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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Level of miR-21-5p, miR-31-5p, and miR-205-5p in control skin and in cSCC was determined by qPCR and normalized to U18 (a), U24 (b), U44 (c), and U48 (d) resulting in variable significances. The mean is indicated by the horizontal line. *$p<0.05$, **$p<0.01$, ***$p<0.0001$.

Figure S2. (A) N-fold induction of miR-21-5p, miR-31-5p, and miR205-5p in cSCC vs. control skin. Difference of specific miRNA expression between cSCC and control skin was determined for each normalization resulting in variable data. Each miRNA was normalized to all snoRNAs resulting in variable significances. The red line marks the value 1. (B, C) Data analysis was additionally performed by qbase PLUS (BioGazelle), which identified the U44 to be not valuable for normalization. Results also showed significant upregulation for the three miRNAs, whereby the high SD points to the variability of the cSCCs.

Figure S3. Expression levels of four miRNAs, which show a significant increase (miR-17-5p, 106a-5p, and 155-5p) or decrease (let-7b-5p) only when normalized against U24 and/or U48. The mean is shown by the horizontal line.

Table S1. Means of $2^{-\Delta C}$T of all investigated miRNAs normalized against four snoRNAs from cSCC and from control skin.

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Letter to the Editor

NADPH oxidase is a novel target of delphinidin for the inhibition of UVB-induced MMP-1 expression in human dermal fibroblasts

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Abstract: We investigated the reported antiphotoaging effects of the major anthocyanidin delphidin and sought to identify its specific molecular target during UVB-induced MMP-1 expression. Delphinidin treatment significantly inhibited UVB-induced MMP-1 expression in primary cultured human dermal fibroblasts (HDF), an effect associated with the suppression of MKK4-JNK1/2, MKK3/6-p38 and MEK-ERK1/2 phosphorylation. Further investigation revealed that delphinidin significantly inhibited UVB-induced ROS production and NOX activity. Interestingly, the inhibitory effect of delphinidin on UVB-induced NOX activity was stronger than that of apocynin, a pharmaceutical NOX inhibitor. Fractioned cell analysis results using a Western blot assay showed that this effect occurred through the inhibition of UVB-induced $P47^{phox}$ (a NOX subunit) translocation from the cytosol to the membrane. Pull down assays demonstrated that delphinidin binds directly to $P47^{phox}$ *in vitro*. Collectively, our results suggest that delphinidin targets NOX, resulting in the suppression of UVB-induced MMP-1 expression in human dermal fibroblasts.

Abbreviations: HDF, human dermal fibroblasts; MAPKs, mitogen-activated protein kinases; MMP-1, matrix metalloproteinase-1; NOX, NADPH oxidase; ROS, reactive oxygen species.

Key words: Delphinidin – MMP-1 – NADPH oxidase – ROS – UVB

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Background

Repetitive exposure to ultraviolet light (UV) is a causative factor for various skin diseases including premature ageing and cancer. UV is composed of three subtypes, by their wavelengths: UVA (320–400 mm), UVB (280–320 nm) and UVC (200–280 nm). While UVC is blocked by the ozone layer, UVA and UVB penetrate the atmosphere and can cause pathological changes in human skin after prolonged exposure. MMP-1 (also known as collagenase-1) degrades dermal collagen and breaks down the collagen matrix, resulting in the formation of wrinkles (1). Numerous studies have demonstrated that UVB exposure can increase MMP-1 expression, leading to the subsequent degradation of dermal collagen (2,3).

Various enzyme systems are associated with endogenous ROS production, including NADPH oxidase (NOX), which plays a key role in ROS production. The classical NOX complex in phagocytes consists of 5 subunits: $P47^{phox}$, $P67^{phox}$, Rac2, $gp91^{phox}$ and $p22^{phox}$. Whereas $P47^{phox}$, $P67^{phox}$ and Rac2 are cytosolic proteins,

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gp91^{phox} and p22^{phox} are membrane-bound proteins. For the activation of NOX, the cytoplasmic p40-p47-p67^{phox} must interact with the heterodimeric p22-gp91^{phox} (4). Therefore, the intracellular location of the NOX subunit plays a critical role in its regulation (4). Several non-phagocytic NADPH oxidases also exist, which are named NPX1, NOX3, NOX4, NOX5, DUOX1/DUOX2 and NOX2 (5–7).

Delphinidin is an anthocyanin present in fruits and vegetables. Several reports have demonstrated the beneficial effects of delphinidin (8–12). Delphinidin suppressed UVB-induced 8-hydroxy-2′-deoxyguanosine (9-OHdG), oxidative stress markers and lipid peroxidation (12). Furthermore, delphinidin was observed to inhibit UVB-induced decreases in cell viability and the induction of apoptosis. However, the molecular mechanisms and direct target of delphinidin in UVB-induced MMP-1 expression has, to date, not been determined.

Questions addressed

In the present study, we demonstrate that delphinin suppresses UVB-induced MMP-1 expression and MAPKK/MAPKs phosphorylation. These effects are a direct result of decreased ROS production via the inhibition of NOX activity.

Experimental design

Cells were treated with delphinidin (10–20 μM) or apocynin (10 –20 μM) for 1 h, followed by UVB (0.02 kJ/m^2) irradiation. Lysate was collected and protein bands were visualized, while zymography was used to determine the enzymatic activity of secreted MMP-2. Gelatin (0.5 mg/ml) was used as a substrate for MMP-2 and gels were stained with 0.5% Coomassie brilliant blue. Intracellular ROS levels were measured using the fluorescent probe DCFH-DA, following a previously described procedure (13). 500 μg of the human dermal fibroblast cellular fraction was incubated with delphinidin-Sepharose 4B beads (100 μl) or Sepharse 4B beads only (as a negative control) for 6 h. Proteins were analysed by Western blotting and NOX activity was measured using a previously described protocol (14). To separate cytosolic and membrane-bound proteins, the cells were washed in phosphate-buffered saline (PBS), before harvest using a detergent-free buffer. To disrupt the cells, the mixture was subjected to four freeze-thaw cycles. Cytosolic proteins were then obtained by centrifugation at 10 000 × g for 5 min at 4°C. The suspension buffer was then added to the pellet and stirred for 1 h at 4°C. Finally, membrane-bound proteins were extracted by centrifugation at 10 000 X g for 5 min at 4°C.

Results

We investigated the effect of delphinidin on UVB-induced MMP-1 expression in human dermal fibroblasts. Delphinidin dramatically inhibited UVB-induced MMP-1 expression in human dermal fibroblasts (Fig. 1a–b). MMP-2 was used as an internal loading control. We confirmed that the concentrations of delphinidin used in the study were not cytotoxic (Figure S1).

We next investigated whether the reduction in UVB-induced MMP-1 expression by delphinidin was associated with UVB-induced MAPKK and MAPK phosphorylation. Treatment with delphinidin significantly inhibited UVB-induced MKK4-JNK1/2 (Fig. 1b), MKK3/6-p38 (Fig. 1c) and MEK-ERK1/2 (Fig. 1d) phosphorylation. These results support that delphinidin suppresses UVB-induced MMP-1 expression by inhibiting the MAPKs signalling pathway.

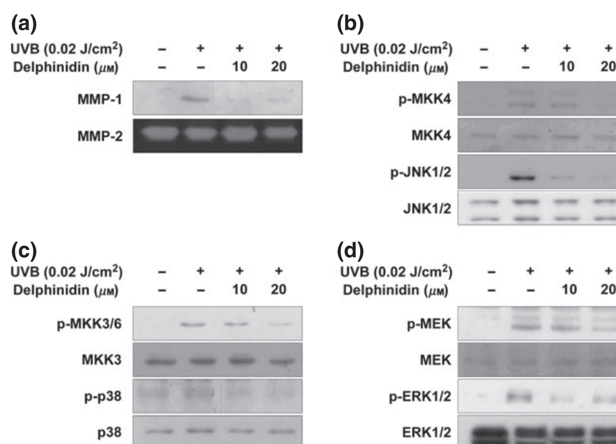

Figure 1. Effect of delphinidin on UVB-induced MMP-1 expression and phosphorylation of MAPK and MAPKK. (a) Delphinidin suppresses UVB-induced MMP-1 expression. Expression levels of MMP-1 were determined by Western blot analysis, using specific antibodies. MMP-2 activity was analysed using zymography. Delphinidin inhibits UVB-induced (b) MKK4-JNK1/2, (c) MKK3/6-p38 and (d) MEK-ERK1/2 phosphorylation. Following incubation in serum free-DMEM for 24 h, cells were pretreated with delphinidin for 1 h, and then irradiated with UVB (0.02 kJ/m^2). Data are representative of three independent experiments which gave similar results.

Previous studies have demonstrated that endogenous ROS production as a result of environmental factors (such as UVB radiation) can induce photoaging through the activation of inflammatory signalling pathways (15,16). Thus, we hypothesized that UVB irradiation increases MMP-1 expression through the promotion of endogenous ROS production, and that delphinidin suppresses UVB-induced MMP-1 expression by reducing endogenous ROS levels. To investigate the effect of delphinidin on UVB-induced ROS production, 2′,7′-dichlorofluorescein diacetate (DCF-DA) dye was used as a ROS marker. Cells stained with DCF-DA were quantified and delphinidin was observed to significantly inhibit UVB-induced intracellular ROS (Fig. 2a).

Previous studies have shown that NOX activation is closely related to ROS-induced skin ageing (17–19). To investigate whether

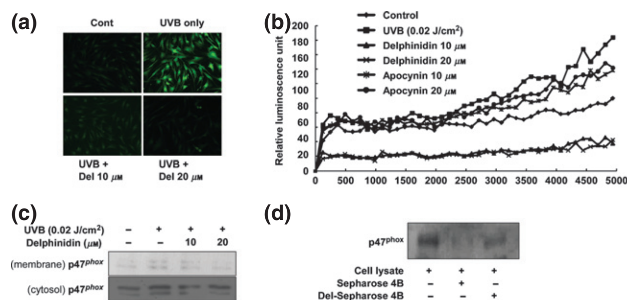

Figure 2. Effect of delphinidin on UVB-induced ROS production and NOX activation. (a) UVB-induced ROS production is inhibited by delphinidin. (b) NOX activity, as measured using a microplate luminometer (described in the Materials and Methods). UVB induces NOX activity in HDF in a time-dependent manner, while delphinidin suppresses UVB-induced NOX activity. (c) Delphinidin reduces UVB-induced p47^{phox} translocation. Cytosolic and membrane proteins were separated, followed by Western blot analysis. (d) Delphinidin directly binds to p47^{phox} in human dermal fibroblasts. p47^{phox}-delphinidin binding in HDF was detected by immunoblotting using a p47^{phox} specific antibody. Data are representative of three independent experiments which gave similar results.

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UVB-induced ROS production is related to NOX inhibition by delphinidin, we examined the effect of delphinidin on UVB-induced NOX activation. UVB irradiation increased NOX activity, while delphinidin was observed to reverse this effect (Fig. 1b).

For NOX activation, complex formation including cytosolic subunits of NOX, p40-p47-p67^{phox} is required (4). Once the complex is formed, it translocates to the membrane and binds to membrane-bound subunits of NOX. This translocation is therefore critical for NOX activation (4). Because delphinidin inhibited UVB-induced NOX activation, we hypothesized that delphinidin may inhibit UVB-induced p47^{phox} translocation to the membrane. We defined the time required for p47^{phox} translocation to occur after UVB radiation. p47^{phox} was observed to translocate to the cell membrane within 15 min after exposure to UVB (Figure S2). We then measured the effect of delphinidin on UVB-induced p47^{phox} translocation within this time frame. Pretreatment with delphinidin was found to significantly suppress UVB-induced p47^{phox} translocation (Fig. 2c). Pull down assay results demonstrated that delphinidin directly binds with p47^{phox} (Fig. 2d).

We compared the inhibitory effect of delphinidin with apocynin, a well-known inhibitor of NOX. Delphinidin treatment exerted similar effects to that of apocynin in relation to UVB-induced MMP-1 expression (Figure S3).

Conclusions

Our study demonstrates that delphinidin significantly inhibits UVB-induced MMP-1 expression in human dermal fibroblasts. The inhibitory effect of delphinidin is associated with the inhibition of NOX activity, mediated by inhibition of P47^{phox} translocation from the cytosol to the membrane as a result of direct binding to P47^{phox}. This underlines the important role of NOX in UVB-induced MMP-1 expression and suggests that the therapeutic inhibition of NOX by delphinidin may prevent photo-aging.

Acknowledgements

T.G. Lim designed the study, performed the research, analysed the data and wrote the paper; S.K. Jung, J. Kim and Y. Kim performed the research; H.J. Lee, T.S. Jang and K.W. Lee supervised the study. This work was supported by the World Class University Program (R31-2008-00-10056-0), the World Class Institute Program and the Leap Research Program Grant (2010-0029233) funded by the National Research Foundation, Ministry of Education, Science and Technology, Republic of Korea. This research was also supported by High Value-Added Food Technology Development Program (311035-3), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Conflict of interests

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Delphinidin exhibits no cytotoxic effect on HDF cells up to 40 μM concentration. Delphinidin cytotoxicity was evaluated using MTT assay as described in Materials and Methods.

Figure S2. UVB induces p47^{phox} translocation. Cytosolic and membrane proteins were separated as described in the Materials and Methods. After 15 min of UVB exposure, p47^{phox} was observed to translocate to the membrane.

Figure S3. Delphinidin and apocynin exert similar effects on UVB-induced MMP-1 expression. MMP-1 expression was detected using specific antibodies. Data are representative of three independent experiments which gave similar results.

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