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HONOKIOL PROTECTS SKIN CELLS AGAINST INFLAMMATION, COLLAGENOLYSIS, APOPTOSIS, AND SENESCENCE CAUSED BY CIGARETTE SMOKE DAMAGE

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Abstract

BACKGROUND—Pollution, especially cigarette smoke, is a major cause of skin damage.

OBJECTIVES—To assess the effects of the small molecule polyphenol, honokiol, on reversing cigarette smoke induced damage in vitro to relevant skin cells.

METHODS—Keratinocytes (HaCat) cultures were exposed to cigarette smoke and, after 48 hours, IL-1 α and IL-8 were measured in cell supernatants. Moreover, TIMP-2 production, apoptosis rate, and senescence β -galactosidase expression were evaluated in primary human fibroblasts (HFF-1) cultures.

RESULTS—Honokiol at 10 μ M reduced IL-1 α production by 3.4 folds ($p < 0.05$), and at 10 and 20 μ M reduced IL-8 by 23.9% and 53.1% ($p < 0.001$), respectively, in HaCat keratinocytes. In HFF-1, honokiol restored TIMP-2 production by 96.9% and 91.9% ($p < 0.001$), respectively, at 10 and 20 μ M, as well as reduced apoptosis by 47.1% ($p < 0.001$) and 41.3% ($p < 0.01$), respectively. Finally, honokiol reduced senescence associated β -galactosidase expression in HFF-1.

CONCLUSION—Honokiol protects both HFF-1 and HaCat against cigarette smoke induced inflammation, collagenolysis, apoptosis, and senescence.

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Adilson Costa: none

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Introduction

Environmental air pollution, especially particulate matter (PM), from both industrial and urban sources are major causes of increasing morbidity and mortality throughout the world.¹ Because of that, according to World Health Organization (WHO), PM 10 μ m must not exceed 20 μ g/m³ (annual mean) and 50 μ g/m³ (24 hours mean); PM 2.5 μ m, the mean of concentration must not exceed 10 μ g/m³ per year and 25 μ g/m³ for 24 hours.¹

PM smaller than the diameter of skin pores can penetrate into the skin,¹ mainly in a transfollicular route.² Because of that, there are indications that PM take part in some skin diseases, such as inflammatory dermatosis, skin aging, androgenetic alopecia, and skin cancer.¹ In fact, the increased PM concentration may play an important role in the increased incidence of skin inflammatory diseases.¹

One interesting model to also evaluate pollution is performing studies with cigarette smoke (CS). According to different reports, CS has important pro-inflammatory effect,^{1–11} acting also on skin pigmentation,^{11; 12} as well as creating strong oxidative stress to different cells presented in the skin.^{5; 7; 13–15} CS acts in part by inducing metalloproteinases (MMP), which are directly related to collagen destruction, as opposed to reducing the collagen protective tissue inhibitors of metalloproteinases (TIMP)^{16–21} thus tying CS to skin aging.^{1; 22–24}

Honokiol (HK) is a phenolic component of *Magnolia officinalis*,^{25–27} with activity *in vivo* and *in vitro* properties, such as antimicrobial,²⁸ anti-inflammatory, antifungal, antioxidative, anticarcinogenic activities.^{25; 28–36}

Given the potential beneficial activities of HK, we examined whether HK could reverse deleterious effects of CS on relevant skin cells. We found that HK was beneficial in reversing many potential deleterious effects of CS on skin cells, thus providing proof of principle of HK as an inhibitor of CS accelerated skin aging.

Methods

1. Test substance

HK (molecular weight: 266.33; 99.8% purity)³⁶ was provided by Jack Arbiser's Laboratory, Department of Dermatology at Emory University Medical School, Atlanta, GA, USA.

2. Cell culture

Human foreskin fibroblasts [HFF-1; American Type Culture Collection SCRC-1041, Rio de Janeiro Cell Bank, RJ, Brazil (RJCB)] and keratinocytes (HaCat; RJCB) were grown in DMEM (*Dulbecco's Modified Eagle Medium*, Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (Cultilab Ltda, Campinas, SP, Brazil), penicillin and streptomycin (Gibco-Life Technologies, Carlsbad, CA, USA) and subcultured at 37° C in 5% CO₂ in a humidified incubator. At 80–90% confluence, cells were seeded into 24-well culture plates (1×10⁵ cells per well) for further treatment and exposure to CS.

3. Treatment protocol

HFF-1 and HaCat cultures were incubated with 2 non-cytotoxic concentrations of HK (10 and 20 μ M) which was confirmed by the cytotoxicity assay (MTT assay – data not shown). After a period of 48 hours of treatment, culture medium was replaced by Hanks' Balance Salt Solution (HBSS; Gibco-Life Technologies, Carlsbad, CA, USA), and cells were exposed to CS. Then, fresh culture medium was added to the wells and they were maintained for an additional 24-hour culture.

In parallel, two groups from each HFF-1 and HaCat cells were also evaluated to work as control groups. Since it was performed with the treatment groups, control groups were treated with DMEM for 48 hours; from then, DMEM was replaced by HBSS, but one of the control groups (positive control) was exposed to CS, while the remaining group was not (negative control). In these groups, fresh culture medium was also added to the wells and they were maintained for an additional 24-hour culture.

Cell-free supernatants and lysates were collected from all groups for further evaluation of the proposed mediators.

4. CS study – a pollution simulator

To simulate pollution, HFF-1 and HaCat cells were subjected to CS using a suitable closed plastic-based chamber that allows complete combustion of the cigarette. (FIGURE 1) It was used 4 Marlboro cigarettes (Marlboro Philip Morris Brazil, Curitiba, PR, Brazil), which were consumed by the filter at once in around 10 minutes.

5. Measurement of mediators

Tissue inhibitor of metalloproteinase 2 (TIMP-2), β -galactosidase, and apoptosis were performed in HFF-1 cultures. Interleukin-1 alpha (IL-1 α) and IL-8 was measured in HaCat supernatants. TIMP-2, IL-1, and IL-8 were quantified by using a commercially available enzymatic immunoassay kit (R&D Systems, Minneapolis, MN, USA; BD Biosciences, San Diego, CA, USA). Detection of apoptotic cells was performed using a commercially available kit (Biocolor, Carrickfergus, Northern Ireland, UK) based on a dye binding to externalized phosphatidylserine at the cell surface. The presence of the dye indicates that the cell is in the process of apoptosis. The dye was extracted from the cells and the absorbance was read in a spectrophotometer Multiskan GO (Termo Fisher Scientific, Vantaa, Finland). The measurement of the enzyme β -galactosidase was performed using a cytochemical assay using commercially obtained kit (BioVision, Milpitas, CA, USA). The cells were photographed under a light microscope (Nikon Eclipse, Melville, NY, USA; power 20 $\times$) using Image-Pro Plus 7 software (Media Cybernetics, Inc., Silver Spring, MD, USA). Each experiment was conducted in triplicate of three independent experiments.

6. Statistical analysis

For statistical evaluation, ANOVA test was used to measure the variation of the results, comparing the data between all the groups. We applied the Bonferroni post-test, which strengthened and made the result presented in the ANOVA more precise (GraphPad Prism v6, La Jolla, CA, USA). A 5% significance level was used.

Results

CS causes an inflammatory stress by raising the production of both IL-1 α and IL-8 (respectively, 5.9 fold and 18.6 %, compared to respective control group). However, cultures pre-treated with HK prevented increase in these pro-inflammatory mediators. In particular, CS induced increases in IL-1 α was prevented in cells treated with 10 μ M of HK (reduction of 3.4 fold, compared to the group only exposed to CS; $p<0.05$), FIGURE 2. In relation to IL-8, 10 μ M and 20 μ M of HK promoted significant reductions in IL-8 levels compared to the group only exposed to CS (23.9 and 53.1%, respectively; $p<0.001$), FIGURE 3.

FIGURE 4 shows TIMP-2 production in cultures of HFF-1 HK treated with concentrations of 10 and 20 μ M and exposed to CS. As expected, CS promoted a marked reduction ($p<0.001$) of 52.46% in the production of TIMP-2, compared with the control group. On the other hand, treatment of the cultures with HK was able to restore TIMP-2 production at the two concentrations tested, compared to only exposed group (96.9 and 91.9%, respectively; $p<0.001$).

FIGURE 5 shows the effect of HK on the apoptosis process in culture of HFF-1 exposed to CS. As it can be seen, exposure of HFF-1 to CS promotes 32.1% increase in apoptosis rate ($p<0.001$), when compared to the baseline control. However, cellular stress was prevented by pretreatment of HFF-1 cultures with both concentrations of HK (reduction of 47.1 and 41.3% at concentrations of 10 and 20 μ M, $p<0.001$ and $p<0.01$, respectively).

Consistent with the results observed in the increased apoptotic rate, exposure to CS also induced cellular senescence process, visualized by the β -galactosidase marker in cultures of HFF-1 (FIGURE 6). Treatment of the cultures with 20 μ M of HK promoted a decrease in senescent cells.

Discussion

The impact of CS on the skin is an unmet need in clinical dermatology. CS is associated with poor wound healing after surgical procedures, including a greatly increased risk of graft failure and necrosis.^{37–40} In addition, CS has profound cosmetic effects on skin and oral mucosa, with a phenotype of accelerated aging.^{15; 41–44} While cessation of smoking is the optimal treatment of this type of skin aging, even cessation may not fully reverse the effects of CS on the skin.

Pro-inflammatory signaling molecules and pathways take part in many subjects of interest in skin basic science other than aging, such as oncogenesis. They act on several tumor cellular compartments that lead to cancer enhance and metastasis through modulation of IL-33-dependent PDGF-BB-SOX7⁴⁵ and TNF α -TNFR1-macrophage axes,^{46; 47} the last one by modulating the VEGF-C-VEGFR3 signaling.^{45; 47}

The pro-inflammatory effects of CS, are mediated in part by induction of IL-8 from keratinocytes.⁴ Such mechanisms seem to rely in part on an aryl-hydrocarbon-receptor (AhR)-dependent pathway, inducing cytochrome P450 enzyme family,^{5; 6} such as subfamily A polypeptide 1 (CYP1A1), which generates reactive oxygen species (ROS)^{5; 7} and

contributes to the severity of inflammatory skin conditions.^{8–10} *In vitro* tests suggest that AhR, which is also involved in skin melanogenesis,¹¹ plays an important role in tobacco-induced skin hyperpigmentation.¹² Indeed, urban areas with high PM levels stimulate increased facial melanin synthesis, which is further augmented by exposure to ultraviolet (UV) rays.^{23; 48}

Once ROS are induced, a cascade of pathways are driven to transcribe IL-1 α and IL-8, as well as IL-1 receptor agonist (IL-1RA),¹ IL-6,^{1; 49} tissue necrosis factor- α (TNF- α),^{1; 49} fibroblast growth factor beta (bFGF), monocyte chemoattractant protein 1 (MCP-1), insulin-like growth factor binding protein 4 (IGFBP4), and several metalloproteinases (MMP), such as -1, -2, -3, -9, and -12.¹ When CS's combined to diesel exhaust, Th17 differentiation, via AhR ligand polyaromatic hydrocarbon (PAH),⁵⁰ occurs, which also can increase IL-8 levels.⁵¹ This creates a pro-inflammatory and collagenolytic environment also involved in skin aging. However, a potential unifying mechanism of HK may be through the recent finding that it normalizes mitochondria through induction of the mitochondrial enzyme sirtuin 3 (Sirt3), causing an increased activation of the enzyme manganese superoxide dismutase (MnSOD).⁵²

Our study shows the inflammatory stress of CS on HaCat culture. IL-1 α and IL-8 were statistically increased in comparison to non-exposed cells. However, HK offers protection from CS induced inflammatory stress at concentrations of 10 μ M (3.4 fold less; $p < 0.05$) for IL-1 α and at both 10 μ M and 20 μ M (23.9 and 53.1%, respectively; $p < 0.001$) for IL-8. Our data suggest that HK should be further explored for human use.

Another point of concern for CS is its collagenolytic capability, previously demonstrated by a number of studies: 1) heavy smokers have increased MMP-2 and MMP-9, as well as decreasing of tissue inhibitors of TIMP-1 and TIMP-2 gene expressions in saphenous vein conduits before coronary artery bypass grafting, despite a long-term smoking cessation period;⁵³ 2) in smoke-exposed rats, lung function improved by reducing MMP-1, when exposed to inhaled budenoside;¹⁶ and 3) in human gingiva, CS degraded collagen,^{17; 18} increased TIMP-1,¹⁷ TIMP-2,^{17; 18} MMP-14,^{17; 18} MMP-1,¹⁷ MMP-2.^{17; 18}

In human osteoarthritis chondrocytes, HK treatment resulted in suppression of IL-17, MMP-3, MMP-9, MMP-13,⁵⁴ inhibiting collagen II destruction.^{54; 55} In addition, HK inhibition of MMP-2^{19; 20} and MMP-9^{19; 21} are dependent on TNF- α blockage.^{19; 20} TNF- α disrupts DNA repair and allows accumulation of DNA mutations,⁵⁶ as well as inhibits collagen synthesis through MMP-9 upregulation.^{57; 58}

Since our data showed that HK prevents TIMP-2 reduction when HFF-1 were exposed to CS, keeping its levels near the control's, we believe that HK presents a good protective effect against pollution. In fact, these results are the first report suggesting HK as a protector against CS' effects on collagen and may increase commercial interest.

In addition, HK could also be an important tool against photoaging. *In vitro* studies show that solar radiation produces TNF- α , IL-1 α , IL-11, and IL-6, inducing lipolysis and decreasing lipogenesis, which contribute to photoaging.⁵⁹ However, treatment with HK inhibits UVB-induced expression of COX-2, prostaglandin E2 (PGE-2), proliferating cell

nuclear antigen and pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 in the skin,²⁵ as well as UVB-induced skin cancer in a dose-dependent manner.^{25; 26}

In regards to the apoptosis of fibroblasts,^{14; 60} CS may act through either caspase-3/7 production¹³ or MAPK-STAT1-dependent pathway, since it increases the Bax/Bcl-2 mRNA ratio, activating cleaved caspase-3 protein.¹⁴ Others have suggested that phosphorylation of STAT1 at Tyr701/Ser727¹⁴ and activation of ERK1/2, p38,^{14; 61} p21,⁶¹ and JNK in the MAPK pathway as the mechanism of apoptosis.¹⁴ In addition, p21 could also take a role in a p21-PARP-1 pro-apoptotic pathway.⁶²

Although HK's anti-inflammatory action has already been linked to caspase-3,^{63–66} -6,⁶⁶ -8,^{63; 64} and -9,^{63–66}, as well as the Bax/Bcl-2 pathway⁶⁷ it is also known that HK can induce apoptosis via both caspase-dependent and -independent manner.^{68; 69}

HK prevents carcinogenesis in part through G1 cell cycle arrest,^{25; 64; 65; 70; 71} as it induces p53 phosphorylation and p21 augmentation.^{64; 67; 70} Further, it inhibits cyclin D1, D2, and E; associated cyclin-dependent kinases (CDK)2, CDK4 and CDK6;^{25; 65; 70} downregulates survivin;⁶⁵ upregulates Cip/p21 and Kip/p27; and inhibits phosphatidylinositol 3-kinase (PI3K) and the phosphorylation of Akt at Ser(473) in UVB-induced skin tumors.²⁵

Interestingly, unlike the HK anti-apoptotic profile, our results also showed that HK reduced fibroblasts apoptosis under CS, protecting them in almost 41 to 47%. Our assumption is that HK should be doing that as acting as an anti-ROS agent, since HK reduced endothelial cell injury and apoptosis by regulating the expression of inducible NO synthase and endothelial NO synthase, as well as the generation of NO.⁷²

Airway epithelial cells undergo CS-dependent senescence^{13; 73} by upregulating GDF15, p21,^{62; 73–78} p16, and^{73; 77} high mobility group box 1 (HMGB1).⁷³ GDF15 protein's capable to bind to the ALK1 receptor and promotes senescence via activation of the Smad1 pathway.⁷³ In a high microscopic magnitude, an increase in senescence-associated β -galactosidase activity,^{73; 78} and inhibition of cell proliferation,⁷³ as well as increasing cell size and lysosomal mass, accumulation of lipofuscin, and irreversible growth arrest is observed.⁷⁸

Senescent cells are easily observed through β -galactosidase activity,^{15; 74; 75; 77; 79} which is why it is widely used to evaluate skin aging.^{80–86} This study is the first to describe HK activity protecting skin fibroblasts against CS-dependent senescence, observed by reduction of β -galactosidase activity features in HFF-1 culture. Indeed, blockade of senescence by HK may also decrease inflammation, as senescent cells are often pro-inflammatory, a phenomenon known as senescence associated secretory phenotype.⁸⁷

In fact, our findings showed that the most significant results were obtained with 10 μ M HK, with no added effect in production of IL-1 α and TIMP-2, as well as in apoptosis rate under 20 μ M HK. Such pattern has already been observed in other studies,^{45; 88–90} with no definitive explanation for it, yet. However, since concentration of 20 μ M HK has not shown direct cytotoxicity, we believe 10 μ M HK is the optimum amount of HK that avoids stimulation other mechanisms of cell survival, leading to a slow, continuous, and pace-

marked pathways that blocks inflammation, stimulates collagenolysis, and circumvent skin cell death.

Indeed, we can affirm that HK can be an excellent anti-inflammatory, anti-collagenolytic, anti-apoptotic, and anti-senescent agent for skin epithelial and fibroblasts against one of the most important pollution representatives: CS. We strongly believe that such results open different opportunities in research as well as for effective commercial products.

Conclusion

Based on the results presented here, HK has important anti-inflammatory, anti-collagenolytic, anti-apoptotic, and anti-senescence properties against CS, given that HK reduces production of IL-1 α and IL-8 in keratinocytes, prevents reduction TIMP-2 in fibroblasts, protects fibroblasts against apoptosis, and diminishes β -galactosidase expression in fibroblasts when these cells are exposed to CS. These properties present HK as an interesting compound not only for basic science, but also for clinical use.

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FIGURE 1.
CS plastic-based chamber that was the pollution simulator device use to exposure cells cultures.
CS: cigarette smoke.

FIGURE 2.

Effects of HK on the production of IL-1α in HaCat cultures exposed to CS.

HK: honokiol; CS: cigarette smoke. Cell cultures were treated with 10 and 20μM of HK for 48 hours and exposed to CS. After 24 hours, IL-1 production was measured in the culture supernatants. The data represent the mean ± standard deviation of three biological replicates (ANOVA, Bonferroni).

FIGURE 3.

Effects of HK on the production of IL-8 in HaCat cultures exposed to CS.

HK: honokiol; CS: cigarette smoke. Cell cultures were treated with 10 and 20µM of HK for 48 hours and exposed to CS. After 24 hours, IL-8 production was measured in the culture supernatants. The data represent the mean $\pm$ standard deviation of three biological replicates (ANOVA, Bonferroni).

FIGURE 4.

Effects of HK on the production of TIMP-2 in HFF-1 cultures exposed to CS.

HK: honokiol; CS: cigarette smoke. Cell cultures were treated with 10 and 20 μ M of HK for 48 hours and exposed to CS. After 24 hours, TIMP-2 production was measured in the culture supernatants. The data represent the mean $\pm$ standard deviation of three biological replicates (ANOVA, Bonferroni).

FIGURE 5.

Effects of HK on the apoptosis rate in HFF-1 cultures exposed to CS.

HK: honokiol; CS: cigarette smoke. Cell cultures were treated with 10 and 20 μ M of HK for 48 hours and exposed to CS. After 24 hours, the apoptotic rate was measured. The data represent the mean $\pm$ standard deviation of three biological replicates (ANOVA, Bonferroni).

FIGURE 6.

Effects of HK on the β -galactosidase expression in HFF-1 cultures exposed to CS.

HK: honokiol; CS: cigarette smoke. Cell cultures were treated with 10 and 20 μ M of HK for 48 hours and exposed to CS. After 24 hours, the senescence marker, β -galactosidase, was measured (A – Basal; B – CS-exposed; C and D – treated with HK 10 and 20 μ M, respectively, and exposed to CS; 20X magnitude). Arrows point to senescence figures in greenish staining.