JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Protective Effect of Structurally Diverse Grape Procyanidin Fractions against UV-Induced Cell Damage and Death

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ABSTRACT: UV radiation leads to the generation of reactive oxygen species (ROS). These molecules exert a variety of harmful effects by altering key cellular functions and may result in cell death. Several studies have demonstrated that human skin can be protected against UV radiation by using plant-derived antioxidants. Here we evaluated the *in vitro* capacity of several antioxidant polyphenolic fractions from grape, which differ in their degree of polymerization and percentage of galloylation, to protect HaCaT human keratinocytes against UV-induced oxidative damage. These fractions inhibited both basal and UVB- or UVA-induced intracellular ROS generation in this cell line. Consequently, the same fractions inhibited p38 and JNK1/2 activation induced by UVB or UVA radiation. The highest protective effect was for fractions rich in procyanidin oligomers and gallate esters. These encouraging *in vitro* results support further research and should be taken into consideration into the clinical pharmacology of plant-derived polyphenolic extracts as novel agents for skin photoprotection.

KEYWORDS: grape, polyphenols, antioxidant, UV radiation, reactive oxygen species (ROS), mitogen-activated protein (MAP) kinases (MAPKs)

INTRODUCTION

UV radiation from sunlight is the main environmental cause of skin damage.[1] Excessive exposure to UV radiation has several adverse effects on human health, such as skin carcinogenesis, immunosuppression, solar erythema and premature skin aging.[2] Many harmful effects of short-wavelength UVB (290−320 nm) and long-wavelength UVA (320−400 nm) are associated with the generation of reactive oxygen species (ROS), for instance superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). An integrated defense system comprising nonenzymatic and enzymatic antioxidants, including catalase, glutathione, and superoxide dismutase, is thus crucial in protecting the skin from oxidative stress.[3] Severe depletion of endogenous skin antioxidants during oxidative stress caused by prolonged exposure to UV radiation results in insufficient protection and hence extensive cellular damage and eventual cell death by apoptosis.[4,5] Excessive levels of ROS not only damage cells directly by oxidizing macromolecules such as DNA and lipids but also indirectly by triggering stress-sensitive signaling pathways such as that of c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (MAPK).[6] The activation of these intracellular cascades increases expression of various proteins related to the induction of apoptosis.[7] Moreover, activation of JNK and p38 pathways has been described in keratinocytes in response to exposure to UVB[8] and UVA.[9,10]

The current approach to protecting human skin against solar UV-induced oxidative damage relies heavily on the avoidance of excessive exposure to sunlight and the use of sunscreens. Topical and oral supplementation of phytochemicals may complement these strategies.[11,12] Several studies have recently demonstrated the efficacy of naturally occurring botanical antioxidants, such as green tea polyphenols,[13] rosmarinic acid,[14] resveratrol,[15] genistein,[16] and grape seed proanthocyanidins, against the adverse effects of UV radiation on skin. In particular, dietary grape seed proanthocyanidins have been reported to inhibit photocarcinogenesis in SKH-1 hairless mice,[17] to show anti-inflammatory activity in mouse skin,[18] and to reduce UV-induced oxidative stress by inhibiting MAPKs and NF-κB signaling in human epidermal keratinocytes and in mice.[19] However, several questions remain as to the relevance of the polyphenolic structure of grape extracts and their photoprotective capacity. In this study, we tested the polyphenolic fractions from a grape by-product. These contained monomers and oligomers of catechins with some galloylation and mainly polymerized procyanidins.[20] Lizárraga and colleagues described that the mean degree of polymerization and the percentage of galloylation of grape polyphenolic fractions affect their antiproliferative potential and their scavenging capacity in colon cancer cells.[21] Moreover, the most widely studied natural catechin, epigallocatechin-3 gallate, is a potent antioxidant and skin photoprotector.[19] These and other results suggest that the degree of polymerization and the percentage of galloylation in natural extracts are crucial chemical characteristics for biological activity.[22,23] These parameters may be useful indicators to evaluate the potential of natural plant extracts to protect skin against UV-induced damage.

Here we studied the relationship between key structural characteristics of grape procyanidins, such as the mean degree

Received: November 17, 2010
Accepted: March 15, 2011
Revised: March 9, 2011
Published: March 15, 2011

of polymerization and percentage of galloylation, and the capacity of these compounds to protect skin against photodamage. To examine whether these polyphenols, which are potent free radical scavengers,[24,25] exert a protective effect against UV-induced oxidative stress in human keratinocytes, we used immortalized, but not tumorigenic, HaCaT cells from human adult skin keratinocytes.[26] This cell line provides a suitable experimental model to assess the response of epidermal components of the skin to UV-induced oxidative stress.

Thus, we examined the potential protective effects of grape-derived phenolic fractions against ROS formation and against the activation of JNK1/2 and p38 MAPKs induced by UVB and UVA radiation. At concentrations between 5 and 20 μg/mL, these fractions had a protective effect against UV-induced ROS generation and MAPK activation. Moreover, we demonstrate that the degree of polymerization and percentage of galloylation are crucial to the protective effect of these fractions.

MATERIALS AND METHODS

Natural Products. The polyphenolic fractions were obtained from pressing destemmed Parellada grapes (*Vitis vinifera*) as described before.[27] Fractions contained flavanols (catechins) that differed in the mean degree of polymerization (mDP) and percentage of galloylation (% G). Total fraction OW, which was soluble in ethyl acetate and in water, was obtained by solvent fractionation, and it contained gallic acid (GA), (+)-catechin (Cat), (−)-epicatechin (EC), glycosylated flavonols and procyanidin oligomers (the latter with mDP 1.7 and % G 15%). The other fractions were derived from OW by column chromatography using either reverse-phase, absorption/exclusion, or a combination of both techniques. These derived fractions contained mainly flavanols and were as follows: IV (formed by flavanol oligomers with mDP = 2.7, % G = 25); XI (formed by flavanol oligomers with mDP = 3.7, % G = 31); and V (formed only by nongalloylated flavanol monomers). To analyze their effects, the fractions were dissolved in sterilized PBS at concentrations of 5 mg/mL and subjected to nitrogen gas immediately prior to use.

Materials and Chemicals. The UV source was a BIO-SUN system illuminator (Vilbert Lourmat; Torcy, France) consisting of two UV lamps that delivered uniform UVA (365 nm) and UVB (312 nm) radiation at a distance of about 10 cm.

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

Cell Culture. The HaCaT cell line comprising spontaneously transformed but nonmalignant human skin keratinocytes was used.[26] Cells were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) containing 25 mM glucose (Cambrex Bioscience; Verviers, Belgium) and supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories GmbH; Pasching, Austria), L-glutamine 2 mM, Hepes 10 mM and 0.2% antibiotic (Gibco-BRL; Eggenstein, Germany). 22,600 cells/cm^2 were grown for 24 h to 80−90% confluence and fed with standard medium (without serum) for 48 h to induce quiescence and basal levels of phospho-p38 and JNK 1/2.

Treatment with Polyphenols and UV Radiation. Quiescent keratinocytes, cultured as described above, were pretreated with various concentrations of polyphenolic fractions (5, 10, and 20 μg/mL for the total fraction OW and 5 μg/mL for the derived fractions IV, V and XI) for 6 h. After washing with PBS, plates without cover were placed in the BIO-SUN system and cells in PBS were UVB-irradiated at 312 nm and doses of 0.03 or 0.05 J/cm^2 (less than 1 min of radiation) or UVA-irradiated at 365 nm and doses of 10, 20, or 30 J/cm^2 (radiation times were 30 min, 1 or 2 h respectively). The conditions used for UVA and UVB radiation were adapted from previous publications.[28,29] After UV exposure, cells were fed with fresh serum-free medium and postincubated for 24 h for viability assays or for 30 min for the analysis of MAPK activation and ROS release. Furthermore, control nonirradiated cells were treated in the same way.

Detection of Intracellular ROS. Intracellular ROS in UVB- or UVA-irradiated cells pretreated or not with the polyphenolic fractions was analyzed by flow cytometry using dihydrorhodamine 123 (DHR) as a specific fluorescent dye probe, since the intracellular release of ROS irreversibly oxidizes DHR, which is then converted to the red fluorescent compound rhodamine 123.[30−32] Thus, 83,000 cells were cultured in 12-well microtiter plates in standard culture medium. After 24 h this medium was replaced by another without serum, and cells were incubated for 48 h prior to the 6 h treatment with the corresponding polyphenolic fraction. Cells were loaded with 5 μM DHR for 30 min and washed in PBS before being exposed to UVB or UVA radiation at 0.05 or 20 J/cm2, respectively. After 30 min of postincubation, cells were washed in PBS, trypsinized and collected by centrifugation at 500*g*. Pellets were then washed in PBS before fixing cells with 400 μL of 0.5% formaldehyde in PBS. Finally, cells were placed on ice and analyzed by measuring the fluorescence intensity of 10,000 cells at 488 nm in an Epics XL flow cytometer. The results were expressed as a percentage of mean fluorescence intensity of nonirradiated DHR-stained cells, considering them as 100%.

Western Blot Analysis of MAPK Activity. Cells cultured in 22.1 cm^2 plates were pretreated with or without the corresponding polyphenolic fraction, and irradiated as described in the previous section. After UV radiation, cells were postincubated at 37 °C and 5% CO2 for a range of times (30 min, 1 and 2 h) for the activation time course assay or collected at 30 min for the protection assays. Thus, cells were washed in PBS and extracted with 300 μL of lysis buffer containing 81.5 mM Tris pH 6.8, 2% (w/v) SDS and protease inhibitors (10 μg/mL leupeptin, aprotinin and PMSF and 1 μg/mL ortovanadate). After sonicating the cells, we quantified the protein content using the Lowry assay.[33] Equal amounts of protein (25 μg) in loading buffer containing 50 mM Tris pH 6.8, 2% (w/v) SDS, 10 mM DTT, 10% (v/v) glycerol and 0.2% (w/v) bromophenol blue were boiled for 5 min, separated by SDS−PAGE (4% stacking and 10% resolving) and transferred to PVDF membrane (Bio-Rad Laboratories, CA, USA). After blocking in TBS-Tween (0.1%) and 5% (w/v) of BSA for 1 h at room temperature, blots were incubated overnight at 4 °C with the corresponding primary antibody in TBS-T with 5% (w/v) BSA at 1:1000 dilutions. For phosphorylated MAPK analysis, we used polyclonal antiphospho-p38 and monoclonal antiphospho-JNK antibodies (Cell Signaling, Beverly, MA, USA). For total MAPK analysis, polyclonal anti-p38 and anti-JNK antibodies were used (Cell Signaling). Afterward, the blots were washed in TBS-T three times for 5 min each, and incubated with HRP-conjugated goat anti-rabbit (Amersham Biosciences AB, Uppsala, Sweden) or HRP-conjugated rabbit anti-mouse (DAKO, Copenhagen, Denmark) for polyclonal and monoclonal primary antibodies respectively. Secondary antibodies were prepared in TBS-T and 2% (w/v) dry milk at 1:3000 dilutions and incubated for 1 h at room temperature. After incubation with secondary antibody, blots were again washed three times for 5 min in TBS-T, followed by one washing step in TBS. They were then visualized on film by enhanced chemiluminescence with an ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel).

RESULTS

UV-Induced and Basal ROS Generation Is Inhibited by the Polyphenolic Fractions. The polyphenolic fractions lowered baseline ROS levels in a significant manner in non-UV-irradiated cells (Figure 1A). In addition, this reduction was dose-dependent as observed for the total fraction OW and was lower for the fraction V at 5 μg/mL with respect to IV and XI at the same concentration (about 20% vs 40−50% reduction for IV and XI) (Figure 1A). Moreover, the decrease in ROS levels observed for

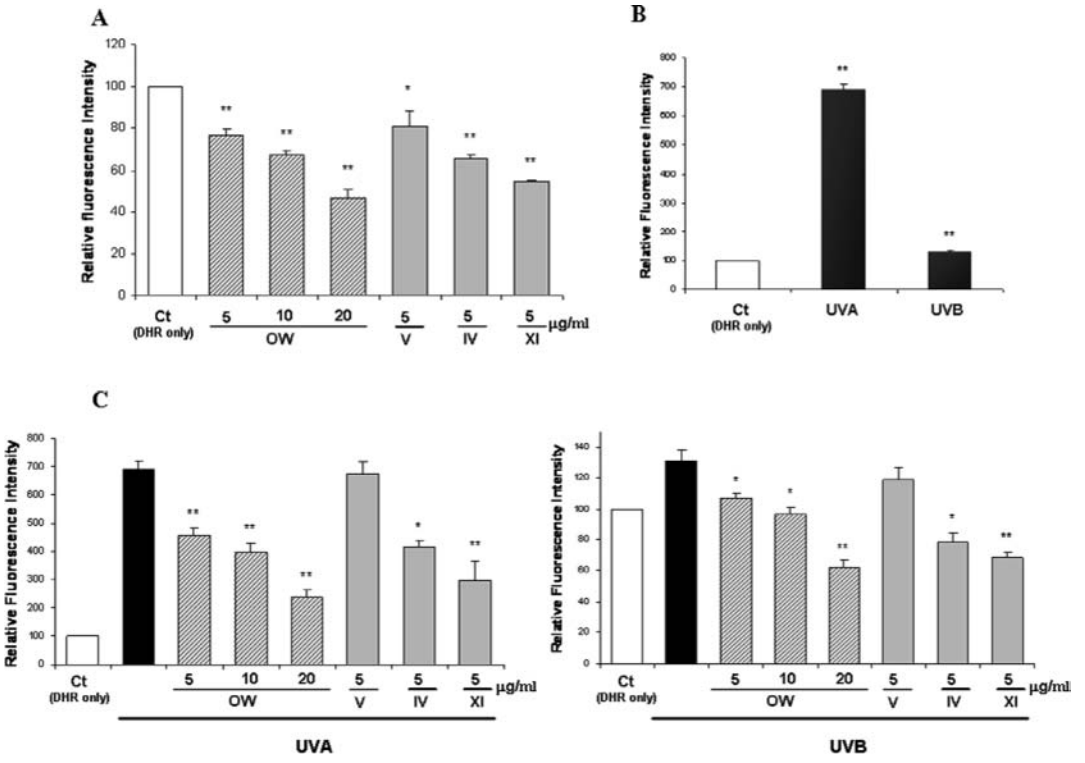

Figure 1. Inhibition of basal and UV-induced ROS production by polyphenolic fractions. (A) To determine intracellular ROS generation, HaCaT keratinocytes treated with total or derived polyphenolic fractions (OW, IV, V and XI) at the indicated concentrations for 6 h were incubated with DHR, followed by 30 min of postincubation, and analyzed by flow cytometry as indicated in Materials and Methods. (B) UV-induced ROS production in nontreated HaCaT cells after exposure to 20 J/cm^2 UVA or 0.05 J/cm^2 UVB. (C) Cells were pretreated with total or derived polyphenolic fractions (OW, IV, V and XI) at the indicated concentrations for 6 h previous to UVA or UVB irradiation, and ROS production was determined as stated above. Results are representative of three independent experiments (mean ± SEM). * $p < 0.05$, ** $p < 0.001$ versus control.

5 μg/mL of fractions IV and XI was achieved only by 10−20 μg/mL of the total fraction OW (Figure 1A). UV-irradiated HaCaT cells showed an increase in intracellular ROS after UV radiation. This increase was about 6-fold higher after UVA exposure and less than 2-fold higher after UVB radiation compared to the baseline levels of ROS. (Figure 1B) Pretreatment of cells with the polyphenolic fractions reduced ROS levels in a significant manner after UVB and UVA radiation, regardless of the concentration tested, except fraction V, which failed to exert a protective effect at 5 μg/mL (the concentration studied for the derived fractions) (Figure 1C). Moreover, ROS generated after UVA and UVB exposure decreased in the total fraction OW in a dose-dependent manner. A reduction in ROS of about 50−60% was achieved with 10−20 μg/mL of this fraction. A similar decrease (50−60%) was induced by only 5 μg/mL of the fractions IV and XI (Figure 1C). In addition, when cells were exposed to UVB radiation, all the fractions, except V, reduced the generation of ROS to lower values than the control nonirradiated cells (Figure 1C, right section). Since the increase in ROS production was much higher after UVA radiation, the extent of the decrease exerted by fractions OW, IV and XI for this source of radiation was not as large as for UVB radiation (Figure 1C).

Time Course of p38 and JNK1/2 Activation after UVA or UVB Irradiation. Activation of the MAPKs p38 and JNK1/2 was analyzed at a range of time points after exposure to 20 J/cm^2 UVA or 0.05 J/cm^2 UVB. A 30 min incubation of cells after exposure to the two kinds of UV radiation was sufficient to activate p38 and JNK1/2 (Figure 2). Moreover, this activation was more accentuated after UVA radiation (Figure 2). The phospho levels of the two MAPKs (phospho-p38 and phospho-JNK1/2) decreased with longer postirradiation incubation times, the reduction being greater after UVA radiation (Figure 2).

Inhibition of UV-Induced p38 and JNK1/2 Activation by the Polyphenolic Fractions. Having determined that the optimum postirradiation incubation time with the fractions was 30 min, we studied the capacity of the total polyphenolic fraction OW and the derived fractions V, IV and XI to prevent the activation of p38 and JNK1/2. For OW, we tested 5, 10, and 20 μg/mL while 5 μg/mL was used for all the other fractions.

A 6 h pretreatment with the fractions protected cells against p38 and JNK1/2 activation. When cells were irradiated with UVA, this effect was clearer for the highest concentration of OW (20 μg/mL) and 5 μg/mL for fractions V, IV and XI (Figure 3A, right). Similar results were obtained for UVB radiation, except for fraction V at 5 μg/mL, which did not inhibit p38 activation (Figure 3A, left). In addition, the amount of total MAPKs was identical for nonirradiated cells and cells irradiated with UVB and UVA (Figure 3A). Given this observation, the decrease observed in protein phosphorylation levels was specific.

The active forms of MAPKs (phospho-p38 and -JNK1/2) (Figure 3B) were quantified by referring the density of phospho-p38 or -JNK1/2 to that of the corresponding α-actin bands and considering the density of UV-radiated nontreated cell bands as 100% activation. The OW fraction demonstrated a dose-dependent protective effect against p38 activation. This effect was

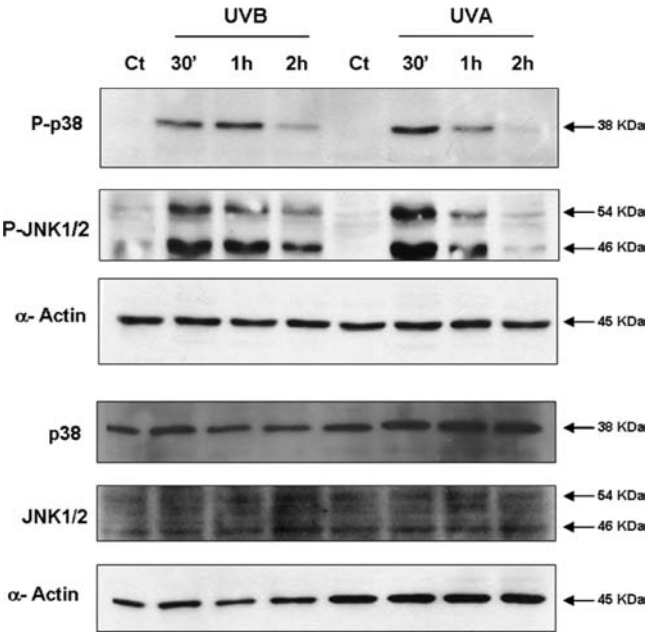

Figure 2. Time course of p38 and JNK1/2 phosphorylation induced by UV radiation. HaCaT cells were cultured for 48 h in the absence of serum followed by exposure to 20 J/cm^2 UVA or 0.05 J/cm^2 UVB. Cells were collected at a range of times, as indicated. The levels of phosphorylated and total p38 and JNK1/2 were determined by Western blot analysis using phospho- and total-specific antibodies. Actin expression was used as loading control.

higher for UVB radiation, where all the concentrations tested decreased activation in a significant manner (Figure 3B, upper section). Moreover, the protective effect of fractions V, IV and XI was quite similar for UVB- and UVA-irradiated cells. Fractions IV and XI showed the greatest protection, presenting about a 50−60% reduction in p38 activation at 5 μg/mL, while the same concentration of fraction V resulted in only a 25% decrease (Figure 3B, upper section). Furthermore, the 50−60% decrease in UVB-induced p38 activation achieved by 5 μg/mL of IV and XI was obtained only at a concentration 2-fold higher of OW (Figure 3B, upper section). When cells were UVA-irradiated, the highest concentration of OW (20 μg/mL) did not reduce p38 activation to the same extent as IV and XI at 5 μg/mL (Figure 3B, upper section). As observed for p38, protection against UV-induced JNK1/2 activation by the fractions was generally higher for UVB-irradiated cells and was dose-dependent, as observed for OW (Figure 3B, lower section). However, in contrast to the findings for p38 activation, the protective effect of OW and V, IV and XI did not differ greatly, showing a 20−40% decrease in JNK1/2 activation at 5 μg/mL for all the fractions and about a 60% reduction only for the highest concentration of OW (20 μg/mL) (Figure 3B).

DISCUSSION

UV radiation-induced oxidative stress in skin cells caused by the generation of intracellular ROS results in cell damage and ultimately in apoptosis. Several studies using *in vitro* and *in vivo* systems have demonstrated that grape proanthocyanidins prevent UV-induced skin damage.[19] However, much less is known about the relationship between the structure of polyphenols and their photoprotective capacity. Here we tested the protective effect of several grape-derived polyphenolic fractions, previously characterized in our group,[27] against UV-induced oxidative damage. Particular attention was paid to the differences in polymerization and galloylation, measured as mean degree of polymerization and percentage of galloylation. Thus, we used fractions (IV and XI) composed of large oligomers (mDP 2.7 and 3.7, respectively) and a high percentage of galloylation (% G 25 and 31, respectively), a fraction (V) that contained only monomers without gallate groups, and a fraction with moderate characteristics from which the other fractions were generated (OW, mDP 1.7 and % G 15%). We demonstrate a statistically significant increase in intracellular ROS in keratinocytes HaCaT exposed to either UVA or UVB radiation, although this increase was much more marked after UVA exposure. Our results show that the polyphenolic fractions acted through a free radical scavenging dependent pathway to inhibit UV-induced oxidative stress. Thus, a 6 h pretreatment of cells with these fractions resulted in a decrease in UVA- or UVB-induced ROS generation, except for the monomeric nongalloylated fraction V, which failed to exert a protective effect. Similar results were obtained for nonirradiated cells pretreated with the fractions. This observation indicates the powerful radical scavenger capacity of these grape polyphenolic fractions, which also decreased the baseline levels of intracellular ROS. We propose that the level of photoprotection is related to the procyanidin size and galloylation from inactive nongalloylated monomers (fraction V) to the most efficient oligomers (mDP 3.7) with 31% galloylation (fraction XI) (Figures 1A and 1C). Fractions IV and OW, having intermediate galloylation and polymerization levels, showed a moderate capacity to decrease intracellular ROS. We detected a clear relationship between radical scavenging activity and the prevention of ROS formation. Therefore, exogenous supplementation of grape fractions may reverse the oxidative imbalance produced by UV-radiation through the scavenging and quenching of ROS. The concentrations of polyphenolic fractions used (5, 10, and 20 μg/mL for the total fraction OW and 5 μg/mL for the derived fractions IV, V and XI) are more efficient at protecting HaCaT cells than other natural products. For instance, 30 μg/mL of a red orange extract protects human keratinocytes from UVB-induced damage[34] and 15 μg/mL of quercetin has been proved to protect these cells from UVA-induced oxidative stress.[35] Moreover, the concentrations assayed can be easily reached *in vivo*. Since 1 to 5% of topically applied catechin samples penetrate the epidermis,[36] preparations containing 1% of catechins would deliver the active concentrations used here. Nonetheless, the observation that the monomers did not protect keratinocytes from radiation may indicate that they do not penetrate the cellular membrane as efficiently as the oligomers. This notion is consistent with previous experiments that stressed the relevance of procyanidin size for surface effects.[37]

JNK and p38 are activated by oxidative stress, thus suggesting that ROS serve as second messengers.[38] These intracellular cascades trigger many transcription factors, such as the activator protein-1 (AP-1), the signal transducers and activators of transcription-1 (STAT-1) and the tumor protein p53, which regulate the expression of genes implicated in cell differentiation, survival and apoptosis.[39,40] Here we demonstrate that UVA and UVB radiation induced p38 and JNK1/2 activation in HaCaT and that this activation was higher after UVA exposure (Figure 2), correlating with their capacity to induce ROS generation (Figure 1B). As the total amount of MAPKs did not decrease during the postirradiation incubation times, we propose that the

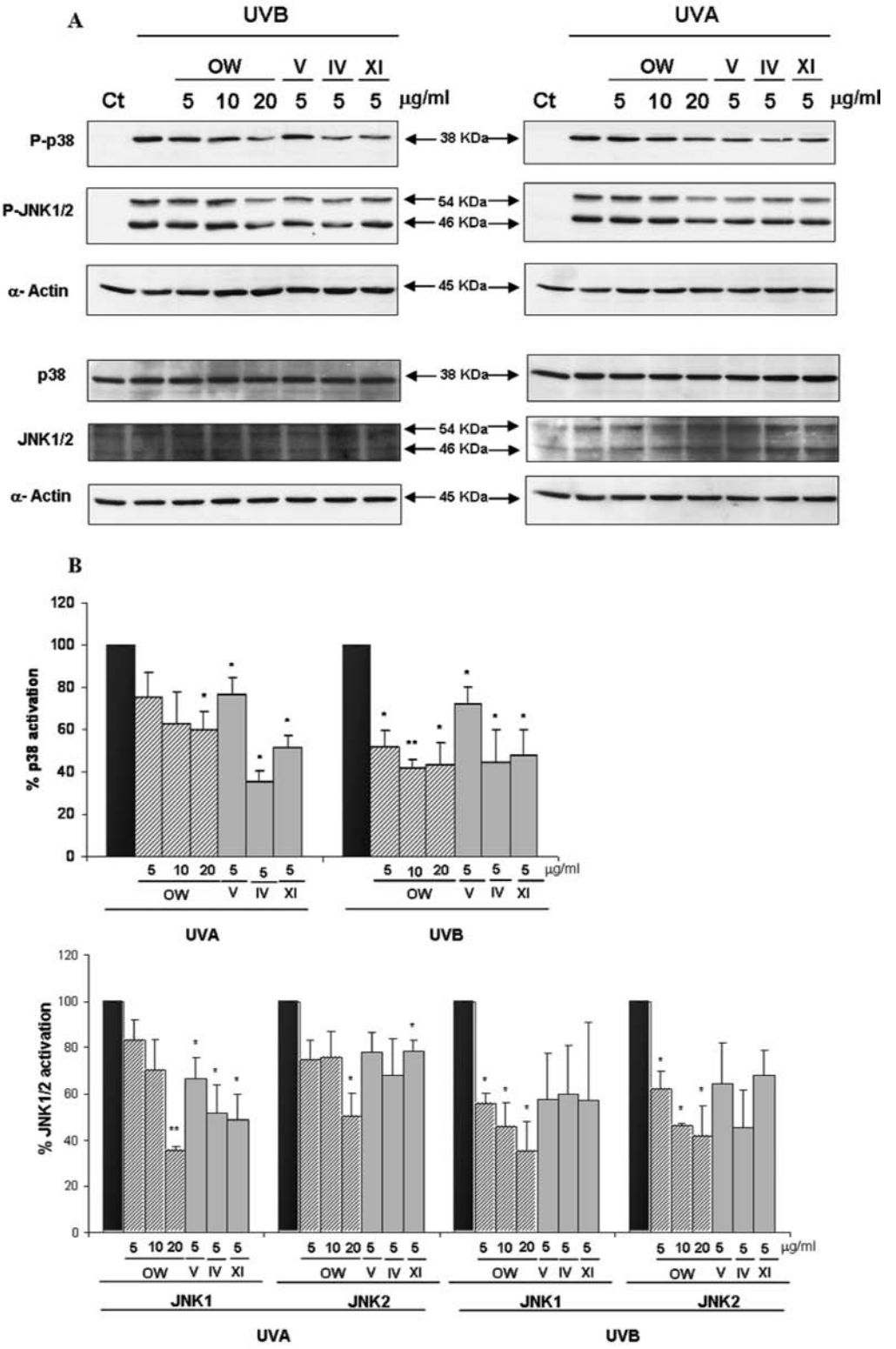

Figure 3. Effect of the polyphenolic fractions on UV-induced p38 and JNK1/2 activation. Serum-deprived HaCaT cells were pretreated with total or derived polyphenolic fractions (OW, IV, V and XI) at the indicated concentrations for 6 h before exposure to 20 J/cm^2 UVA or 0.05 J/cm^2 UVB. (A) To detect phosphorylated and total p38 and JNK1/2, Western blot analysis using phospho- and total-specific antibodies was performed, using actin expression as loading control. (B) The levels of phosphorylated and total p38 and JNK1/2 were quantified by densitometric scanning and percentage of activation was calculated by referring the density of phospho-p38 or -JNK1/2 to density of the corresponding actin bands and considering the density of UV-radiated nontreated cell bands as 100% activation.

reduction in the phosphorylation levels is specific (Figure 2). Once the stress-activated MAPKs act by triggering several transcription factors, which regulate the repair mechanisms and cell death, they are deactivated by a range of mechanisms. For instance, the

dx.doi.org/10.1021/jf103692a |J. Agric. Food Chem. 2011, 59, 4489–4495

wild-type p53-induced phosphatase (Wip1) is induced by p53 in response to stress, which results in the dephosphorylation of proteins such as p38. Interestingly, Wip1 is activated via the JNK-c-Jun and p38-p53 signaling pathways.[41] A 6 h pretreatment of cells with the grape fractions caused a decrease in the active forms of p38 and JNK1/2 (Figure 3). The same concentration (5 μg/mL) of oligomeric grape procyanidins rich in gallate groups (fractions IV and XI) reduced the activation of these MAPKs more efficiently than the monomeric nongalloylated fraction V and the OW fraction, which had moderate polymerization and galloylation (Figure 3). In addition, dose-dependent inhibition was observed for the total fraction OW (Figure 3). The decrease in UV-induced ROS produced by the polyphenolic fractions inhibited p38 and JNK1/2 activation, which may in turn inhibit the activation of nuclear transcription factors downstream of these pathways, thus attenuating the transcription of proteins involved in proapoptotic responses and, thus, protecting skin from UV-induced cell damage and death.[42] It is important to emphasize that our data indicate that the grape fractions did not directly inhibit the stress-activated MAPKs, but the oxidative cell levels. Otherwise, lack of JNK and p38 activation in the presence of high ROS would prevent the activation of repair mechanisms or apoptosis and would be harmful for the organism.

In summary, our results provide evidence that galloylated procyanidin oligomers are more effective protective agents than nongalloylated monomers against oxidative damage induced by UVA and UVB radiation on human keratinocytes and epidermis. The total scavenging potential provided by the number of phenoxyl groups is directly related to the photoprotective capacity of grape polyphenols. In addition, small, flexible and amphiphilic procyanidin oligomers (mDP around 3) may contribute to the overall action of their hydroxyl moieties by facilitating interactions with biological membranes. Thus, our findings highlight the relevance of specific structural characteristics, such as polymerization and galloylation, for the photoprotective effects exerted by grape-derived polyphenols. These results support further research and should be taken into consideration into the clinical pharmacology of photoprotective plant-derived agents.

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Funding Sources

The authors would like to thankfully acknowledge the research project grants SAF2008-00164 and AGL2006-12210-C03-02/ALI from the Ministerio de Ciencia e Innovación, and from Red Temática de Investigación Cooperativa en Cáncer, Instituto de Salud Carlos III, Spanish Ministry of Science and Innovation & European Regional Development Fund (ERDF) "Una manera de hacer Europa" (ISCIII-RTICC Grants RD06/0020/0046 and RD06/0020/0010). It has also received financial support from the AGAUR-Generalitat de Catalunya (grant 2009SGR1308, 2009 CTP 00026 and Icrea Academia award 2010 granted to M. Cascante).

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