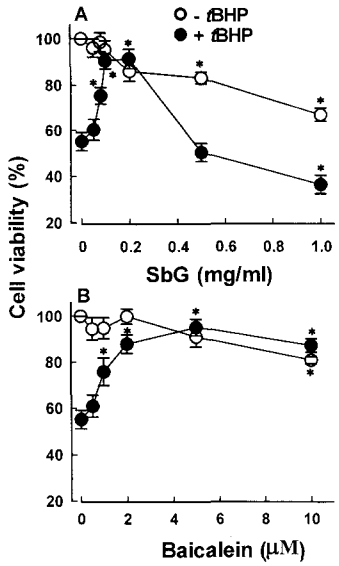

Fig. 3. Dose-dependency of Protective Effect of *Scutellaria baicalensis* Georgi Extract (SbG) (*A*) and Baicalein (*B*) on *t*BHP-induced Cell Death in Ospossum Kidney Cells. Cells were pretreated with various concentrations of SbG and baicalein for 30 min and incubated in medium with or without 50 M *t*BHP at 37℃ for 24 hr. Data are mean±SE of four experiments. *p<0.05 vs. -SbG.

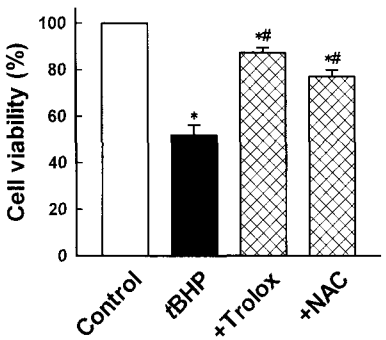

Fig. 4. Effects of Antioxidants on *t*BHP-induced Cell Death in Ospossum Kidney Cells. Cells were treated with 50 M *t*BHP at 37℃ for 24 hr in the presence or absence of 1 mM Trolox and 2 mM N-acetylcysteine (NAC). Data are mean±SE of four experiments. *p<0.05 vs. control; #p<0.05 vs. *t*BHP alone.

indicating that *t*BHP-induced apoptosis is associated with lipid peroxidation.

3. Role of MAPK signaling pathways in tBHP-induced apoptosis

Role of MAPK signaling pathways in *t*BHP-induced apoptosis was investigated by measuring activation of each MAPK subfamily using a Western blotting analysis. A time-dependent activation of ERK was observed as illustrated in Fig. 5A. ERK activation increased above base-line level at 1 hr after exposure to 50 M *t*BHP and peaked after 12 hr of exposure. The ERK activation during exposure to *t*BHP was attributed to an increase in kinase activity and not secondary to elevation of ERK protein levels, as Western blot analysis of ERK proteins showed no significant changes in protein levels when ERK activity was increased. By contrast, expression levels of phospholyated forms of JNK and p38 were not evident in cells exposed to *t*BHP (data not shown).

The *t*BHP-induced ERK activation was completely inhibited by U0126, a specific inhibitor of MEK1/2 (the immediate upstream kinases of ERK1/2) and DFO, an iron chelator. By contrast, Trolox, SbG and baicalein were not effective in

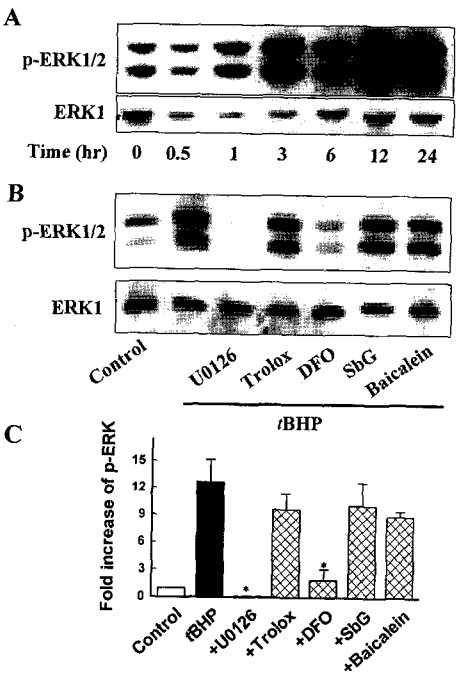

Fig. 5. *A*, Time-dependent Activation of Extracellular Signal-regulated Kinase (ERK1/2) in Cells Treated with *t*BHP in Opossum Kidney Cells. Cells were treated with 50 M *t*BHP for various times and protein levels of phosphorylated (active) ERK1/2 and total ERK1 were measured by Western blotting with anti-phospho-ERK1/2 and anti-ERK1 antibodies, respectively.
B, Effects of Various Compounds on *t*BHP-induced ERK Activation. Cells were treated with 50 M *t*BHP for 12 hr and protein levels of phosphorylated (active) ERK1/2 and total ERK1 were measured by Western blotting with anti-phospho-ERK1/2 and anti-ERK1 antibodies, respectively. Cells were pretreated with 10 M U0126, 1 mM Trolox, 0.2 mM deferoxamine (DFO), 0.1 mg/ml *Scutellaria baicalensis* Georgi extract (SbG), and 2 μM baicalein.
C, fold increases in p-ERK determined by densitometry and calculated as the ratio of treated cells to untreated cells. Data are mean±SE of four experiments. *p<0.05 vs. *t*BHP alone.

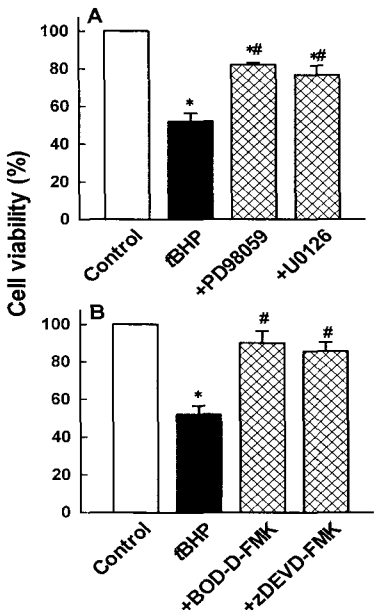

Fig. 6. Effects of ERK Inhibitors (*A*) and Caspase Inhibitors (*B*) on *t*BHP Induced Cell Death in Ospossum Kidney Cells. Cells were treated with 50 M *t*BHP at 37℃ for 24 hr in the presence or absence of 20 M PD98059, 10 M U0126, 20 M BOD-D-FMK, and 20 M zDEVD-FMK. Data are mean±SE of five experiments. *p<0.05 vs. control; #p<0.05 vs. *t*BHP alone.

inhibiting the ERK activation (Fig. 5B and C). Pretreatment of DFO prevented *t*BHP-induced cell death (86.93 ± 0.88 vs. 47.09 ± 3.65% for *t*BHP alone).

To determine if ERK activation is involved in *t*BHP-induced apoptosis, effect of inhibitors of ERK activation on cell viability in cells exposed to *t*BHP was examined. Exposure of cells to *t*BHP caused loss of cell viability, which was significantly prevented by PD98059 and U0126, inhibitors of ERK activation (Fig. 6A).

4. Role of caspase activation in tBHP-induced apoptosis

Although a family of cysteine proteases, the caspases, plays a central role in the initiation and execution of apoptosis induced by various stimuli[22], the signaling pathways mediating apoptosis and the role of caspase activation in oxidant-induced apoptosis are less well defined. To evaluate the role of caspase activation in *t*BHP-induced apoptosis, effect of caspase inhibitors on cell viability was examined. As shown in Fig. 6B, *t*BHP-induced loss of cell viability was prevented by BOD-D-FMK, a general caspase inhibitor, and zDEVD-FMK, a caspase-3 inhibitor. These data suggest that caspase activation plays is an important role in *t*BHP-induced apoptosis.

Ⅳ. Discussion

In the present study, SbG prevented apoptosis caused induced by *t*BHP at 0.1 mg/ml (Fig. 1). SbG attenuated *t*BHP-induced cell death as estimated by trypan blue exclusion assay over concentrations of 0.8-0.2 mg/ml, but it did not affect cell death at 0.5

mg/ml (Fig. 3). Similarly to SbG, baicalein also prevented apoptosis and loss of cell viability caused by *t*BHP in a dose-dependent manner (Figs. 1-3). SbG itself at higher conentrations (0.5 and 1 mg/ml) resulted in loss of cell viability. Exposure of control cells to 10 μM baicalein also caused a reduction in cell viability to approximately 14%. These data suggest that SbG and baicalein exert anti-apoptotic effect at lower concentrations and pro-apoptotic effect at higher concentrations. Induction of apoptosis by SbG and baicalein has been reported in several cell types[23,24]. Although the underlying mechanism of cell death induced by higher concentrations of SbG and baicalein in the present study remains to be defined, apoptotic action of baicalein has been reported to be related to the decreased levels of anti-apoptotic protein Bcl-2 and increased levels of pro-apoptotic protein bax in breast cancer cells[24].

Since the cytoprotective effects of flavonoids have been reported to be associated with antioxidant capacity[10], the effect of SbG on *t*BHP-induced apoptosis may be attributed to its antioxidant action. This possibility may be supported by the data that antioxidants prevented *t*BHP-induced cell death (Fig. 4), suggesting that *t*BHP induces apoptosis through a lipid peroxidation-dependent mechanism.

Oxidants can trigger the activation of MAPK signaling pathways[25]. The role of individual membranes of the MAPK family in induction of apoptosis remains incompletely defined and is likely to vary from one cell type to another[26]. H_2O_2 induces JNK activation associated with apoptosis induced by H_2O_2 in various cell types[18,19,27]. Role of ERK activation in H_2O_2-induced apoptosis is controversial. ERK activation inhibits H_2O_2-induced apoptosis in HeLa[28] and primary cultured rat renal tubular cells[29], but other investigators have shown that ERK activation promotes apoptosis caused by H_2O_2 in mesangial[18,27] and renal proximal tubular epithelial cells (LLC-PK1)[30]. In the present study, we observed that *t*BHP induced ERK activation (Fig. 5) and pretreatment of cells with ERK inhibitors protected cell from the apoptotic cell death (Fig. 6), suggesting that ERK activation plays an important role in H_2O_2-induced apoptosis of OK cells.

Since flavonoids have been known to inhibit various enzymes including tyrosine protein kinases and serine/threonine proein kinases[31] and protein kinase C[10]. The anti-apoptotic effect of SbG would be attributed to inhibition of ERK activation. Indeed, baicalein has been reported to inhibit MAPK signaling pathways induced by various stimuli in nonrenal cells[32-34]. However, the present study showed that SbG and baicalein did not produce significant effect on *t*BHP-induced ERK activation (Fig. 5B and C). These results suggest that anti-apoptotic effects of SbG and baicalein are not due to inhibition of MAPK signaling pathways. Recently, it has been reported that baicalein inhibits apoptosis caused by lipopolysaccharide in brain microglia[35] and by TGF-1 in human hepatoma cells[36] through modulation of NF-B activation. Baicalein has also been shown to modulate cytochrome c release from mitochondria, the levels of anti-apoptotic proteins Bcl-2 and Mcl-1, and the levels of pro-apoptotic protein bax[24]. Therefore, the action site of SbG and baicalein may be the downstream of MAPK signaling pathways. Since apoptosis caused by *t*BHP was dependent on caspase activation as evidenced by significant protective effects of caspase inhibitors (Fig. 6B), SbG may attenuate apoptosis through inhibition of caspase activation.

Although the antioxidant Trolox exerted significant protective effects against *t*BHP-induced

cell death, the ERK activation by *t*BHP was not affected by the antioxidant (Fig. 5B). These data suggest that *t*BHP causes the ERK activation through a lipid peroxidation-independent mechanism. *t*BHP may induce apoptosis via two signaling pathways: ERK-dependent and lipid peroxidation-dependent mechanisms.

Previous studies have demonstrated that flavonoids have iron chelating peroperties[37]. *t*BHP also causes cell death through an iron-dependent mechanism in renal epithelial cells[38]. In the present study, the iron chelator DFO inhibited apoptotic cell death and ERK activation induced by *t*BHP, unlike SbG (Fig. 5B), suggesting that the anti-apoptotic effect of SbG is not attributed to its iron chelating activity.

In conclusion, the present study demonstrated that SbG and its major flavonoid constituent baicalein prevented apoptotic cell death induced by *t*BHP. ERK activation is involved in *t*BHP-induced apoptosis. However, their anti-apoptotic effects were not attributed to inhibition of ERK activation. Although antioxidants prevented *t*BHP-induced apoptosis, the ERK activation was not affected by the antioxidants. These results indicate that SbG has the potential for inhibiting apoptotic death of OK cells and may exert anti-apoptotic effects through a mechanism other than inhibition of MAPK signaling pathways. Although the precise mechanism of anti-apoptosis of SbG remains to be explored, the results of the present study provide information that SbG may be useful in treatment and prevention of various renal diseases mediated by oxidants.

Ⅴ. Conclusion

This study was performed to evaluate the effects of *Scutellaria balicalensis* Georgi extract (SbG) and its major flavonoid constituent baicalein on oxidant-induced apoptotic cell death in renal epithelial cells using opossum kidney (OK) cells, an established renal proximal epithelial cell line. The organic hydroperoxide *t*-butylhydroperoxide (*t*BHP) was employed as a model oxidant.

*t*BHP resulted in apoptotic cell death in a dose-dependent manner. SbG and baicalein prevented *t*BHP-induced cell death in a dose-dependent fasion at lower concentrations, but they caused cell death at higher concentrations. *t*BHP-induced cell death was prevented by antioxidants Trolox and N-acetylcysteine and an iron chelator defroxamine, suggesting that *t*BHP induced apoptosis through a lipid peroxidation-dependent mechanism. Western blot analysis showed that *t*BHP induced activation of extracellular signal-regulated kinase (ERK), which was inhibited by an ERK inhibitor U0126 and an iron chelator deferoxamine. However, Trolox, SbG and baicalein had little effect. The ERK inhibitors PD98059 and U0126 prevented *t*BHP-induced cell death. *t*BHP-induced cell death was prevented by caspase inhibitors.

These data suggest that SbG prevented apoptotic cell death induced by *t*BHP and may exert the anti-apoptotic effects through a mechanism other rather than the inhibition of MAPK signaling pathways, antioxidant action, and iron chelator activity. The present study suggests that SbG may be employed as a useful candidate herb for drug development to prevent and treat oxidant-mediated renal failure.

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