Modulation of Apoptosis in HaCaT Keratinocytes via Differential Regulation of ERK Signaling Pathway by Flavonoids*

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The exact molecular mechanisms underlying the cellular effects associated with various flavonoids have yet to be fully explained. In the present study, we have administered several flavonoids to human HaCaT keratinocytes and determined that 3,4′-dihydroxy flavone (3,4′-DHF) exerts a slight stimulatory effect on cell growth, although other flavonoids, including kaempferol, quercetin, and isorhamnetin, exhibited growth inhibitory properties. 3,4′-DHF was found to exert an anti-apoptotic effect on etoposide-induced cell death of HaCaT keratinocytes. We were also able to determine that sustained ERK activation was intimately associated with the etoposide-induced apoptosis of HaCaT cells, and treatment with 3,4′-DHF induced a significant suppression of etoposide-induced ERK activation, concomitant with the repression of poly(ADP-ribose) polymerase or the cleavage of pro-caspase 3. ERK overexpression significantly overrode the anti-apoptotic function of 3,4′-DHF, but this was not true of ERK-DN. Moreover, treatment with 3,4′-DHF resulted in the protection of cells from H2O2-induced cell death and exerted an apparent suppressive effect on the stress-induced generation of reactive oxygen species (ROS). Finally, we showed that 3,4′-DHF almost completely abolished kaempferol-induced apoptosis, coupled with a concomitant suppression of both intracellular ROS generation and the activation of ERK. Taken together, our data clearly indicate that a host of phytochemicals, including etoposide and a variety of flavonoids, differentially regulate the apoptosis of human HaCaT keratinocytes via the differential modulation of intracellular ROS production, coupled with the concomitant activation of the ERK signaling pathway. According to these results, we are able to conclude the distinct structure-activity relationship between several flavonoids.

Apoptosis is not only a fundamental cellular activity, which allows for the maintenance of physiological balance within an organism, but it also constitutes a protective mechanism against carcinogenesis, via the elimination of damaged cells or abnormal excess cells (1). It is also involved in the machinery of immune defense and the pathogenesis of a host of diseases associated with imbalances between positive and negative cell

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Apoptosis is not only a fundamental cellular activity, which allows for the maintenance of physiological balance within an organism, but it also constitutes a protective mechanism against carcinogenesis, via the elimination of damaged cells or abnormal excess cells (1). It is also involved in the machinery of immune defense and the pathogenesis of a host of diseases associated with imbalances between positive and negative cell survival regulatory factors. Apoptotic processes occur under the tight control of many pro- and anti-apoptotic molecules and are typically executed by the caspase family of cysteine proteases (2). A series of recent studies has established that the phosphorylation and dephosphorylation of key regulatory proteins are two of the most essential cellular events with regard to both cell growth and apoptosis.

One of the primary phosphorylation-dephosphorylation apoptotic signaling cascades has been shown to be composed of mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases that is composed of three distinct components of the protein kinase family; the MAPK kinase kinases (MAPKKKs), the MAPK kinases (MAPKKs), and the MAPKs (3). Currently, several distinct MAPKs, including the p42/p44 extracellular signal-related kinases (ERK1 and -2) (4), c-Jun N-terminal protein kinase (JNK)/stress-activated protein kinase (5), p38 MAPK (6), and big MAPK (BMK1/ERK5) have been demonstrated to function in these independent signaling pathways (7). The activated MAPKs affect the phosphorylation of Ser-Pro and Thr-Pro motifs in the substrate proteins and have also been shown to be regulated by a variety of extracellular stimuli, including growth factors, mitogens, cytokines, and environmental stresses (8). The specificity of the activation and function of the MAP-kinase signaling modules is determined by the activities of several apoptotic regulators, including scaffold, adaptor, and inhibitory proteins, which engage in interactions with the kinases in the MAPK signaling cascade.

The flavonoids comprise a broadly distributed class of plant pigments, all of which can be synthesized from phenylalanine. Recently, considerable scientific and therapeutic interest has focused on the structure and functions of these flavonoids (9, 10), including the well known compounds etoposide and taxol in cancer chemotherapy. They appear to be ubiquitous in the cells of green plants, and are responsible for much of the coloring of plants in nature. Most emit brilliant fluorescence when excited by UV light.

Flavonoids also exert a host of regulatory functions on plant growth, via inhibition of the exocytosis of the auxin indolyl acetic acid, as well as by inducing gene expression, and have been shown to modulate other biological functions, as well. Moreover, the flavonoids have been demonstrated to either inhibit or kill many bacterial strains, as well as viral enzymes such as reverse transcriptase and protease. They have also
been observed to destroy some pathogenic protozoans, but their toxicity to animal cells is generally low. Flavonoids have been used in the treatment of many important common diseases, due to their well proven ability to inhibit specific enzymes, to stimulate some hormones and neurotransmitters, and to scavenge free radicals (11).

The flavonoids are phenolic compounds and are characterized by a diphenylpropane (C6C3C6) skeleton. This structure (and the appellation “flavonoid”) is shared by the monomeric flavonols, flavones, flavanols, and flavanones, and all of these compounds have been subjected to intensive study, to determine their possible roles in human health, including cancer prevention. Several previous studies have indicated that some flavonoids exhibit potent anti-tumor properties and can modulate apoptosis, differentiation, and the cell cycle, probably by virtue of their anti-oxidant functions (12). However, only a few cases have exhibited their anti-oxidant activities in vitro, and some controversy has arisen with regard to the possible anti-oxidant properties possessed by several flavonoids, which have been reported to function as either pro-oxidant or anti-oxidant compounds, depending on their concentration (13). Despite the many studies that have been conducted regarding the function of flavonoids, the pro-oxidant/anti-oxidant properties of flavonoids remain somewhat debatable, and the detailed molecular mechanisms of their effects remain largely unknown.

The objective, then, of the present work was to assess the apoptosis-modulating effects of several flavonoids, and to clearly elucidate the molecular mechanisms underlying their activity on the apoptosis of human keratinocytes. Epidermal keratinocytes, which comprise the barrier between the body and the environment, are routinely and continuously exposed to a variety of environmental and physiological stresses, including radiation and chemicals.

Keratinocytes respond to such sometimes-noxious stimuli via the activation of distinct signaling pathways which result in either apoptosis or survival (14). In the present study, we treated experimental HaCaT keratinocytes with a variety of flavonoids, and determined that several flavonoids, including kaempferol, quercetin, and isorhamnetin, exerted distinct pro-apoptotic effects. However, some of the tested flavonoids, including 3,4’-dihydroxy flavone (3,4’-DHF), appeared to exert an anti-apoptotic influence. We also determined that 3,4’-DHF suppresses etoposide- and kaempferol-induced apoptosis via the reverse modulation of the ROS-mediated ERK signaling pathway, clearly showing the structure-activity relationship inherent to the flavonoids. Our findings indicate that apoptosis is differentially modulated by various flavonoids, and this appears to be dependent on the specifically different hydroxyl (OH) substitutions in their diphenylpropane (C6C3C6) skeletons.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Transfection**—The spontaneously immortalized human keratinocyte HaCaT cell line was cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) and 100 units/ml penicillin/streptomycin. For the treatment of many important common diseases, due to their well proven ability to inhibit specific enzymes, to stimulate some hormones and neurotransmitters, and to scavenge free radicals (11).

**Antibodies and Materials**—The 2’’,7’’-dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR), and the electrophoresis reagents and Bio-Rad protein assay kit were purchased from Bio-Rad. Antibodies against pro-caspase-3, actin, and cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the ERK1/2 and phospho-ERK (Thr-202/Tyr-204) antibodies were acquired from Cell Signaling (Beverly, USA).

**Determination of Cell Viability**—The HaCaT cells were plated at a density of 5 × 10⁶ cells in 96-well plates, and cell viability was evaluated via conventional MTT reduction assays. After incubation, the cells were treated with MTT solution (final concentration, 0.25 mg/ml) for 2 h at 37 °C. Dark blue formazan crystals forming in the intact cells were then dissolved with Me₂SO, and the absorbance was measured at 570 nm with an enzyme-linked immunosorbent assay reader. The results were then expressed as percentages of MTT reduction, with the absorbance exhibited by the control cells being arbitrarily set at 100%.

**DAPI Staining**—We also conducted DAPI staining for the identification of apoptotic nuclei. HaCaT cells were collected at 2000 rpm for 5 min, washed once with cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 5 min, then stained with 0.8 mg/ml 4,6-diamidino-2-phenylindole (DAPI) in darkness (15). Morphological changes in the apoptotic cells were assessed visually under a Zeiss Axiovert 200 microscope, at the fluorescence DAPI region (excitation, 351 nm; emission, 380 nm). To count the apoptotic nuclei of the transiently transfected cells, the cells were co-transfected with EGFP (Clontech) and the indicated plasmids. After 48 h of transfection, the cells were either left unexposed or were exposed to the indicated agents. The cells were fixed with 4% paraformaldehyde, and then stained with DAPI. The number of cells containing apoptotic nuclei among the green fluorescent protein-expressing cells was counted via fluorescence microscopy. Apoptotic cell death was then calculated as a percentage of apoptotic cells over the total green fluorescent protein-positive cells (16, 17).

**Western Blot Analysis**—Cells in 100-mm dishes were washed three times in ice-cold phosphate-buffered saline (PBS), scraped from the dishes, then collected in extraction buffer (1% Triton X-100, 100 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM p-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride). After the cells had been incubated on ice for 15 min, the lysates were centrifuged, and the proteins in the cleared lysates were quantitated with Bradford Protein Assay Reagent (Pierce). An equal amount of proteins was then separated on 10–12% SDS-PAGE gel, and then transferred electrophotorectively onto nitrocellulose membranes (0.2 μm, Schleicher and Schuell).

**Measurement of Intracellular ROS Levels**—Intracellular ROS levels were measured using the oxidant-sensitive fluorescent probe, DCFH-DA, under inverted microscopy. Cells grown at 1 × 10⁶ cells per 35-mm culture dish were maintained for 24 h in growth medium, then exposed for 30 min to 5 mM DCFH-DA. The cells were washed with PBS, and a coverglass was placed atop the dish. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was then imaged on an inverted fluorescence microscope. The production of intracellular peroxide was also measured using DCFH-DA coupled with Spectrofluorometry. Fluorescence was quantified with a Shimadzu RF5301 PC spectrofluorometer, set at an excitation of 404 nm and an emission of 524 nm (18). The cells were then exposed to RBlight, and a 5 μM stock solution of DCFH-DA dissolved in 20 μl of Me₂SO was added to each culture dish 30 min prior to the assay. After another incubation at 37 °C, the cells were washed twice in ice-cold PBS, resuspended in 200 μl of PBS, and disrupted by three 10-s cycles of low output sonication. The supernatants were then acquired after 10 min of centrifugation in a microcentrifuge, and the crude extract (500 μg of protein) were suspended in PBS, after which the fluorescence was recorded.

**RESULTS**

**Effects of Several Flavonoids on the Cell Viability of HaCaT Keratinocytes**—The body’s initial line of defense is often the skin, which is composed of the epidermis and the dermis. Keratinocyte are the principal cell type comprising the epidermis, and constitute ~90% of total epidermal cells. The HaCaT human keratinocyte cell line is a spontaneously immortalized human epithelial cell line, which was developed by Dr. Norbert Fusenig and coworkers (19), and harbors specific p53 mutations (20). However, HaCaT cells are not tumorigenic when inoculated into mice. To evaluate the effects of various flavonoids on the cell viability of human HaCaT keratinocytes, we treated these cells with differing quantities of several flavonoids, including isorhamnetin, kaempferol, 3,4’-dihydroxy...
flavone (3,4'-DHF), and quercetin.

We then determined cell viability at several time points, via MTT assay. The MTT assay relies principally on the mitochondrial metabolic capacities of the viable cells and, hence, reflects the intracellular redox state. Most flavonoids have been shown to reduce the cell viability of the HaCaT cells, and kaempferol and isorhamnetin exhibited the most dramatic effects (Fig. 1, A and B). However, some flavonoids exhibited no negative effects on cell viability and, interestingly, 3,4'-DHF was shown to induce a slight but significant increase in the cell viability of the HaCaT keratinocytes.

3,4'-Dihydroxy Flavone Suppresses Etoposide-induced Apoptosis—To evaluate the differential effects of flavonoids in a more apparent manner, we initially attempted to induce apoptosis in the HaCaT cells using etoposide, which is another phenolic phytochemical and one of the better-known apoptosis-inducing agents. As expected, etoposide effected a reduction in the viability of the HaCaT cells, in a dose-dependent manner (Fig. 1C). In a further experiment to determine the differential effects of flavonoids on etoposide-induced cell death, we used 10 μM etoposide, at which concentration we observed a cell viability of ~50%. At this concentration, etoposide was shown to induce the cleavage of PARP and the activation of caspase 3, both of which constitute markers for the etoposide-induced apoptosis of HaCaT keratinocytes (Fig. 1D).

We then treated the cells with flavonoids, combined with 10 μM etoposide. Whereas the additional treatment of kaempferol, quercetin, and isorhamnetin was determined to enhance the negative effects of etoposide on cell viability, 3,4'-DHF was shown to significantly suppress the etoposide-induced abnegation of cell viability (Fig. 1, E and F). We also assessed morphological changes in the amount of apoptotic nuclei in the

**Fig. 1.** Effects of flavonoids and etoposide on viability and apoptosis of HaCaT cells. The HaCaT cells were treated with indicated amounts of flavonoids, and cell viability was determined via MTT assay. A, HaCaT cells were exposed to different concentrations (5–30 μM) of the indicated flavonoids for 48 h. As a control, cells were treated with an equal amount of Me2SO in the absence of flavonoids. Data are expressed as the means ± S.E. of values from three independent experiments. B, cells were incubated with the indicated flavonoids (10 μM) for the indicated time periods. C, the HaCaT cells were treated with different concentrations (5–30 μM) of etoposide for 48 h, and cell viability was analyzed via MTT assay. D, the cells were incubated with etoposide (10 μM) for the indicated time periods. Proteins were separated on 10% SDS-polyacrylamide gel (30 μg/lane), and transferred to nitrocellulose membranes. The cleavage of PARP and pro-caspase-3 was analyzed via Western blotting with mouse monoclonal antibodies. The blots were then re-probed with anti-actin antibody, to confirm an equal amount of protein loading. E, the cells were preincubated with the indicated flavonoids (10–30 μM) for 1 h, followed by 24 h of stimulation with etoposide (10 μM). F, the cells were pretreated with the indicated flavonoids (10 μM) for 1 h, then exposed to etoposide (10 μM) for the indicated time periods.
cells, via DAPI staining, after the exposure of the cells to 10 μM etoposide, and/or 3,4’-DHF.

Etoposide induced nuclear fragmentation in the HaCaT cells, but this etoposide-induced apoptosis was suppressed by 3,4’-DHF (Fig. 2A). Addition of 3,4’-DHF also protected the etoposide-induced PARP and procaspase 3 cleavage (Fig. 2B). Taken together, these data strongly suggest that a variety of flavonoids exhibit differential effects on cell death, and that 3,4’-DHF, a flavonoid which has not been thoroughly studied, exerts an anti-apoptotic effect on etoposide-induced cell death in human HaCaT keratinocytes.

The ERK Signaling Pathway Is Involved in the Anti-apoptotic Effect of 3,4’-DHF—Despite the many previous studies that have been conducted with flavonoids, the exact molecular mechanisms underlying the observed cellular effects of flavonoids remain largely unclear. Several reports, however, have reported that the MAPK signaling pathway may play an important role in the activities and effects of chemotherapeutic drugs (21). MAPK signaling pathways are clearly involved in a host of cellular functions, including cell growth, differentiation, development, and apoptosis. They are, in general, subdivided into three different pathways, namely the ERK, p38 kinase, and JNK signaling pathways (22).

Therefore, to determine whether the MAPK signaling pathway is, indeed, involved in the anti-apoptotic effects of 3,4’-DHF, we treated the cells with several kinase inhibitors, each of which blocks a specific MAPK pathway. We initially treated the cells with kinase inhibitors, such as PD98059, SB202190, or SP600125, to ascertain which MAPK signaling pathway was involved in the etoposide-induced apoptosis of HaCaT keratinocytes. Both SB202190 (p38 kinase inhibitor) and SP600125 (JNK inhibitor), were found to have little, if any, influence on the etoposide-induced loss of cell viability in the HaCaT cells (Fig. 3A). Notably, treatment with PD98059 (MEK inhibitor) was shown to suppress etoposide-induced cell death. The effects of PD 98059, a specific ERK signaling pathway inhibitor, was demonstrated to be similar to those associated with 3,4’-DHF. We then evaluated nuclear fragmentation and caspase activation, both of which are fairly reliable indicators of apoptotic cell death. Pretreatment with an MEK inhibitor clearly antagonized both etoposide-induced nuclear breakdown and caspase activation (Fig. 3, B and C). However, sustained ERK1 and ERK2 activation was observed in cells that had been exposed to etoposide (Fig. 3D). This clearly indicates that sustained ERK activation may be involved in etoposide-induced apoptosis in human HaCaT keratinocytes.

ERK activation is generally thought to inhibit apoptosis, but recent studies have demonstrated that sustained ERK activation is also involved in the apoptotic process (23). To the best of our knowledge, in human HaCaT keratinocytes, we can surmise that the observed sustained ERK activation is associated with etoposide-induced apoptosis. It is significant that 3,4’-DHF treatment was shown to induce the suppression of etoposide-induced ERK activation, concomitantly with the repression of PARP or procaspase 3 cleavage (Fig. 3E). According to these results, the ERK signaling pathway appears to perform an important function in the etoposide-induced apoptosis of HaCaT keratinocytes and, particularly, in the anti-apoptotic effects associated with 3,4’-DHF.

To confirm the involvement of ERK stimulation in the anti-apoptotic effects associated with 3,4’-DHF treatment, we transfected our HaCaT keratinocytes with wild-type ERK, or with the kinase-inactive and dominant negative ERK2 mutant, ERK2-DN (K52R). ERK overexpression was shown to significantly override the anti-apoptotic functions of 3,4’-DHF, but this was not seen in the cells treated with ERK2-DN (Fig. 4). When ERK was overexpressed, we registered a significant level of recovery with regard to both ERK phosphorylation and concomitant caspase activation (Fig. 4A).

Our DAPI-staining nuclear fragmentation analysis also clearly showed that ERK overexpression exerted an antagonistic effect on the anti-apoptotic effects of 3,4’-DHF (Fig. 4B). These data clearly demonstrate that the ERK signaling pathway is intimately involved in the anti-apoptotic effects associated with 3,4’-DHF.

Suppression of ROS-ERK Signaling Pathway by 3,4’-DHF Plays an Important Role in the Modulation of Apoptosis in HaCaT Cells—Several reports have suggested that a host of phenolic phytochemicals, including etoposide and flavonoids, can induce variations in the levels of cellular ROS (24).

To further elucidate the molecular basis of the anti-apoptotic effects of 3,4’-DHF, we therefore attempted to determine whether 3,4’-DHF exerts a suppressive effect on ROS-induced apoptosis. We initially assessed the effects of 3,4’-DHF treatment on cell viability after the exposure of HaCaT keratinocytes to hydrogen peroxide. Hydrogen peroxide was shown to induce cell death in about 50% of the cells at a concentration of 300 μM. However, the addition of 3,4’-DHF significantly protected the cells from hydrogen peroxide-induced cell death (Figs. 5, A and B).
We then measured intracellular ROS production using the oxidant-sensitive fluorescent dye, DCFH-DA, after the cells were treated with either etoposide or flavonoids. In the presence of intracellular peroxides, the esterase-mediated deacylation of DCFH-DA to DCFH occurs within the cells, and the non-fluorescent DCFH is subsequently oxidized, resulting in the formation of the highly fluorescent DCF. Recently, DCFH has been reported to be sensitive to oxidation by peroxynitrite, hydrogen peroxide (in combination with cellular peroxidases), peroxidases alone, and hydroxyl radicals. However, DCFH appears to be unsuitable for the measurement of NO, hypochlorous acid, or superoxide radicals in biological systems (25).

Etoposide treatment was shown to strongly induce the generation of fluorescent DCF in the HaCaT keratinocytes, and etoposide-induced ROS generation was wholly antagonized as the result of the addition of N-acetyl cysteine, a well-known anti-oxidant and free radical scavenger (Fig. 5C). The administration of N-acetyl cysteine was also shown to suppress both etoposide-induced caspase activation and ERK phosphorylation. This suggests that etoposide induces both apoptosis and ERK phosphorylation in HaCaT keratinocytes, via the generation of intracellular ROS (Fig. 5D).
Most importantly, the addition of 3,4'-DHF had an obviously repressive influence on the etoposide-induced generation of ROS (Fig. 5C). By way of contrast, kaempferol exhibited an additive effect on etoposide-induced ROS generation. In a consistent result, kaempferol alone induced intracellular ROS generation in the HaCaT cells, although 3,4'-DHF did not. These results indicate that 3,4'-DHF protects keratinocytes from stress-induced apoptosis via the suppression of intracellular ROS generation and the concomitant activation of the ERK signaling pathway.

Differential Regulation of ROS-ERK Signaling Pathway by Different Flavonoids Plays Distinct Roles in Modulation of Apoptosis in HaCaT Keratinocytes—Kaempferol, a compound that occurs naturally in fruits and vegetables, has been shown to exert anti-proliferative effects in a variety of living systems, on the basis of its striking repressive effects on the diverse cellular events associated with tumor initiation, promotion, and progression (26). Because two different flavonoids, namely kaempferol and 3,4'-DHF, were shown to exert differential effects on both cell viability and ROS production (Figs. 1A and 5C), we attempted to determine whether 3,4'-DHF also antagonized effects of kaempferol.

MTT and DAPI staining assays also revealed that 3,4'-DHF resulted in the almost complete abolition of the apoptotic effects associated with kaempferol treatment (Fig. 6, A and B). The addition of 3,4'-DHF was also shown to suppress ROS generation, as well as the associated caspase activation and ERK phosphorylation (Fig. 6, C and D). Taken together, our present findings suggest that many phytochemicals, including etoposide and a variety of flavonoids, can induce apoptosis, mediated by both ROS and the ERK signaling pathway. Also, other specific flavonoids, including 3,4'-DHF, have been clearly demonstrated to block apoptosis in the human HaCaT keratinocytes, via the modulation of the ROS-ERK signaling pathway (Fig. 6E).

**DISCUSSION**

Recently, the use of naturally occurring compounds in the development of anti-tumor agents has become a critical topic in the scientific and industrial communities. Several phytochemicals, most notably taxol and etoposide, have already been put into use as clinical anti-tumor drugs (27).

The flavonoids are a group of natural products currently receiving a great deal of attention. Several studies have reported that some flavonoids, including kaempferol and quercetin, exert anti-oxidant effects and can also inhibit carcinogenesis (10, 11). Besides these anti-tumor activities, some flavonoids have been observed to exhibit a variety of beneficial biological activities, including anti-hypertensive, anti-viral, and anti-inflammatory properties. A few flavonoids have also
FIG. 5. 3,4’-DHF protects HaCaT cells from stress-induced apoptosis by suppressing intracellular ROS production and concomitant activation of ERK activation. **A** and **B**, HaCaT cells were pretreated for 1 h with 3,4’-DHF (10 μM), then exposed to H₂O₂ (300 μM) for 48 h (**A**) or for the indicated time periods (**B**). Cell viability was the evaluated via MTT assay. **C**, after the pretreatment of the cells with 3,4’-DHF, kaempferol, or N-acetylcysteine (NAC) for 1 h, the cells were exposed to etoposide (10 μM) for 12 h. The intracellular ROS levels were then measured via fluorescence microscopy with the oxidant-sensitive probe, DCFH-DA. **D**, DCF fluorescence in the cells was quantified with a spectrofluorometer (excitation, 504 nm; emission, 524 nm). The results represent the means ± S.E. of values obtained from three separate experiments. **E**, equal amounts of cell lysates were resolved via SDS-PAGE, and analyzed via Western blotting with anti-ERK, anti-phospho-ERK, anti-PARP, and anti-pro-caspase-3 antibodies.

FIG. 6. 3,4’-DHF suppresses kaempferol-induced apoptosis in HaCaT cells. **A** and **B**, cells were pretreated with 3,4’-DHF (10 μM) for 1 h, then exposed to kaempferol (10 μM) for 48 h. **A**, cell death was analyzed via MTT assay. Data are expressed as the means ± S.E. of values obtained from three independent experiments. **B**, the cells were stained with DAPI, and fluorescence images were obtained via fluorescence microscopy. The apoptotic cells exhibiting nuclear condensation and fragmentation were then counted, and the percentage of cells undergoing apoptotic death was presented in the figure. **C** and **D**, 3,4’-DHF-pretreated cells were exposed to kaempferol (10 μM) for 12 h, and then intracellular ROS levels were evaluated by fluorescence microscopy with an oxidant-sensitive probe, DCFH-DA. **D**, DCF fluorescence was then determined with a spectrofluorometer (excitation, 504 nm; emission, 524 nm). The results were expressed as the means ± S.D. of three separate experiments. **E**, equal amounts of cell lysates were then analyzed via immunoblot analysis using anti-ERK, anti-phospho-ERK, anti-PARP, and anti-pro-caspase-3 antibodies.
been reported to harbor anti-apoptotic properties (28). Despite the many studies that have been conducted to determine the variety of biological functions associated with the flavonoids, the precise molecular mechanisms underlying their cellular effects remain largely unknown. In the present study, we demonstrated that flavonoids exert differential effects on the cell viability of human HaCaT keratinocytes. Several flavonoids, including kaempferol, quercetin, and isorhamnetin, have been shown to exert inhibitory effects on growth, but 3,4'-DHF was demonstrated to induce cell growth and suppress cell death induced by etoposide (Figs. 1 and 7).

Currently, several flavonoids have been reported to induce cell cycle arrest and apoptosis in a variety of cancer cells (29, 30). However, only a few studies have focused on any significant degree on the anti-apoptotic effects of specific flavonoids. To the best of our knowledge, no previous studies have focused on the anti-apoptotic effects of 3,4'-DHF. The fact that 3,4'-DHF protects the cells from apoptotic cell damage appears to be applicable to the protection of keratinocytes, which are the primary cell type in the epidermis, and play a key role in the body's initial line of defense.

We have also demonstrated that sustained ERK activation is involved in the etoposide-induced apoptosis of the HaCaT cells, and that the anti-apoptotic effects associated with 3,4'-DHF were attributable to the suppression of etoposide-induced ERK activation (Fig. 3). The MAPK signaling pathways are expressed ubiquitously, and have been shown to respond to a broad variety of external stresses and drugs.

The ERK cascade is, in general, activated by mitogenic stimuli, and is believed to mediate both cell proliferation and survival, whereas the JNK and p38 MAPK modules are activated in response to cellular stress, and appear to exert both protective as well as pro-apoptotic effects. The ERK signaling pathway appears to involve multiple signal transduction pathways, which are used to accomplish a variety of functions (31, 32). The activation of ERK by extracellular growth signals is mediated via the activation of a small G protein, Ras, and enhances cell proliferation (33).

Several studies have shown that the activation of ERK stimulates proliferation via the induction of cyclin D expression (34), or via the inactivation of the p27KIP cell cycle inhibitor protein (35). Moreover, ERK has been shown to prevent apoptosis via caspase activation (36, 37), and the induction of expression of anti-apoptotic factors, including Mcl-1 (38) and the inhibitor of apoptosis proteins (23). The activation of ERK has been demonstrated to inhibit apoptosis, as was shown in studies of the apoptosis of rat PC12 cells under growth factor withdrawal conditions (39). However, recent reports have maintained that the sustained activation of ERK is also involved in the apoptotic process (40–42). Robust ERK stimulation has been reported to suppress the cell cycle, via the induction of the expression of cell cycle inhibitor proteins, including p21Cip/Waf and p27KIP (43), and DNA damage-induced ERK activation was assessed with regard to the stimulation of apoptosis (44, 45). ERK activation has also been studied with regard to the mediation of apoptosis in T-cells, which occurs via the up-regulation of Fas ligand expression (46), which leads to ceramide-induced apoptosis, by repressing the inactivation of the pro-apoptotic BAD (47).

Here, we have initially demonstrated that sustained ERK activation is involved in the etoposide-induced apoptosis of human HaCaT keratinocytes. Our results were consistent with the findings associated with T cells, Jurkat T cells, and neutrophils treated with hydrogen peroxide or other oxidants (40, 48). As suggested in these studies, sustained ERK activation in HaCaT cells after treatment with etoposide was expected to
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result from a combination of MEK activation and the inhibition of cysteine-containing proteins, specifically the tyrosine phosphatases.

In this study, 3,4′-DHF was shown to protect the cells from hydrogen peroxide-induced cell death, and exerted an obvious repressive effect on etoposide-induced ROS generation (Fig. 5). These data strongly suggested that etoposide-induced ERK activation could be attributed to intracellular ROS generation, which is readily suppressed by treatment with 3,4′-DHF.

We also determined that 3,4′-DHF almost completely suppresses kaempferol-induced apoptosis via the ablation of intracellular ROS production, and the concomitant activation of the ERK signaling pathway (Fig. 6). ROS-dependent redox cycling is considered to be critical for the regulation of protein interactions, which modulate the activity of important proteins in signal transduction and carcinogenesis, including the activity of protein kinase C (49) and MAPKs, especially ERK. The MAPKs have been shown to be activated in response to oxidant-induced alterations in the redox state (50). Also, after activation, each MAPK has been observed to phosphorylate a distinct spectrum of substrates, including key regulatory enzymes, apoptosis regulators, cytoskeletal proteins, nuclear receptors, and a host of different transcription factors (40).

In addition, endogenous ROS generation has been reported to support sustained MAPK activation via the inhibition of CD45 and other tyrosine phosphatases, and has also been shown to contribute to the induction of distinct MAPK activation profiles via differential signaling pathways (40, 48). In our study, both etoposide-induced and kaempferol-induced cell death were inhibited as the result of the addition of 3,4′-DHF and PD98059, both specific chemical inhibitors of the MEK/ERK signaling route. This suggests that the ROS-mediated MEK/ERK signaling pathway contributes to the modulation of apoptosis in the keratinocytes by various phytochemicals, including etoposide and flavonoids.

Flavonoids have been used in the treatment of several common diseases, due to their ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters, and to scavenge free radicals (10, 11). Although a majority of studies have been focused firmly on the anti-oxidant effects of flavonoids, they have also been reported to function in either a pro- and anti-oxidant manner, depending on both their concentration and structure (13, 51).

Moreover, several reports have demonstrated that a variety of flavonoids can differentially modulate the apoptosis of specific cells. This differential effect appears to be due, at least in part, to their structure, suggesting the structure-activity relationship inherent to these compounds. Previous papers have suggested that the number of hydroxyl (OH) substitutions in a flavonoid is a critical factor in its ability to scavenge reactive oxygen species (ROS) (52, 53). Flavonoids with more OH groups were suggested to possess more potent anti-oxidant properties and to exert more robust anti-inflammatory effects (52, 53). However, the effects of OH substitution have yet to be determined in detail, and further study will be required to characterize the effects of each OH substitution on specific sites in the diphenylpropane (C6C3C6) skeletons of specific flavonoids. In addition, previous studies have shown that the methoxyl (OCH3) group exerts a negative influence on the anti-inflammatory and anti-oxidant properties of the flavonoids. The 3-OH flavone has been shown to inhibit epidermal growth factor-induced proliferation, and the addition of glycoside was shown to suppress the apoptotic effects generally associated with flavonoids (51, 54).

In our study, kaempferol, isorhamnetin, naringenin, taxifolin, and querce tin exerted negative effects on the HaCaT keratinocytes, but 3,4′-DHF exhibited a positive effect on the cells (Fig. 7). In a consistent finding, kaempferol was shown to induce intracellular ROS generation in the HaCaT cells, but 3,4′-DHF suppressed this kaempferol-induced ROS production (Fig. 6). These results underscore the distinct structure-activity relationship inherent to several flavonoids, including 3,4′-DHF and kaempferol. According to our results, we can surmise that OH substitutions on the 5 or/and 7 carbons in the diphenylpropane (C6C3C6) skeleton of the flavonoids might significantly affect the anti-oxidant properties of these compounds, because 3,4′-dihydroxy flavone featured differential OH substitutions in this region (Fig. 7).

Our findings represent a significant step forward in our understanding of the structure-activity relationship of flavonoids and shed new light on the molecular mechanisms underlying the differential effects of structurally different flavonoids. However, more study will be required to clearly elucidate the related structure-activity relationship of other flavonoids. Based on the results of the current study, we plan to compare the apoptotic properties of other flavonoids, whether naturally obtainable or chemically synthesized, to clarify the ramifications of the structure-activity relationship with regard to the differential modulation of apoptosis.

In conclusion, this study may provide helpful information, allowing us to further address the controversial pro-oxidant/anti-oxidant properties evidenced by various flavonoids. Our results also augment our current understanding of the molecular mechanisms underlying the differential cellular functions associated with flavonoid treatment.

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