Thesis for the Degree of Master of Science

Cytoprotective effect of phlorofucofuroeckol-A on UVB-induced oxidative stress in mouse epidermal JB6 cells

By

JI-Young Park

Department of Food and Life Science

The Graduate School

Pukyong National University

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UVB로 산화적 스트레스를 유도한 JB6 세포에 대한 PFF-A의 세포보호효과

Advisor: Prof. Hyeung-rak Kim

By
Ji-Young Park

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By
Ji-Young Park

Approved by:

_____
Eun-Soon Lyu(chairman)

_____
Hyeung-Rak Kim (member)

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Taek-Jung Nam (member)

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박 지 영

부경대학교 대학원 식품생명과학과

요약

피부는 태양광선에 노출되어 있어 자외선에 의한 표적기관이 될 수 있다. 태양광선이 포함하는 여러 가지 전자기파 중에 지표에 도달하여 피부에 생물학적인 영향을 가장 많이 주는 광선은 자외선이다. 특히 UV는 일광화상, 피부노화, 피부암 등의 생물학적 변화를 일으키는 가장 중요한 역할을 하는 파장으로 알려져 있다. UVB 조사에 의해 발생되는 reactive oxygen species (ROS)는 지질과 단백질 그리고 DNA를 산화시키는 원인으로 다양한 피부 질병과 관련이 있다. 본 연구에서는 UVB 조사에 의하여 JB6 세포에 산화적 스트레스를 유도한 후 이에 관련된 mitogen-activated protein kinase (MAPK)의 활성화에 대한 phlorofucofuroeckol-A (PFF-A)의 세포보호효과를 분석하였다. PFF-A를 JB6 세포에 처리한 결과 UVB 조사에 의해 생성된 free radical을 효과적으로 제거하였다. PFF-A를 JB6 세포에 처리한 결과 UVB조사에 의해 활성화된 MAPK family인 ERK1/2, JNK, 그리고 p38 단백질의 인산화를 억제시켰다. 그리고 UVB 조사 전 PFF-A의 처리는 항산화 또는 해독화 전사인자인 NF-E2 related factor-2 (Nrf2)를 활성화시켜 catalase, superoxide dismutase 및 heme oxygenase-1 (HO-1) 전사를 활성화시켰다. 따라서 PFF-A 처리에 의하여 항산화 효소의 발현을 조절하는 전사인자의 활성화로 인하여 항산화 효소의 발현을 증가시킴을 알 수 있었다.

1. Introduction

Well-established epidemiological evidences indicate that ultraviolet (UV) radiation of the sunlight is the major environmental carcinogen responsible for the development of skin cancer (Armstrong et al., 2001). UV radiation has proved to be a potent carcinogen as it can induce skin cancer in the absence of any other potential tumor promoter. The process of skin cancer induction can be divided into three overlapping stages: initiation, promotion, and progression of tumors (Digiovanni, 1992). Initiation of skin cancer by UV involves the induction of an irreversible DNA damage and formation of photoproducts in critical genes. If this damage remains unrepaired, it may result in the mutation of proto-oncogenes and tumor suppressor genes. Chronic exposure to UV light and clonal expansion of the initiated cells leads to tumor promotion and the development of a benign tumor. It has been documented that UV radiation-induced skin tumor promotion is closely associated with alterations in the induced signal transduction pathways, including the activation of mitogen-activated protein kinases (MAPKs) that lead eventually to the transcription of specific set of genes as part of a general UV response (Bode et al., 2003). Progression of skin cancer entails the transformation of the benign tumors into malignant skin cancers (Madronich et al., 1998).

UV irradiation is potent inducer of reactive oxygen species, which have been implicated in cutaneous aging as well as in skin cancer and various cutaneous inflammatory disorders (Devary et al., 1992; Bender et al., 1997). UV irradiation have been reported to upregulate expression of genes such as c-fos and c-jun (Karin, 1995; Su et al., 1996). Transcription of many genes is mediated by the sequential activation of cytoplasmic protein kinases, and the mitogen-activated protein kinase (MAPK) plays a major role in triggering and coordinating these gene response (Cobb et al., 1995). MAPKs, a group of serine/threonine-specific, proline-directed protein kinases are known to modulate transcription factor activities. Three structurally related but biochemically and functionally distinct MAPK signal transduction pathways have been identified and include the extracellular signal regulated kinases (ERK), c-jun N-terminal kinases (JNK), and p38 (Guyton et al., 1996). Transient activation of ERK is

responsible for proliferation and differentiation (Chen et al., 1996) and has also been shown to be involved in tumor promotion processes especially stimulated by the oxidant state (Ip et al., 1998). Stimulation of JNK and p38 can mediate differentiation, inflammatory response, and cell death (Robinson et al., 1997; Wang et al., 1998; Wilmer et al., 1997). There is evidence that antioxidants can attenuate MAPK activation (Kensler et al., 1997; Chen et al., 2004).

UV radiation cause activation of the phosphatidylinositol 3-kinase (PI3K) pathway as well as the activation of tyrosine receptors and Ras (Kabuyama et al., 1998; Whitman et al., 1998). A family of PI3K enzymes phosphorylates the number 3 position of the inositol ring of some different phosphoinositides (Cantley et al., 1991). Among the phosphoinositides, phosphatidylinositol diphosphate is believed to be the preferred substrate in vivo generating the second messenger phosphatidylinositol (3,4,5) triphosphate (Stephens et al., 1991; Carpenter et al., 1996). PI3K plays a central role in a broad range of biological effects such as cell growth, apoptosis, intercellular vesicle trafficking/secretion, regulation of actin, cell migration, and integrin function (Keely et al., 1997; Stambolic et al., 1999). In addition, accumulating evidence suggests the importance of PI3K signaling in carcinogenesis (Krasilnikov et al., 2000; Sugimoto et al., 1984). Initially PI3K was a subject of interest because of its known ability to form complexes with some viral oncoproteins (Macara et al., 1984; Klippel et al., 1998) and also because of its involvement in the viral transformation process (Shayesteh et al., 1998). The oncogenic transformed phenotype was observed in mammalian fibroblasts transfected with the constitutively active 110a (Phillips et al., 1998). In fact, alterations or amplification of PI3K have been detected in a number of human malignances (Marte et al., 1997; Dudek et al., 1997). UV irradiation induces the activation of Akt. In a wide range of cellular systems, Akt has been shown to control intracellular pathways responsible for preventing cell death in response to a variety of extracellular stimuli (Kulik et al., 1997; Medema et al., 2000; Muise-Helmericks et al., 1998). Furthermore, several reports has shown that Akt is not only a “cell survival” kinase but it may play an important role in protein synthesis, glycogenesis, and regulation of cell cycle progression (Bellacosa et al., 1991; Cerutti, 1994). In contrast, identification of the gene encoding Akt as a transforming oncogene that causes thymic lymphomas in mice suggests a role for Akt in tumorigenesis (Darr et al., 1994).

Nrf2, NF-E2-related factor 2 is sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Kelch-like ECH associated protein 1 (Keap1). Dissociation of Nrf2 from the inhibitory protein Keap1 is a prerequisite for nuclear translocation and subsequent DNA binding of Nrf2. After forming a heterodimer with small Maf protein inside the nucleus, the active Nrf2 binds to cis-acting ARE or EpRE, also alternatively known as Maf recognition element (Lee et al., 2005a). Besides the dissociation of the Nrf2-Keap1 complex that is facilitated by covalent modification or oxidation of critical cysteine residues contained in Keap1 may facilitate the dissociation of Keap1-Nrf2 complex or increase the stability of Nrf2. Cysteine residues present in Keap1 serve as a molecular sensor for recognizing the altered intracellular redox-status triggered by electrophiles or ROS (Lee et al., 2005b). Besides the direct oxidation or covalent modification of thiol groups contained in Keap1, the Nrf2-Keap1 signaling can be modulated directly by post-transcriptional modification of Nrf2. Phosphorylation of Nrf2 on its serine and threonine residues by several kinases such as protein kinase C (PKC), phosphoinositol 3-kinase (PI3K), and mitogen activated protein kinases (MAPKs) has been demonstated to activate Nrf2 (Owuor et al., 2002).

Upon nuclear translocation, Nrf2 not only binds to the specific consensus *cis*-element called ARE or EpRE present in the promoter region of genes encoding many antioxidant enzymes but also to other *trans*-acting factors such as small Maf-F/G/K as well as the coactivators of ARE including cAMP response element binding protein(CREB)-binding protein (CBP)/p300 that can coordinately regulate the ARE-driven antioxidant gene transcription. AREs have been found in the 5'-flanking region of many genes involved with cytoprotection from oxidative stress, such as catalase, superoxide dismutase (SOD), heme oxygenase 1 (HO-1), and glutathion (Jaiswal, 2004; Juan et al., 2005).

HO-1 is the inducible form of three isozymes of heme oxygenase, a microsomal enzyme which catalyzes the rate-limiting step in heme catabolism (Tyrrell, 1991). Three isoforms of HO that are the products of separate genes have been identified in mammals (Maines et al., 1997; Tenhunen et al., 1969; Yoshidat et al., 1978). HO-1 is transcriptionally upregulated as a sensitive anti-inflammatory protein by various types of oxidative stress, such as oxidized LDL (Maines et al., 1974.), UV radiation (McCoubrey et al., 1994), thiol scavengers (Shibahara et al., 1993), and hypoxia

(McCoubrey et al., 1997; Wang et al., 1998), as well as substrate heme (Keyse et al., 1998) in the cardiovascular system. A common feature of these HO-1 inducers is their ability to regulate the intracellular redox state. HO-1 is also transcriptionally activated through several regulatory mechanisms. Studies on the promoter region of HO-1 have revealed transcriptionally responsive elements, including activator protein I, activator protein II, nuclear factor-kB, interleukin-6-responsive elements, and an antioxidant response element (ARE) (Maines et al., 1997; Lee et al., 1997; Morita et al., 1995). HO-2 is constitutively expressed in many organs throughout the body, although it is particularly high in the brain and testes, but is unresponsive to any of the inducers of HO-1 (Shibahara et al., 1979). The third isoform, HO-3, is nearly devoid of catalytic activity (Alam et al., 1992), However, Hayashi and colleagues (Hayashi et al., 2004) recently reported that HO-3 is a pseudo gene derived from HO-2 transcripts.
Moreover, HO-1 is a sensitive marker for oxidative stress induced by the substate heme itself, as well as a wide variety of cellular stressors including reactive oxygen species such as hydrogen peroxide, hydroxyl radical, nitric oxide, and $1O_2$ (Maines et al., 1986; Applegate et al., 1991). In dermal fibroblasts, photochemically generated $1O_2$ seems to be the main effector species for UVA-mediated HO-1 up-regulation (Basu-Modak et al., 1993).

Ecklonia stolonifera OKAMURA (Fig. 1) is a perennial brown alga, belonging to the family Laminariaceae. It is abundant in the Southern coast of Korea and Japan. It is popular as a health food and is occasionally used as a gynecopathy in Japan (Sugiura et al., 2006). Several compounds from *Ecklonia* species exhibits radical scavenging activity (Kang et al., 2003), anti-plasmin inhibiting activity (Fukuyama et al., 1990), anti-mutagenic activity (Han et al., 2000), tyrosinase inhibitory activity (Park et al., 2003). Phlorotinnins which is oligomeric polyphenol of phloroglucinol units are responsible for the biological activities of *Ecklonia*.

Mouse keratinocyte JB6 cells are well stuited to study tumor promotion, as these cells are sensitive to tumor promoter-mediated cell transformation and promotion (Suzukawa et al., 2002). In this study, the cytoprotective effect of PFF-A on the UVB-induced oxidative stress was evaluated on the activation of Nrf2 transcription factor and the activations of its up-stream proteins and expression of its down-stream proteins.

2. Materials and methods

2.1 Materials

MEM (Minimum essential medium), penicillin-streptomycin mixture, 0.25% tripsin-EDTA, fetal bovine serum (FBS) were purchased from HyClone Laboratory Inc. (Logan, UT), sodium pyruvate, non-essential amino acid were purchased from Gibco BRL (Gaithersburg, MD), Celltiter 96 AQ one solution Cell Proliferation Assay kit was purchased from Promeaga (San Luis Obispo, CA) and DMSO (dimethyl sulfoxide) were purchased from Sigma Chemical (St. Louis, MO). Culture dish, 6-well plate, 96-well plate, centrifuge tube and scrapper were purchased from Corning Incorporated (Corning, NY), Protein standard marker was purchased from Amersharm Pharmacia (Piscataway, NJ) and the enhanced chemiluminescence (ECL) detection kit was purchased from Perkin Elmer Life Science (Wellesley, MA). Polyclonal antibodies against PI3K, pAkt, Actin, pERK1/2, pJNK, pp38, HO-1, SOD, catalase, Nrf2, β-tubulin and secondary antibodies, horse radish peroxidase conjugated anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Methods

2.2.1 DPPH Radical Scavenging Assay

DPPH radical scavenging activities of PFF-A were tested according to the modified method of Nanjo et al. Twenty microtiter of sample solution (or DMSO as vehicle) were added to 40 ul of DPPH (100 uM) in a 96-well microtiter plate were mixed and incubated at room temperature for 30 min. The absorbance at 520 nm was measured with microplate reader (Ultraspec 2100 Pro, Amersham Biosciences, Piscataway, NJ). EC_{50} values mean effective concentration to exert half of antioxidant and were calculated by three different concentrations of sample.

2.2.2 Cell Culture

Mouse epidermal keratinocyte JB6 cells (ATCC 2010, Parklawn, MD) were maintained in MEM containing 2.0 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin in 0.85% saline) and 5% heat-inactivated FBS at 37℃ in 95% air-5% CO_2.

2.2.3 UVB Irradiation

JB6 cells were grown to 80% confluence and then starved in 0.1% fetal bovine serum in MEM at 37°C in a 5% CO_2 incubator. After 48 hours of starvation, the cells were pretreated with PFF-A for 6 hours or as indicated in the figures or DMSO alone. Before UVB irradiation, the medium was removed from culture plates; cells were washed with PBS twice and then covered with a thin layer of PBS followed by UVB irradiation. Control cultures were identically processed but not irradiated. The UVB light source was equipped with a UVB meter. The strength of UVB was monitored by a UVB meter (Dr. Hönle GmbH, D-82152 planegg) transmitting wavelengths of 315-380 nm.

2.2.4 MTS assay

Cell viability was determined by MTS assay using Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, WI, USA) according to the manufacturer's manual. JB6 cells ($1x10^5$ cells/well) were seeded into 96-well plate and cultured for 12 h. Cells starved with medium for 12 h were treated with indicated doses of PFF-A. Thereafter, cells were exposed to 400 mJ/cm^2 dose of UVB. The culture medium was removed and replaced by 95 ul of fresh culture medium and 5 ul of MTS solution. After 1 h, the absorbance was measured using micoplate reader (Ultraspec R 2100pro, Amersham Biosciences, Piscataway, NJ) at 490 nm.

2.2.5 Measurement of Intracellular ROS

The intracellular ROS scavenging activity of the PFF-A was measured using the oxidant-sensitive fluorescent probe DCFH-DA.

DCFH converted from DCFH-DA by deacetylase within the cells is oxidized by a variety of intracellular ROS to DCF, a highly fluorescent compound. The cells were incubated with 0-20 uM of PFF-A in the absence or presence of UVB for 30 min. Cells were harvested by trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in PBS) and washed with PBS twice. The cells were treated with 20 uM DCFH-DA for 30 min at 37 ^{o}C. The fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 528 nm using fluorescence microplate reader (Dual Scanning SPECTRA max, Molecular Devices Corporation, Sunnyvale, CA).

2.2.6 Western Immunoblot

JB6 cells were washed twice with ice-cold PBS, lysed with a buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet-40, 1% Tween-20, 0.1% SDS, 10 ug/mL leupeptin, 50 mM NaF, 1 mM PMSF) on ice for 1 h. After centrifuge at 18, 000 g for 10 min, protein content of supernatant was measured, and aliquots (20 μg) of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk in TBST buffer for 1 h and incubated for 2 h with primary antibody in TBST buffer containing 5% non-fat dried milk. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST buffer containing 5% non-fat dried milk for 1 h, and immune complex was detected using ECL detection kit.

2.2.6.1 Preparation of Cytosolic and Nuclear Extracts

Nuclear extracts were prepared as described previously (Nanjo et al., 1996). Cell were washed twice with PBS and harvested by centrifugation (1,200 rpm for 5 min). Cell pellets were resuspended in hypotonic buffer (10 mM HEPES/KOH, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), stood on ice for 15 min, vortexed, and centrifuged at 13,000 rpm for 1 min. The nuclear pellet were gently resuspended in 20 μl of ice cold saline buffer (50 mM Hepes/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, and

0.5 mM PMSF, pH7.9), left on ice for 30 min, vortexed, and centrifuged at 13,000 rpm for 20 min at 4^{o}C.

2.2.7 Statistical analysis

All data are expressed as the mean ± SEM. Data were analyzed using one-way ANOVA. Differences were considered significant of $P < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL).

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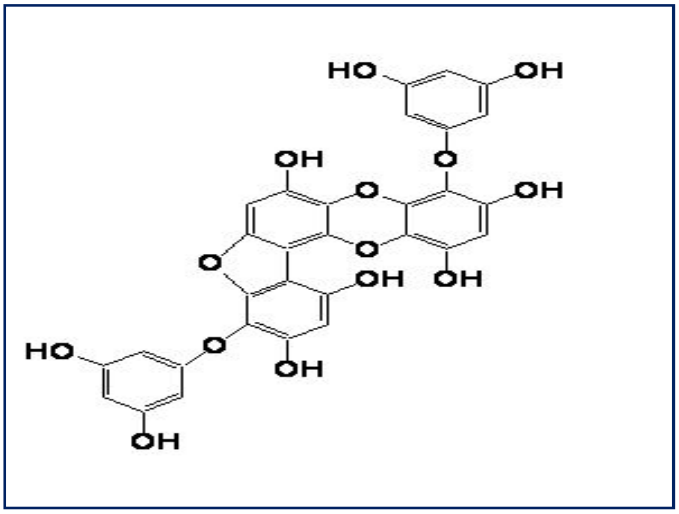

Figure 1. Chemical structure of PFF-A from *E. stolonifera.*

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3. Results

3.1 Effects of PFF-A on DPPH radical scavenging activity

The isolated phlorotannins from *E. stolonifera* were assayed for their antioxidant activity by measuring their ability to scavenge free radicals with DPPH. The antioxidant capacities of the isolated compounds were compared with those shown by a known antioxidant, L-ascorbic acid, treated in the same assay. As shown in Table 1, EC_{50} values for PFF-A was estimated to be 4.7 μM, which is lower than L-ascorbic acid as positive control.

Phlorotannins isolated from showed DPPH-radical scavenging activities and the EC_{50} values of the eckol, dieckol and 8'8'-bieckol were determined to be 26, 13 and 15 μM, respectively. (Toshiyuki et al., 2008) In contrast, those of catechin, EGCG and α-tocopherol were 32, 7.4 and 52 μM, respectively. (Toshiyuki et al., 2008)

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Table 1. DPPH radical scavenging activity of PFF-A

Compounds	EC_{50} (μM± SD)
Phlorofucofuroeckol A	4.7 ± 0.3
Dieckol	6.2 ± 0.4
Dioxinodehydroeckol	8.8 ± 0.4
L-ascorbic acid	10.3 ± 0.5

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3.2 Effect of PFF-A on UVB-induced JB6 cells

To examine cytoprotective effect of PFF-A on UVB-induced JB6 cells, cell viability was evaluated by MTS assay. Pretreatment of JB6 cells with PFF-A (5 to 40 μM) before UVB (400 mJ/cm^2) exposure resulted no change of cell viability as shown in Fig. 2.

Treatment with quercetin, red wine extract (REW) and myricetin has no effect on cell viability in TNF-α-induced JB6 cells. Also, treatment with eckol and triphlorethol-A from Ecklonia cava has cytotoxic effect on H_2O_2-induced in V79-4 cells (Hwang et al., 2009; Kim et al., 2009; Kang et al., 2005a; Kang et al., 2005b).

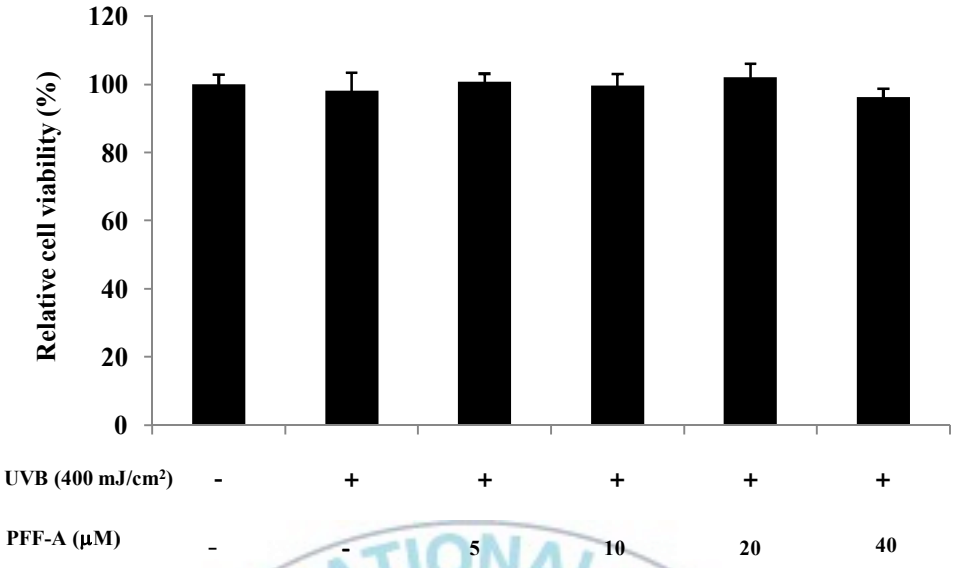

Fig. 2. The Effect of PFF-A on JB6 cell viability. JB6 cells were treated with 5 to 40 μM PFF-A for 12 h before expose with 400 mJ/cm^2 UVB for 12 h, and then MTS solution was added. After 1 h incubation with MTS solution, the absorbance was measured at 490 nm.

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3.3 PFF-A inhibit UVB-induced ROS in JB6 cell

JB6 cell exposed with 400 mJ/cm^2, UVB strength, resulted in induction of ROS productionas shown in Fig. 3, which was caused by UVB-induced oxidative stress. Pretreatment of JB6 cells with PFF-A (10 and 20 μM) before UVB (400 mJ/cm^2) exposure resulted in suppression of UVB-induced ROS production. This data indicated that PFF-A possess antioxidant activity and confirmed that PFF-A have the capability to inhibit UVB radiation-induced oxidant stress in target cells.

UV exposure to the skin results in generation of reactive oxygen species (Inal et al 2001; Thiele et al., 2001). ROS comprise a number of active metabolites including hydroxyl radical, superoxide anion and peroxyl radical and their active precursors namely singlet oxygen, hydrogenperoxide and ozone. Nitric oxide and nitric dioxide, reactive nitrogen species (RNS), are also generated by UV irradiation. Excess of free radicals results in a cascade of events mediating progressive deterioration of cellular structure and function, and this can lead to a loss of cellular integrity by modification of DNA and also to abnormal expression of cellular genes. ROS damage cell membranes by peroxidation of fatty acids within the phospholipid structure of the membrane. During this process, lipid peroxide radicals, lipid hydroperoxides and other fragmentation products, that are themselves active oxidizing agents, are formed. The lipid peroxides are comparatively longer-lived species and can initiate the chain reactions that enhance oxidative damage. Previous study reported that phlorotannins isolated from Ecklonia cave showed antioxidant and cytoprotective effects against oxidative stress : triphlorethol-A and eckol protected cell damage from H_2O_2 and radiation induced oxidative stress via radical quenching effect (Kang et al., 2006; Kang et al., 2005). Also (-)-epigallocatechin-3-gallate (EGCG) and grape seed proanthocyanidins (GSPs) inhibited UVB-induced intracellular production of ROS generation, thus providing a possible mechanism for the photoprotection (Sudheer et al., 2006; Praveen et al., 2003). Therefore, suppression of UVB-induced ROS production by PFF-A treatment would result to

inhibition of the biological changes caused by UVB radiation.

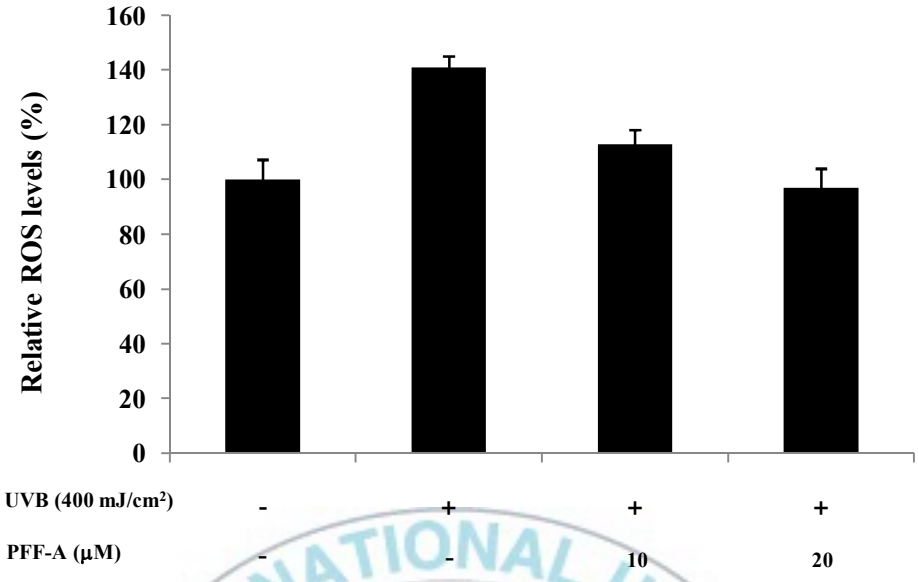

Fig. 3. Treatment of JB6 cell with PFF-A inhibits UVB-induced ROS. Cells at ~80% confluence were serum starved for 12 h, and during the last 1 h of starvation were treated with indicated doses of PFF-A. Thereafter, cells were exposed to 400 mJ/cm^2 dose of UVB ; 30 min thereafter. After 30 min incubation with DCFH-DA solution, the absorbance was measured at 428/528 nm.

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3.4 PFF-A inhibit UVB-induced PI3K and Akt signaling

The effect of the PFF-A on UVB-induced phosphorylation of Akt and PI3K in JB6 cell were determinate by Western bolt. The results showed that pretreatment of cell with 20 μM PFF-A inhibits UVB-induced phosphorylation of Akt and PI3Kas shown in Fig. 4.

A number of studies have demonstrated important roles for AKT as downstream effectors of PI3K. Akt is suggested to be implicated in tumor promotion or carcinogenesis (Coffer et al., 1998; Hemmings, 1997; Cheng et al., 1992). PI3K is an important regulatory protein that is involved in different signaling pathways and in the control of cell growth, survival, and malignant transformation (Macara et al., 1984; Chang et al., 1997; Cheng et al., 1996). PI3K forms complexes with some viral or cellular oncoproteins (Cheng et al., 1992), which have transforming activities, and by itself also has oncogenic activity (Ananthaswamy et al., 1990). The transforming effect of PI3K is suggested to be related to the activation of its downstream effector kinases, including Akt (Coffer et al., 1995; Carpenter et al., 1996). UVB is a crucial risk factor for skin cancer (Keely et al., 1997) and also induces PI3K activation (Stambolic et al., 1999). Exciting developments have implicated PI3K as an important factor in carcinogenesis (Stambolic et al., 1999; Krasilnikov et al., 2000) and suggested to play a critical role in carcinogenesis induced by UVB radiation. Therefore, suppression of PI3K and Akt phosphylation by 20 μM PFF-A treatment in UVB-induced JB6 cells would suggest that PFF-A has preventive activity on the tumor promotion effects in UVB-induced JB6 cells.

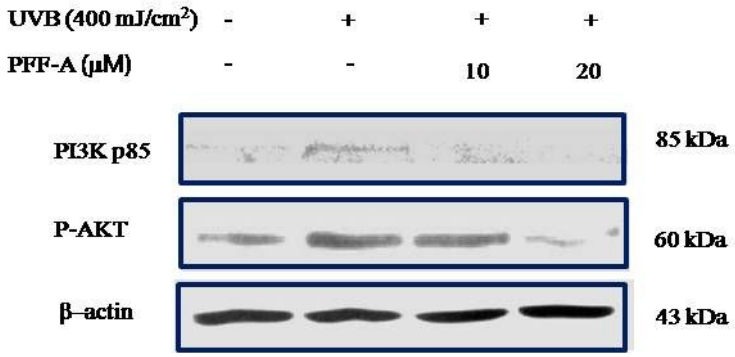

Fig. 4. Effect of PFF-A pretreatment on UVB-caused PI3K and Akt phosphorylation in JB6 cells. Cells at ~80% confluence were serum starved for 48 h, and during the last 6 h of starvation were treated with indicated doses of PFF-A. Thereafter, cells were exposed to 400 mJ/cm^2 dose of UVB ; 30 min thereafter, lysates were prepared and Western blotting was carried out to probe phosphorylated PI3K and Akt.

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3.5 PFF-A inhibits UVB-induced MAPK signaling

The effect of PFF-A (10 and 20 μM) pretreatment on UVB (400 mJ/cm^2)-induced MAPK signaling proteins in JB6 cells were determined with Western blot. UVB strongly upregulated the phosphorylation of ERK1/2, JNK1/2, and p38K as shown in Fig. 5. However, UVB-induced phosphorylation of proteins of the MAPK family, such as ERK1/2, JNK, and p38 in vitro JB6 cell was inhibited by PFF-A treatment. These results suggest that PFF-A inhibits phosphorylation of UVB-induced MAPK in JB6 cells.

MAPKs are important upstream regulators of transcription factor activities and their signaling is critical to the transduction of a wide variety of extracellular stimuli into intracellular events and thus they control the activities of various downstream transcription factors implicated in tumor promotion (Bode et al., 2003). Several studies have shown that UVB irradiation induces the phosphorylation of MAPKs, such as ERK1/2, JNK, and p38, which have been implicated in skin carcinogenesis. The phosphorylation of these proteins has been shown to be mediated through UVB-induced oxidative stress (Gupta et al., 1999). However, their kinetics of activation and inhibition are a little different. ERK1/2 has been shown to be strongly activated by tumor promoters, growth factors, and UV radiation and has a critical role in transmitting signals initiated by them (Cowley et al., 1994). In the present study observed that treatment of JB6 cells with PFF-A inhibits UV-induced activation of ERK1/2. Activation of JNK regulates activator protein-1 (AP-1) transcription in response to environmental stress such as UV radiation (Dunn et al., 2002). Increased AP-1 activity has been implicated in inflammation, metastasis, angiogenesis and also in the promotion and progression of various type of cancers (Chang et al., 2001; McCarty, 1998). This data indicates that treatment of JB6 cells with PFF-A inhibits UVB-induced activation of ERK, JNK and p38 and that inhibition may lead to the inhibition of photocarcinogenesis.

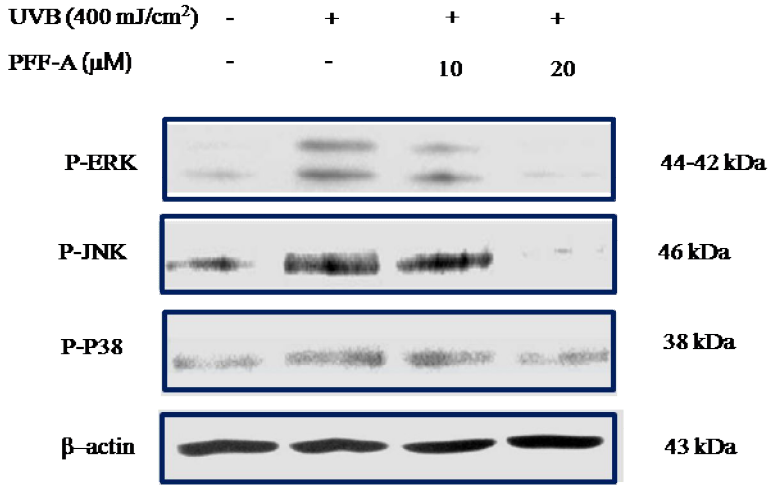

Fig. 5. Effect of PFF-A on UVB-induced MAPK activation. JB6 cells at ~80% confluence were serum starved for 48 h and then either pretreated with PFF-A (10, 20 μM) for 6 h followed by exposure to UVB or exposed to UVB alone. Cells were harvested 30 min thereafter, lysates were prepared, and Western blotting was carried out to probe phosphorylated of MAPKs.

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3.6 Effect of PFF-A on UVB-induced activity of Nrf2

To analyze PFF-A could activate the transcription factor Nrf2, pretreated JB6 cells with different concentration of PFF-A were exposed with 400 mJ/cm^2 UVB. As shown in Fig. 6, treatment of JB6 cells with UVB exhibited increase in Nrf2 expression in cytosol, which might be due to the production of ROS (Fig. 3). However, treatment of JB6 cells with PFF-A induced translocation of Nrf2 into nucleus with dose-dependent manner. This may indicate that Nrf2, upon PFF-A treatment, dissociates form Keap1 protein and subsequently translocate to nucleus.

The transcription factor Nrf2 plays an essential role in the expression of antioxidant enzymes and Phase II detoxifying and in response to oxidative or electrophilic stress (Hayes et al., 2000Chan et al., 1999). The activity of Nrf2 is normally suppressed in the cytosol by specific binding to the chaperone protein Keap1. Keap1 repression of Nrf2 activity is abrogate, Nrf-2 Keap1 complex is dissociated upon stimulation by electrophilic agents, releasing Nrf2 to translocate into the nucleus and potentiate the ARE response (Kobayashi et al., 1999). In addition, other model for activation of Nrf2 includes phosphorylation of serine/threonine residues of Nrf2 by protein kinases, leading to enhanced nuclear accumulation of Nrf2 (Wu et al., 2006; Andreadi et al., 2006). Phosphoryltion has been shown to promote in the release of Nrf2 form Keap1, its nuclear translocation, and its increased stability due to decreased degradation (Huang et al., 2002). It has been reported that flavonoids are the most potent inducers of Nrf2 expression (Lee et al., 2006). The function of Nrf2 and its downstream target genes have been shown to be important for protection against oxidative stress- or chemical-induced cellular damage. Therefore, treatment of 20 μM PFF-A in UVB-induced JB6 cells induced translocation of Nrf2 into nucleus and possibly protected of cell damage through increased production of antioxidant enzymes.

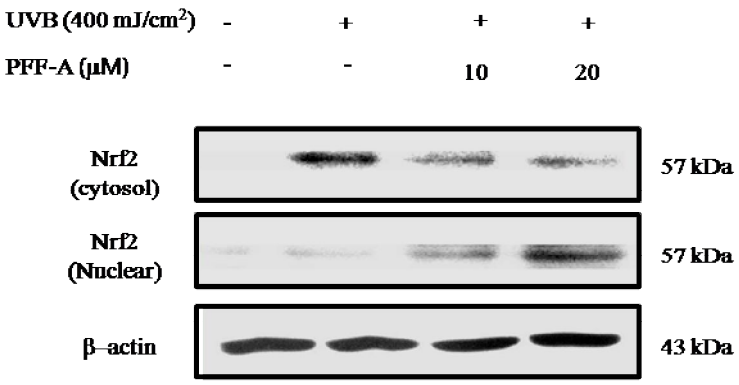

Fig. 6. PFF-A increase on UVB-induced Nrf2 activation. JB6 cells at ~80% confluence were serum starved for 48 h and then either pretreated with PFF-A (10, 20 μM) for 6 h followed by exposure to UVB or exposed to UVB alone. Cells were harvested 1 h thereafter, lysates were prepared, and Western blotting was carried out to probe activity of Nrf2 as mentioned in Materials and Methods.

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3.7 Effect of PFF-A on antioxidant enzyme expression

Since PFF-A treatment induced the activation of Nrf2, the expression of it's down-stream proteins such as HO-1, catalase, and SOD were determined western bolt at various time course. The expression levels of HO-1, catalase, and SOD were increased with time-dependent manner up to 12 h (Fig. 7)

HO-1 can produce the very effective antioxidant bilirubin and stimulate the production of ferritin to reduce the amount of free iron, the stimulation of HO-1 is directly associated with a change in the intracellular ROS levels (Ryter et al., 2002). It is established that UV exposure depletes the antioxidant defense capability of the skin at UV-irradiated site (Katiyar et al., 2001).

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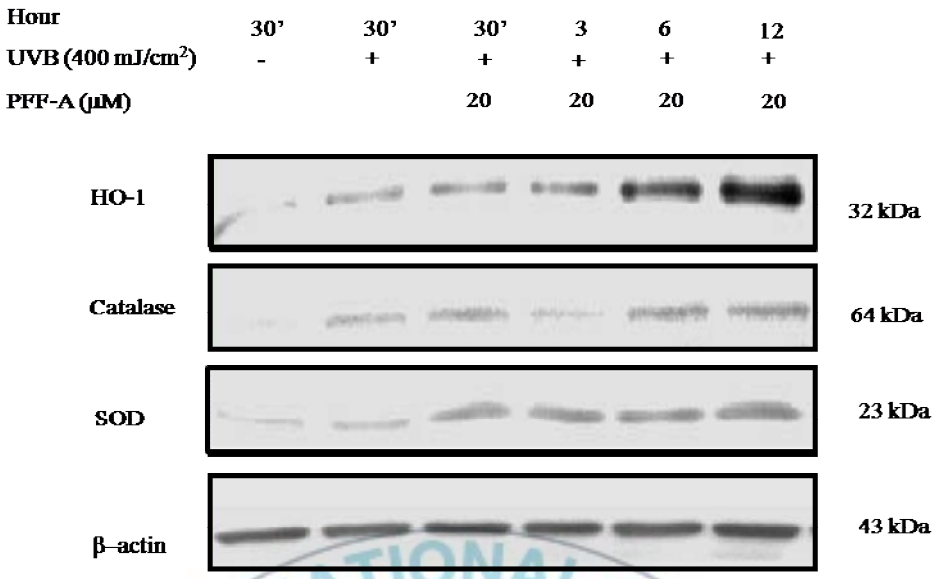

Fig. 7. Effect of antioxidant enzyme expression of PFF-A on time-dependent of UVB irradiation in JB6 cells. Cells at ~80% confluence were serum starved for 48 h and pretreated with DMSO or PFF-A (20 μM) for 6 h and then irradiated with UVB (400 mJ/cm^2). Cells were harvested at the indicated time, lysates were prepared, and Western blotting was carried out to probe protein levels of HO-1, catalase and SOD.

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3.8 PFF-A increase UVB-induced HO-1 protein expression

To determine whether PFF-A induce the expression of HO-1 in UVB-treated JB6 cells, the level of HO-1 expression was analyzed with Western blot. A shown in Fig. 8, pretreatment of JB6 cell with PFF-A before UVB exposure enhanced the HO-1 expression compared to non-treatment of PFF-A in the cells.

Recent research reveals that the HO-1 isoform participates in wound healing, psoriasis, keratinocyte proliferation and, in its role as a heat shock protein, protects against cellular oxidative stress (Chol et al., 1996; Germolec et al., 1996; Clark et al., 1997; Hanselmann et al., 2001). The reaction products of HO-1, biliverdin and bilirubin, are potent cellular antioxidants thereby providing cytoprotection from free radicals (Fang et al., 2004; Ryter et al., 2004; Ryter et al., 2005). UVA produces ROS and upregulates HO-1 in mouse skin, but induction of HO-1 in response to UVA in human keratinocytes is less robust or not detected (Hansselmann et al., 2001; Allanson et al., 2004). In contrast, pronounced induction of HO-1 mRNA is observed in human keratinocytes following exposure to low levels of arsenite (Hamadeh et al., 2002; Rea et al., 2003). Although both UVA and arsenite generate oxidative stress in human keratinocytes (Sander et al., 2004; Shi et al., 2004a; Shi 2004b), the differences reported in HO-1 induction by UVA and arsenite in human keratinocytes suggest involvement of additional regulatory mechanisms.

The cytoprotective properties of antioxidants have been partially attributed to their ability to induce cytoperotective enzymes. Among the various cytoprotective enzymes, HO-1 expression has been considered an adaptive and beneficial response to oxidative stress in a wide variety of cells (Alam et al., 2003; Choi et al., 2003; Jaeschke et al., 2000; Otterbein et al., 2003; Takahashi et al., 2004). This result demonstrated that PFF-A increased HO-1 protein expressions (Fig.8). Increase of HO-1 expression by PFF-A confere conferred cytoprotection against UVB-induced oxidative stress.

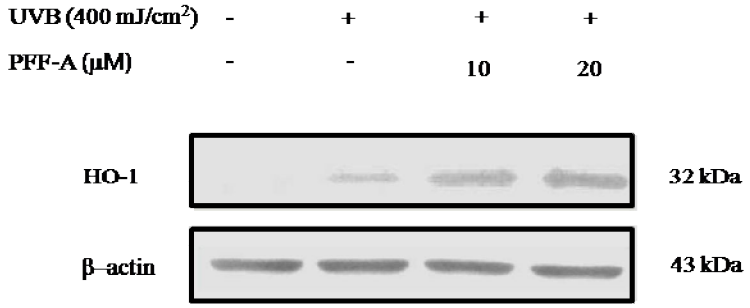

Fig. 8. Effect of PFF-A on UVB-induced expression of HO-1. JB6 cells at ~80% confluence were serum-starved for 48 h and then pretreated with PFF-A (10, 20 μM) for 6 h followed by irradiation with UVB (400 mJ/cm^2) and incubated for another 12 h and then harvested.

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3.9 Effect of PFF-A on UVB-induced depletion of antioxidant enzyme

The treatment of JB6 cells with PFF-A resulted in the prevention of UVB-induced depletion of antioxidant defense enzymes such as catalase and SOD (Fig. 9). Antioxidant enzyme system function cooperatively and any change in one of them may affect the equilibrium state of ROS or oxidative stress. Therefore, prevention of UVB-induced depletion of antioxidant defense enzymes by PFF-A treatment would result in inhibition of the tumor-promoting activity of UV radiation.

It is well recognized that UV exposure depletes the antioxidant defense capability in an in vivo system (Vayalil et al., 2003). By using JB6 cells as an in vitro model the further confirmed that UVB irradiation depletes the levels of endogenous antioxidant defense enzymes (Fig. 9). Catalase is endogenous antioxidant enzyme involved in the catalytic conversion of H_2O_2 to oxygen and water and thus reduces the level of oxidative stress. SOD catalyzes the dismutation of superoxide into oxygen and hydroperoxides, thereby acting as a potent antioxidant. The irradiation of JB6 cells with UVB resulted in decreases of catalase compared to non-UVB-exposed JB6 cells, whereas pretreatment of JB6 cells with PFF-A restored the activities of catalase. Similar to catalase enzyme levels, UVB irradiation of JB6 cells depleted the level of SOD by compared to non-UVB-exposed cells, and pretreatment of JB6 cells with PFF-A slight restored the activity of SOD enzyme, thus indicating photoprotective effects of PFF-A against UVB-induced depletion of antioxidant defense.

This study was undertaken to investigate the effect of PFF-A on molecular events involved in tumor promotion in tumor promoter-sensitive mouse epidermal JB6. Overall, the central finding of the present study is that PFF-A inhibits activation of MAPK and PI3K/Akt and increases Nrf2 and antioxidant enzyme in response to UVB in JB6 cells.

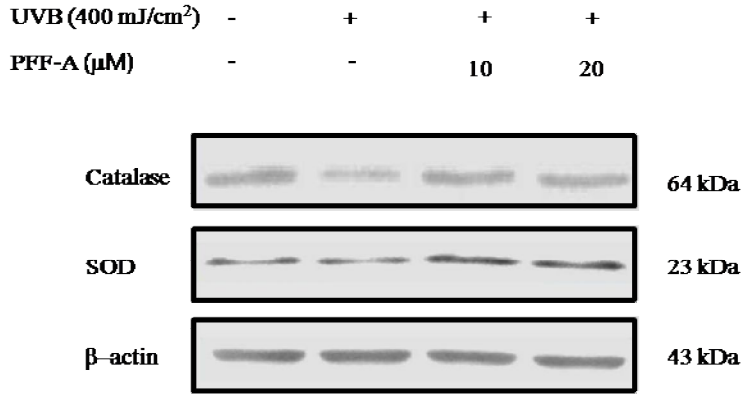

Fig. 9. Effect of PFF-A on UVB-induced expression of catalase, SOD. JB6 cells at ~80% confluence were serum-starved for 48 h and then pretreated with PFF-A (10, 20 μM) for 6 h followed by irradiation with UVB (400 mJ/cm^2) and incubated for another 12 h and then harvested.

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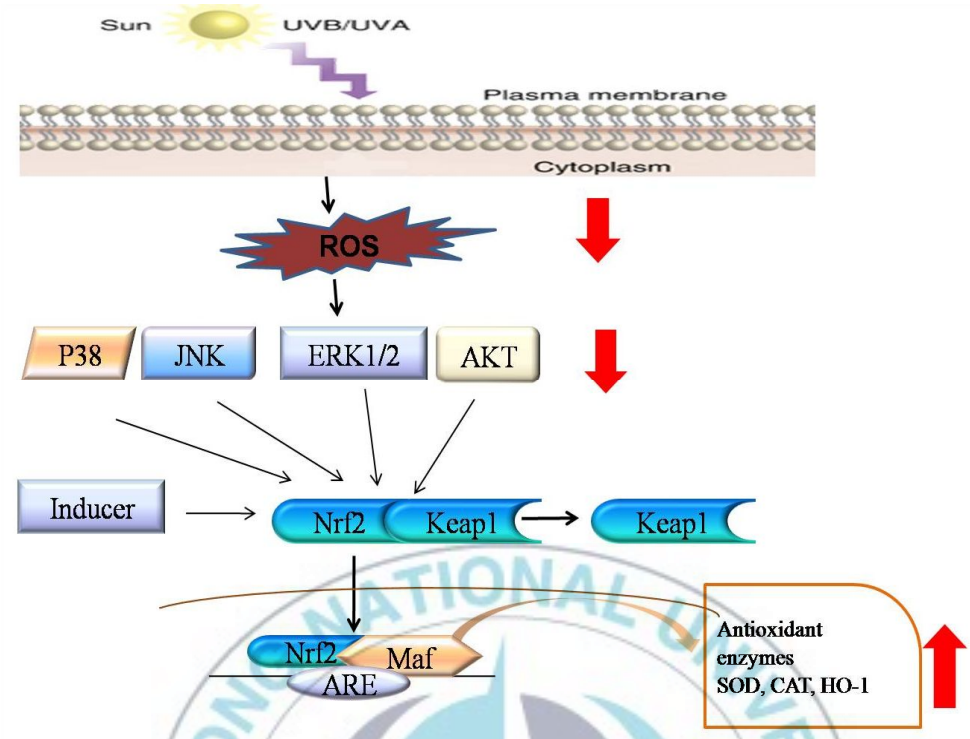

Fig. 10. Possible regulatory mechanisms of PFF-A in the Nrf2 pathway.

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