

A *Polypodium leucotomos* extract inhibits solar-simulated radiation-induced TNF- α and iNOS expression, transcriptional activation and apoptosis

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Abstract: In this report, we have examined the molecular basis of the photoprotective effect of a hydrophilic extract of the fern *Polypodium leucotomos* (PL) *in vitro*, using a solar simulator as the source of UV radiation (SSR). We found that pretreatment of human keratinocytes with PL inhibited SSR-mediated increase of tumor necrosis factor (TNF)- α and also abrogated nitric oxide (NO) production. Consistent with this, PL blocked the induction of inducible nitric oxide synthase (iNOS) elicited by SSR. In addition, PL inhibited the SSR-mediated transcriptional activation of NF- κ B and AP1. Finally, we demonstrated that pretreatment

with PL exerted a cytoprotective effect against SSR-induced damage, resulting in increased cell survival. Together, these data postulate a multifactor mechanism of protection not exclusively reliant on the antioxidant capability of PL, and strengthen the basic knowledge on the photoprotective effect of this botanical agent.

Key words: apoptosis – HaCaT – iNOS – photoprotection – polypodium – TNF-alpha

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Introduction

Single or cumulative exposure to solar ultraviolet radiation (UV) results in severe damage to the skin (1,2). Lesions caused by UV exposure to skin include sunburn, immunosuppression, changes in pigmentation, photoaging and skin cancer (3–9). The general mechanism of photodamage is complex, but some of the responses that cause these deleterious effects are known, such as direct DNA damage and *in situ* generation of reactive oxygen species (ROS) and free radicals leading to inflammation, apoptosis and necrosis (3,8,10). There is also a clear synergy between these mecha-

nisms (i.e. oxidative stress can modulate the inflammatory response, and so forth).

The UV-induced inflammatory response includes inflammation and erythema caused in part by vasodilatation, triggering of cytokine production, which causes infiltration of immune cells and activation of transcriptional pathways (7,11–13). Some of these pathways are aimed to perpetuate inflammation, whereas others are photoprotective countermeasures. Among these, production of nitric oxide (NO) and the enzyme responsible of its synthesis, inducible nitric oxide synthase (iNOS), are key elements in the response against UV aggression. It has been shown that UVB irradiation controls iNOS activity through a mechanism that involves cytokine secretion (14). Increased NO prevents UV-induced apoptosis by inhibiting caspase-3 activity through S-nitrosation (15–18) and/or inducing expression of the anti-apoptotic factor Bcl-2 (19), but exacerbated production of NO may result in additional damage and

Abbreviations: AA, ascorbic acid; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PL, *Polypodium leucotomos* extract; ROS, reactive oxygen species; SSR, solar-simulated radiation; TNF, tumor necrosis factor; UV, ultraviolet (radiation).

apoptosis (20), highlighting the need for a fine regulation of these pathways.

Thus, UV irradiation initiates a complex response that includes direct photodamage and also elicits a powerful transcriptional and immune response that is composed of a multitude of regulatory feedback loops. This complicated picture can be used, however, to assess the effect and molecular mechanisms of photoprotection induced by a wide array of compounds with photoprotective properties, from direct photon acceptors (sunscreens), to antioxidants and immune and transcriptional modulators.

We have previously described the beneficial effect of an extract of the fern *Polypodium leucotomos* (PL), preventing acute sunburn and minimizing photoaging changes (21,22). These results were further supported by its *in vitro* photoprotective effect. PL inhibits the ROS formation induced by UV irradiation (23), prevents *t*-UCA isomerization (24) and possesses anti-inflammatory properties (25). In addition, orally administered PL greatly decreased sunburn and UV-induced mast cell infiltration in the skin, and reduced the loss of epidermal Langerhans cells of the skin associated with UV exposure (26,27). However, the molecular mechanisms of photoprotection were explored only partially (28).

In this report, we describe some of the molecular effects underlying the photoprotective effect of PL on human keratinocyte cell line, HaCaT cells, using solar-simulated radiation (SSR) and UVB. PL counteracts SSR-dependent induction of tumor necrosis factor (TNF)- α expression and production of NO; the latter involves abrogation of iNOS up-regulation induced by UV light. This correlates well with inhibition of the activation of pro-inflammatory, UV-induced transcription factors, such as NF- κ B and AP1. Finally, we demonstrate that the photoprotective mechanisms of PL on human keratinocytes result in decreased damage of cell membrane and cell death.

Materials and methods

Cells and reagents

HaCaT cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified eagle's medium (DMEM) medium supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂ atmosphere. Hydrophilic extract of PL was prepared as follows: Dry *P. leucotomos* leaves were extracted employing water at 55°C as solvent, allowing recirculation until the sample reached a plateau of antioxidant activity [determined by FRAP (29)] and phenolics concentration [determined by the Folin-Ciocalteu method (30)]. Such conditions were generally achieved after approximately 4 h of processing. Finally, the extract was vacuum-concentrated and kept light-shielded at 4°C until use. All other reagents

were from Sigma Chemical Co. (St Louis, MO, USA), unless and otherwise stated.

ELISA and antibodies

The TNF- α ELISA kit was purchased from Endogen (Woburn, MA, USA). Antibodies against iNOS and α -tubulin were from BD Transduction Laboratories (Mountain View, CA, USA), and Sigma respectively. Horseradish peroxidase-labelled goat anti-rabbit IgG and enhanced chemiluminescence (ECL) Western blotting detection reagent were from Amersham (Buckinghamshire, UK).

Plasmids

The reporter plasmids KBF-Luc and AP1-Luc have been previously described (31). The reporter plasmid pRL-null bearing a promoterless *Renilla* luciferase gene was co-transfected when indicated (5 μ g) and used to normalize all the firefly luciferase values obtained.

Solar-simulated UV radiation

A 1000 W xenon arc solar simulator (Oriel, Surrey, UK) equipped with an Oriel 81017 filter ('Colipa') was used. Spectral emission has been described previously (32). UVB and UVA irradiance measurements were performed daily using an IL-1700 radiometer (International Light, Newburyport, MA, USA) equipped with a SED240/UVB-1/TD and SED033/UVA/TD photodetectors. The radiometer was calibrated with a Solar Scope (Solatell, Croydon, UK) spectroradiometre. HaCaT cells (2×10^6 in 0.75 ml of Hank's balanced salt solution) were irradiated with the indicated doses and processed as indicated for every individual experiment.

Nitric oxide determination

Nitrite generation was measured by the modified Griess method (33). HaCaT cells were treated with PL and irradiated with SSR as described. The supernatants were collected and centrifuged to remove cell debris. Samples and sodium nitrate standards were loaded (85 μ l per well) into microtitre plates. We added 5 μ l of nitrate reductase (0.01 U per well) and 10 μ l of 2 mM β -NADH (reduced form) per well. Reactions were incubated at room temperature under continuous stirring for 2 h. Subsequently, 50 μ l N-(1-Naphthyl)-ethylenediamine (NED) and 50 μ l Dapsone were added to each well and incubated for another 10 min at room temperature. The absorbance at 550 nm was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) with reference to 620 nm. NO concentrations were estimated from the standard curve.

Transcriptional activation of NF- κ B and AP1

Cells were transfected with KBF-luc or AP1-luc reporter plasmids and incubated for 36 h prior to treatments and

irradiation. Transfected cells were treated with PL (0.5, 1, or 2 mg/ml) or L-ascorbic acid (AA: 1 mM) and different doses of SSR. The maximum transcriptional activation of the reporter plasmids was obtained with a SSR dose of $\text{UVA} = 2.4 \text{ J/cm}^2 + \text{UVB} = 0.2 \text{ J/cm}^2$, and beyond this dose there was a significant increase in cell death. Therefore the data presented were obtained using this relatively low dose of irradiation. To normalize the firefly luciferase values, in some experiments the reporter plasmid pRL-null bearing a promoterless *Renilla* luciferase gene was co-transfected. Luciferase activity was determined with the Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions and determined in a Lumat LB9501 luminometer (Berthold technologies GmbH & Co, Wildbad, Germany).

Determination of cell viability

Cell viability was determined by trypan blue exclusion and direct counting of viable cells in each condition using a haemocytometry chamber. Cell morphology and presence of cellular debris were assessed by performing the PL pretreatments and SSR irradiation on cells adhered to glass coverslips, and examined in a Leica DMR photomicroscope (Leica, Mannheim, Germany) equipped with a $20\times$ phase-contrast objective, coupled to a COHU 4912-5010 CCD Camera (COHU, San Diego, CA, USA). The acquisition software was Leica QFISH V2.1 (Leica), and images were processed with Adobe Photoshop 7.0.

Determination of cellular apoptosis

Cellular apoptosis was detected using the BD ApoAlert kit (BD Biosciences, San Jose, CA, USA), which determines annexin V binding, according to the instructions of the manufacturer. Briefly, HaCaT cells, pretreated with PL or vehicle, irradiated with SSR or not (5×10^5 cells/ml) were washed and resuspended in binding buffer and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V during 15 min at room temperature. Cells were then stained with propidium iodide ($2 \mu\text{g/ml}$) to identify non-viable cells, and analysed by FACScalibur flow cytometer (BD Biosciences). Results were expressed as the percentage of Annexin V-binding cells.

Results

Polypodium leucotomos inhibits $\text{TNF-}\alpha$, NO production and iNOS upregulation induced by UV light

To ascertain the molecular basis for the *in vivo* beneficial effect of PL upon irradiation with UV light, we assayed the effect of PL on the production of $\text{TNF-}\alpha$ induced by UV light on a well-characterized human keratinocyte cell line, HaCaT. For irradiation, we used a solar simulator lamp

emitting SSR, which we employed at two different doses: low, $\text{UVA} = 6 \text{ J/cm}^2 + \text{UVB} = 0.5 \text{ J/cm}^2$; and high, $\text{UVA} = 11 \text{ J/cm}^2 + \text{UVB} = 1 \text{ J/cm}^2$. We found that SSR induced a 10–20-fold induction in the expression of $\text{TNF-}\alpha$ in untreated cells, whereas PL greatly significantly decreased $\text{TNF-}\alpha$ induced by SSR (Fig. 1). At low SSR, inhibition of $\text{TNF-}\alpha$ expression by PL was almost complete, whereas at high SSR, inhibition reached approximately 50% (Fig. 1). To further investigate the effect of PL on other inflammatory mediators induced by UV light, we irradiated HaCaT cells with SSR and studied NO production by production of nitrite as measured by the Griess method. We found that pretreatment with PL significantly decreased the production of SSR-induced NO by more than 60% (at 48 h after SSR exposure) (Fig. 2), thus suggesting a general role for PL in the limitation of inflammatory responses triggered by UV irradiation. The inhibition of NO production was explained, at least in part, by the effect of PL in iNOS upregulation. Consistent with previous reports (14), we found that UV irradiation increased the amount of iNOS expressed by HaCaT cells (Fig. 3a,b). When the cells were pretreated with PL for 2 h, upregulation of iNOS was significantly decreased (Fig. 3a,b), which provides a molecular basis for the observed reduction of NO production.

Polypodium leucotomos modulates the transcriptional activation of AP1 and NF- κB

Ultraviolet light induces the activation of transcription factors of the NF- κB and AP1 families (12,13), which have been shown to regulate the expression of both $\text{TNF-}\alpha$ and

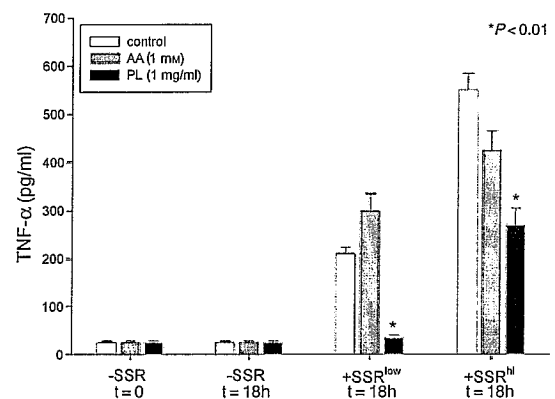


Figure 1. *Polypodium leucotomos* (PL) decreases tumor necrosis factor ($\text{TNF-}\alpha$) induced by solar-simulated radiation (SSR). HaCaT cells were treated with 1 mM L-ascorbic acid (AA) or 1 mg/ml PL for 2 h, irradiated or not with SSR (low, $\text{UVA} = 6 \text{ J/cm}^2 + \text{UVB} = 0.5 \text{ J/cm}^2$; high, $\text{UVA} = 11 \text{ J/cm}^2 + \text{UVB} = 1 \text{ J/cm}^2$), and incubated for another 6 h. $\text{TNF-}\alpha$ production was analysed by specific ELISA. Data are the mean \pm SEM of four independent experiments performed in triplicate. Asterisk represents the level of significance of PL- versus control-treated cells at each irradiation condition.

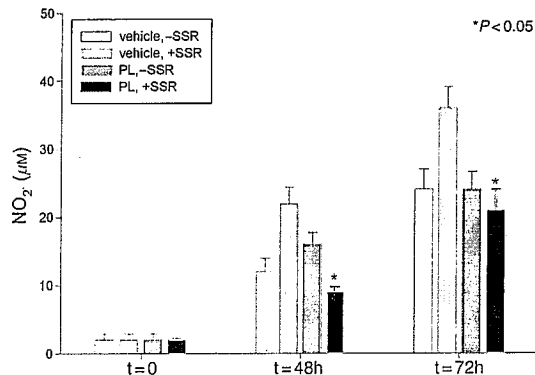


Figure 2. *Polypodium leucotomos* (PL) inhibits nitric oxide (NO) production induced by solar-simulated radiation (SSR). HaCaT cells were treated with 1 mg/ml PL for 2 h, irradiated or not with SSR (UVA = 11 J/cm² + UVB = 1 J/cm²), and incubated for another 6 h. Fresh medium was added, and NO production was analysed using the Griess method at the indicated time points. Data are the mean ± SEM of three independent experiments performed in triplicate. Asterisk represents the level of significance of PL, +SSR versus vehicle-treated, +SSR cells.

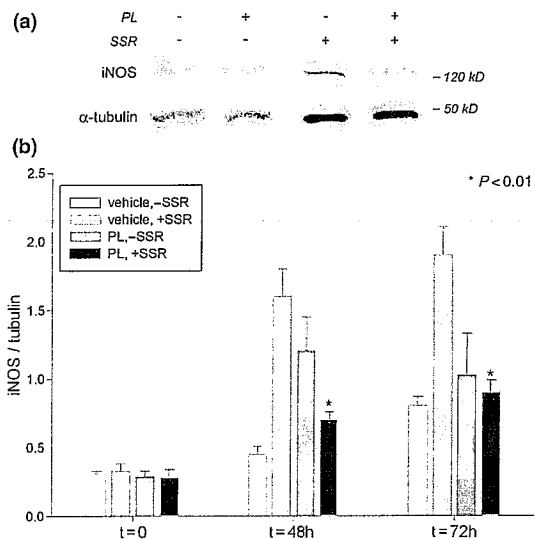


Figure 3. *Polypodium leucotomos* (PL) inhibits inducible nitric oxide synthase (iNOS) up-regulation induced by solar-simulated radiation (SSR). HaCaT cells were treated with 1 mg/ml PL for 2 h, irradiated or not with SSR (UVA = 11 J/cm² + UVB = 1 J/cm²), and incubated for another 6 h. Fresh medium was added, and iNOS expression was analysed by Western blot. α -Tubulin was used as a loading control. (a) Representative blot from samples collected 48-h postirradiation. (b) Quantification of three independent experiments. Data are the mean ± SEM of the densitometric ratio iNOS/ α -tubulin. Asterisk represents the level of significance of PL, +SSR versus vehicle-treated, +SSR cells.

iNOS (34,35). Therefore, we decided to study if the observed downregulation of inflammatory mediators induced by PL upon SSR irradiation was accompanied by

modulation of transcriptional activity. We transfected HaCaT cells with reporter plasmids AP-1-luc or KBF-luc, treated them with PL or AA and irradiated them with SSR (UVA = 2.4 J/cm² + UVB = 0.2 J/cm²). We found that SSR by itself caused a manifold increase in the level of transcriptional activation of both transcription factors (Fig. 4a,b; white bars). This increase was partially inhibited when the cells were pretreated with 2 mg/ml PL (Fig. 4a,b; dark grey bars). On the other hand, AA had no effect in transcriptional activation of either NF- κ B or AP1 under these experimental conditions (Fig. 4a,b). To confirm the specificity of these responses and rule out luciferase-related artifacts, we co-transfected the cells with the reporter plasmids described above and a promoterless plasmid encoding a luciferase from *Renilla*. We then performed experiments similar to those in Fig. 4a,b; for these, we used a concentration of 1 mg/ml PL (Fig. 4c,d). We observed a significant inhibition of SSR-induced activation of both NF- κ B and AP1 even when normalized to *Renilla* luciferase, which confirmed the specificity of our results.

Polypodium leucotomos prevents cytotoxic damage and apoptosis induced by UV light in HaCaT cells

The photoprotective effect of PL in terms of cell viability has been previously documented in different cell types (28). To ascertain its effectiveness in protecting keratinocytes, we pretreated HaCaT cells seeded on glass coverslips coated with 25 μ g/ml collagen I, a previously reported extracellular adhesion protein that supports HaCaT cell adhesion (36). We found that treatment with PL alone induced a morphological change in HaCaT cells, inducing a modest scattering and loss of cell-cell contacts, that however did not represent increased cell death and/or apoptosis (Fig. 5a, compare upper left to lower left panel, and data not shown). On the other hand, irradiation with SSR induced loss of cell-cell contacts, blebbing, rounding and other aberrations in cell morphology, as well as detachment and cell death, as evidenced by increased amounts of debris in irradiated cells (Fig. 5a, upper right panel). Pretreatment of cells with PL partially alleviated these effects; the cells remained spread, their morphology resembled that of PL-treated, non-irradiated cells and the amount of debris was considerably lower (Fig. 5a, lower right panel). Increased viability was confirmed by trypan blue exclusion assay (data not shown).

One of the mechanisms of UV-induced cell death is increased apoptosis (10,37,38). We assessed the cytoprotective effect of PL measuring binding of annexin V to the membrane of UV-irradiated HaCaT cells. SSR irradiation induced a manifold increase in annexin V binding, which was partially counteracted by pretreatment of the cells with PL (Fig. 5b). We conclude that one of the possible protective mechanisms of PL is based on its cytoprotective effects.

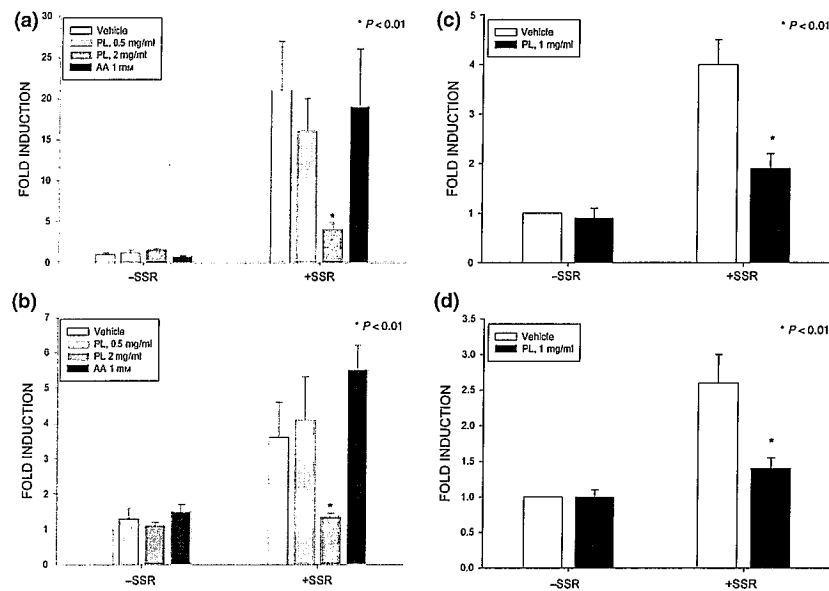


Figure 4. *Polypodium leucotomos* (PL) modulates the activity of AP1 and NF- κ B induced by UV light in HaCaT cells. HaCaT cells were transiently transfected with AP-1-luc (a) or KBF-luc (b) reporter plasmids, pretreated with the indicated doses of PL or AA for 2 h after 36 h post-transfection, irradiated or not with solar-simulated radiation (SSR) (UVA = 2.4 J/cm² + UVB = 0.2 J/cm²), and incubated for another 6 h. Luciferase activity was quantified and normalized to total protein measured by bicinchoninic acid (BCA) assay. Data are the mean \pm SEM of three experiments performed in quadruplicates. (c, d) Cells were co-transfected with AP-1-luc (c) or KBF-luc (d) reporter plasmids, and a promoterless *Renilla* luciferase plasmid, treated and irradiated as in (a) and (b) (PL = 1 mg/ml), and luciferase activities quantified and normalized to *Renilla* luciferase activity and total protein. Data are the mean \pm SEM of three experiments performed in quadruplicates. Asterisk represents the level of significance of SSR-irradiated, PL- versus control-treated cells.

Discussion

Systematic UV irradiation of the skin results in a wide array of injuries, that can be divided into immediate (erythema, swelling, discomfort and pain) and long-term (chronic inflammation, photoaging, skin cancer). One of the goals of modern applied dermatology is to find substances that not only act as UV photon acceptors, e.g. filters or physical screens, but that can also reverse or ameliorate long-term damage to the skin. In this report, we have analysed *in vitro* the molecular bases of the effect of a photoprotective natural product, PL. PL has been previously reported as an efficient and a photoprotective substance (22,24), ameliorating inflammation and photoaging in the animal model and volunteer psoralen and UVA (PUVA)-therapy patients, most notably after oral administration (22,26). However, the molecular mechanisms by which PL exerts its photoprotective effect are far from understood. Using human keratinocytes, we have previously shown that PL efficiently prevents loss of cell viability and proliferation induced by UVA irradiation alone (28). Conversely, this report contains data collected using a SSR system, which allows studying the nature of UV-dependent damage in a more accurate manner, as SSR closely resembles the type of irradiation

that occurs naturally (32). In agreement with previous data, PL exerts a photoprotective effect, inhibiting cell death caused by SSR irradiation.

Ultraviolet irradiation has been shown to activate pro-inflammatory transcription factors, such as NF- κ B and AP1. On one hand, NF- κ B activation includes the phosphorylation, ubiquitination and degradation of I κ B, but the whole mechanism has not been completely deciphered (39). On the other hand, AP1 activation requires activation of the mitogen-activated protein kinase (MAPK) pathway (40). NF- κ B and AP1 activation are part of the defensive response against oxidative stress caused by UV-dependent ROS generation, but aberrant or sustained activation can cause malignant transformation (41). In agreement with the previous observations, SSR induced activation of both NF- κ B and AP1 in HaCaT cells, which was partially inhibited by treatment of the cells with PL. This is a specific effect of PL that cannot be completely ascribed to its antioxidant properties, as treatment of the cells with a *bona fide* antioxidant (AA) did not prevent SSR-induced activation. However, this does not rule out specific antioxidant activities of PL that are not mimicked by AA. Our preliminary results showed that the transcriptional effect of PL does not involve inhibition of the binding of NF- κ B or AP1 to their DNA consensus sequences (data not shown).

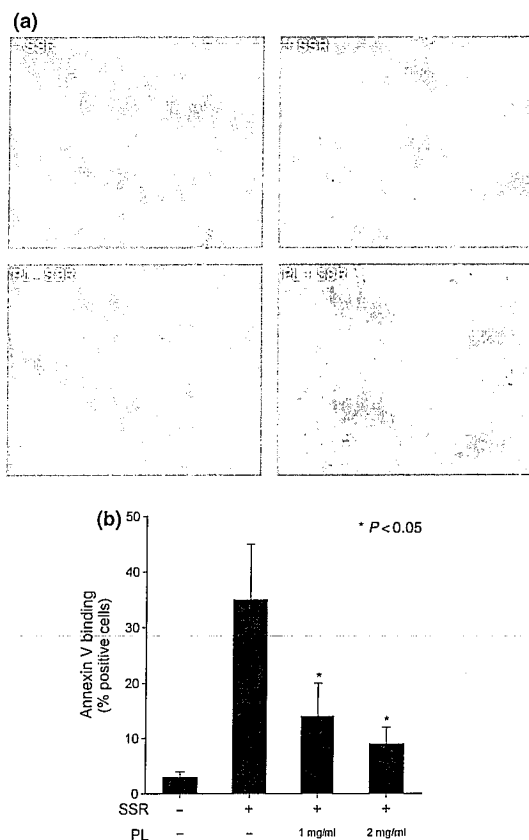


Figure 5. *Polypodium leucotomos* (PL) prevents morphological damage and apoptosis induced by UV light in HaCaT cells. (a) HaCaT cells were seeded on glass coverslips for 24 h, pretreated or not with 1 mg/ml PL, irradiated with solar-simulated radiation (SSR) (UVA = 11 J/cm² + UVB = 1 J/cm²) and incubated for another 6 h. Microphotographs were taken of representative fields. (b) HaCaT cells were pretreated or not with the indicated amounts of PL for 2 h, irradiated as in (a), incubated for another 6 h, and annexin V binding was assessed by flow cytometry. Data are the mean \pm SEM of four independent experiments performed in triplicates. Asterisk represents the level of significance of SSR-irradiated, PL- versus control-treated cells.

In addition, PL downregulates TNF- α and iNOS expression induced by SSR. UV radiation induces TNF- α expression (42). Interestingly, UV irradiation also induces expression of TNF- α receptors (43), which suggests that TNF- α mediates the inflammatory reaction in skin induced by UV irradiation. On the other hand, iNOS is also induced by UV irradiation in HaCaT cells (44). Increased iNOS expression causes elevated levels of NO, which is one of the causal factors of erythema formation. The inhibitory effect of PL on NO production and iNOS expression strongly suggests that NO increase is caused by *de novo* synthesized iNOS. Finally, TNF- α and iNOS up-regulation are linked to increased apoptosis [reviewed in Refs.

(45,46)], thus inhibition of UV-mediated TNF- α and iNOS expression by PL is a likely cause for the observed decrease in apoptosis.

In summary, these findings greatly extend previous observations on the photoprotective effect of PL, and represent a rigorous approach to explain its molecular basis, providing a rationale for increased cell survival, decreased inflammation and changes in pigmentation observed both *in vivo* and *in vitro*.

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