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Inhibition of UVB-induced skin phototoxicity by a grape seed extract as modulator of nitrosative stress, ERK/NF-kB signaling pathway and apoptosis, in SKH-1 mice

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ABSTRACT

Molecular mechanisms concerning the modulation of nitrosative stress, signal transduction and proliferation/apoptosis by a grape seed extract, Burgund Mare variety (*BM*), in SKH-1 mice exposed to UVB, were investigated. The animals were irradiated with single and multiple doses of UVB in 10 consecutive days. In each experiment were used five groups of animals: control, vehicle, UVB irradiated, vehicle + UVB, *BM* + UVB. The extract was applied topically, 30 min before each UVB exposure, in a dose of 4 mg total polyphenols/cm^2. *BM* remarkably inhibited UVB-induced activation of inducible nitric oxide synthase (iNOS) and therefore generation of nitric oxide (NO) and nitrotyrosine, in a UVB single dose regimen. *BM* also suppressed NF-kB activation by UVB but did not affect the activity of total ERK 1/2. In multiple UVB irradiations, *BM* increased NO formation and total ERK 1/2 activity and reduced iNOS activity and nitrotyrosine levels, inhibited cell proliferation, diminished p53 and caspase-3 immunoreactivities and increased the percentage of Bcl-2 positive cells. We concluded that *BM* modulates the apoptotic response of SKH-1 mice skin in UVB irradiation by the inhibition of p53, caspase-3, Bax/Bcl-2 and proliferating cell nuclear antigen expressions, as well as by reducing the activation of iNOS and NF-kB.

1. Introduction

The non-melanoma skin cancers are the most frequently diagnosed cutaneous malignancies in humans, accounting for over 3.5 million cases, each year, in the Unites States (Rogers et al., 2006). Various factors are incriminated in the etiology of these cancers, the leading role being played by the genetic and environmental elements, particularly solar ultraviolet radiation (UV). The experimental and epidemiological data suggest that UVB (λ = 280–320 nm) is the most damaging of solar wavelengths because it acts as a complete carcinogen in initiating and promoting carcinogenetic process (Chilampalli et al., 2011). It has been demonstrated that UVB radiation induces oxidative DNA damage and formation of dimeric photoproducts between adjacent pyrimidine bases (Afaq et al., 2009). In addition, UVB determines mutations in key regulatory genes (Melnikova et al., 2005; De Gruijl and Robel, 2008), inflammation (Nichols and Katiyar, 2010), immunosuppression (Schwarz et al., 2008), cell proliferation causing photoaging and finally skin cancers (Bowden, 2004; Afaq and Mukhtar, 2006).

An essential role, in the cellular response to UVB plays the tumor suppressor protein p53. When the DNA damage is excessive, p53 prevents replication of the damaged DNA until it is repaired. In the case of unsuccessful reparation, p53 induces apoptosis via

Abbreviations: AP-1, activator protein 1; Apaf, apoptotic protease activating factor-1; ABTS, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; CPDs, cyclobutane pyrimidine dimers; DAB, diaminobenzidine tetrahydrochloride; DPPH, 2,2-diphenyl-l-picryl-hydrazyl; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal regulated-kinase 1/2; GSP, grape seeds proanthocyanidins; HPLC, high performance liquid chromatography; IKKα, serine/threonine kinase α; IL, interleukin; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MnSOD, manganese superoxide dismutase; NO, nitric oxide; NF-kB, nuclear factor-kB; $ONOO^-$, peroxynitrite; PBS, phosphate buffered saline; p53, tumor suppressor protein; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDS, sodium dodecylsulfate; TNF, tumor necrosis factor; TP, total polyphenols; UVB, ultraviolet radiation B.

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induction of p21waf1/cip1, Bax, apoptotic protease activating factor-1 (Apaf-1) and also by activation of caspases cascade (Amin et al., 2009).

UVB-generated reactive oxygen species (ROS) affect the regulation of gene expression of signaling molecules such as mitogen activated protein kinases (MAPKs) and transcription factors (Heck et al., 2004) (NF-kB), increase MMP expression and contribute to apoptosis (Fischer and Voorhees, 1998; Kang et al., 2003). Besides ROS, reactive nitrogen species (RNS), including nitric oxide (NO), play an important role in lipids and proteins oxidation and DNA damage with important consequences in cellular homeostasis. Furthermore, NO can react with superoxide radical to produce peroxynitrite anion ($ONOO^-$), a toxic product involved in apoptosis, DNA cleavage (Katiyar and Mukhtar, 2001; Szabo, 2003) and also in mutagenesis and carcinogenesis (Nguyen et al., 1992; Liu and Hotchkiss, 1995). Peroxynitrite molecule nitrates the aromatic amino acid, tyrosine, producing nitrotyrosine (Gow et al., 1996), which is used as a marker of the *in vivo* peroxynitrite production (Giorgio et al., 1996; Hattori et al., 1996; Rawlingson et al., 2000). Recent studies have shown that peroxynitrite indirectly activates Bad and down-regulates Bcl-2 genes, this way promoting apoptosis (Tagawa et al., 2008) and induces activation of MAPK and transcription factors, such as NF-κB and AP-1 (Cooke and Davidge, 2002).

The main enzyme involved in the generation of large amounts of NO following UVB exposure, is iNOS (Lee et al., 2000). Data in literature, concerning the role of NO in apoptosis process, are rather contradictory and generally assert that NO has a dualistic role, protecting either the keratinocytes against apoptosis (Deliconstantinos et al., 1996; Weller et al., 2003) or inducing cellular toxicity by proteins nitration with further alteration of their functions (Poderoso et al., 1996). Therefore, inhibition of NO production by iNOS expression or blockage of its activity may be a useful tool in the neutralization of NO-related undesired conditions. Some experimental data suggest the existence of connections between NF-kB and iNOS (Sasaki et al., 2000). NF-kB, on the one hand, regulates the expression of different genes involved in the inflammatory response including the iNOS and, on the other the promoter region of the gene encoding iNOS contains two NF-kB binding sites (Xie et al., 1994).

All these events require the development of new strategies aiming to protect the skin against the noxious effects of UVB. In recent years, grapes have received considerable interest (Aziz et al., 2005; Shrikhande, 2000) due to their antioxidant properties and potential benefits in preventing degenerative diseases and different types of cancers (Jung et al., 2006; God et al., 2007). Several studies have shown that polyphenols from grape seeds have anti-inflammatory, antimutagenic effects (Mittal et al., 2003; Shi et al., 2003; Vayalil et al., 2004), and inhibit the oxidative stress-mediated activation of MAPK and NF-kB pathways in mouse skin carcinogenesis (Katiyar et al., 2001; Vayalil et al., 2003). The pleiotropic effects of grape seeds are considered to be due to a complex mixture of various flavonoids, especially (+)-catechins, (−)-epicatechin and procyanidin polymers (Amico et al., 2004; Shrikhande, 2000).

In our previous works, we found that crude grape seed extract, Burgund Mare variety (*BM*), protected SKH-1 hairless mice skin against UVB-induced damage by reducing the lipid peroxidation and nitric oxide production, and also by diminishing caspase-3 activity (Filip et al., 2011b) and cytokine levels (IL-6 and TNF-α) (Filip et al., 2011a). In addition, *BM* extract inhibited formation of sunburn cells and cyclobutane pyrimidine dimers (CPDs) induced by single or multiple doses of UVB irradiation (Filip et al., 2011c). At a low dose (2.5 mg total polyphenols-TP/cm^2), the *BM* extract applied topically before each UVB exposure, for 10 consecutive days, restored the superoxide dismutase (MnSOD) activity and reduced the IL-1β levels. These effects were preserved even if *BM* extract was applied post UVB exposure (Filip et al., 2011c).

Although, the research on grape seeds extracts has been concentrated on their effects as a chemopreventive agent, the action mechanisms, especially in chronic exposures, are still not well understood.

In the present study, we explored comparatively the mechanisms involved in the modulatory effect of *BM* grape seeds extract in acute and 10 days UVB-mediated activation of intracellular signaling pathways in connection with proliferation, apoptosis and nitrosative stress, in mice skin. This animal model of multiple UVB exposures corresponds to a human exposure to a daily solar UV irradiation in the sunny time of the year.

2. Materials and methods

2.1. Reagents

Sodium dodecylsulfate (SDS), Triton X-100, 2,2-diphenyl-l-picryl-hydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS), NADPH-dependent nitrate reductase, β-NADPH, N-(1-naphthyl)ethylenediamine hydrochloride, glucose-6-phosphate, glucose-phosphate dehydrogenase, sulfanilamide, Bradford and Folin–Ciocalteu reagents were from Sigma–Aldrich Chemicals GmbH (Germany). ELISA tests for total p44/42MAPkinase (ERK 1/2) and phospho-NF-kB p65 (ser536) measurements were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). ELISA tests for evaluation of iNOS activity were from USCN Life Science Inc. (China) and for nitrothyrosine levels were obtained from Blue Gene (China). Polyclonal rabbit p53 antibody (NCL-p53-CM5p, 1:500), Peroxidase Block, diaminobenzidine tetrahydrochloride (DAB) and Novostain Universal Detection Kit reagents were purchased from Novocastra Laboratories Ltd. (Newcastle upon Tyne, United Kingdom). Polyclonal rabbit anti-Bax antibody (ab7977, 1:50) and monoclonal mouse anti-Bcl-2 antibody (ab692, 1:50) were from Abcam (Cambridge, UK). Polyclonal rabbit anti-caspase-3 antibody (PAK0488, 1:50) was obtained from Linaris (Wiesbaden, Germany) and monoclonal mouse anti-PCNA antibody (M0879, 1:200) from DAKO.

2.2. Vegetal materials and high performance liquid chromatography (HPLC-MS) analysis

The hydroethanolic extract from grape seeds (*Vitis vinifera* L.), variety Burgund Mare (Romania), was prepared, as previously described, from 1:20 w/v mixture of finely powdered dried seeds and water/ethanol 50/50 (v/v) (Postescu et al., 2007). Its total polyphenolic content was measured spectrophotometrically by Folin–Ciocalteu method (Singleton et al., 1999). The antioxidant capacity of *BM* was higher than in gallic acid, measured both by electron paramagnetic resonance technique (k_{BM} = 0.017, $k_{gallic\ acid}$ = 0.013) (Filip et al., 2011c) or by using ABTS (52.89 ± 0.02 vs. 6.3 ± 0.4 eq. mM Trolox) (Filip et al., 2011a) and free radical DPPH tests (0.072 ± 0.002 vs. 0.083 ± 0.004 mmol/mmol DPPH) (Dicu et al., 2010). The previously published HPLC data (Perde-Schrepler et al., 2013) indicated the presence of procyanidin B, catechin hydrate, epigallocatechin, epicatechin and gallic acid as main compounds.

For topical use, the extract was evaporated (in vacuo) to dryness and resuspended in 40% acetone, then adjusted to the desired concentration and stored at 4 °C prior to use.

2.3. Animals and experimental protocol

Female SKH-1 hairless mice (8 weeks old) from Charles River (Germany) were acclimatized for a 1 week under the following conditions: 12-h light/12-h dark cycle, 35% humidity, free access to water and fed by a normocaloric standard diet (VRF 1). In order to evaluate the effects of *BM* extract two series of experiments were performed. In experiment I, the mice were exposed to a single dose of UVB and in experiment II to multiple doses of UVB irradiation within 10 consecutive days. In experiment I, five groups of 12 animals each, were treated as follows: Group 1 – untreated control; Group 2 – treated with vehicle (acetone 40%); Group 3 – UVB irradiated (240 mJ/cm^2); Group 4 – vehicle + UVB irradiated; and Group 5 – *BM* + UVB irradiated. In experiment II were used 8 animals exposed to multiple doses of UVB (240 mJ/cm^2, for 10 days) and groups pretreated with vehicle and respectively *BM* extract before UVB irradiation (n = 8). The results were compared with control and vehicle treated groups. Before any treatment the animals were anaesthetized with an i.p. injection of ketamine xylazine cocktail (90 mg kg^{-1} b.w. ketamine, 10 mg kg^{-1} b.w. xylazine). The *BM* extract was applied topically on the skin in a dose of 4 mg TP/cm^2, in vehicle (acetone 40%), 30 min before each UVB exposure. UVB irradiation was performed with Waldmann UV 181 broadband UVB source as described before (Filip et al., 2011a,b). The UVB emission was monitored with a radiometer Variocontrol radiometer (Waldmann GmbH, Germany). At 24 h after the last treatment, fragments of dorsal skin from animals were excised under