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Mild Mitochondrial Uncoupling Prevents Premature Senescence in Human Dermal Fibroblasts

Journal of Investigative Dermatology (2014) **134,** 540–543; doi:10.1038/jid.2013.352; published online 19 September 2013

TO THE EDITOR

Mitochondrial uncoupling, an increased permeability of the inner membrane to protons not coupled to ATP synthesis, dissipates mitochondrial membrane potential. Mild mitochondrial uncoupling is believed to prolong lifespan of certain model organisms by reducing the production of reactive oxygen species (ROS) and preventing oxidant damage (Brand, 2000). Indeed, long-lived *Caenorhabditis elegans* strains have a lower membrane potential compared with wild-type strains (Lemire *et al.*, 2009). Overexpression of the uncoupling protein UCP1 in murine skeletal muscle offers protection from age-related damage and disease (Gates *et al.*, 2007). In addition, uncouplers extend the lifespan of yeast (Longo *et al.*, 1999) and mice (Caldeira da Silva *et al.*, 2008), suggesting that mild mitochondrial uncoupling might mitigate at least some deleterious aspects of aging. However, little is known about the effect of mitochondrial uncoupling on the aging of human skin. In this current study, we investigated the protective effect of mild mitochondrial uncoupling against oxidative stress-induced premature senescence in human dermal fibroblasts (HDFs).

Oxidative stress is a pivotal mechanism leading to skin aging (Masaki, 2010). Accumulation of ROS elicits premature cellular senescence and deleterious alteration of collagen homeostasis, which can contribute to the development of characteristics of aged skin such as coarse, rough, and wrinkled appearance (Varani *et al.*, 2006; Velarde *et al.*, 2012). HDFs undergo cellular senescence by addition of 200 μM H_2O_2 for 2 hours and prolonged subculture (Chen and Ames, 1994; Ido *et al.*, 2012), which can be detected by changes in cellular morphology and senescence-associated β-galactosidase (SA-βgal) staining at 12 days after addition of H_2O_2 (Figure 1a). In order to determine whether mild mitochondrial uncoupling could inhibit oxidative stress–induced premature senescence, we pretreated HDFs with 60 nM carbonyl cyanide (*P*-trifluoromethoxy)-phenylhydrazone (FCCP) for 30 minutes before addition of H_2O_2. In a pilot study, the effective concentration of FCCP that would reduce the ROS production and mitochondrial membrane potential but have no discernible effect on cell viability was determined (Supplementary Figures S1 and S2 online). We found that cells pretreated with FCCP showed delayed senescence (Figure 1a), increased proliferative capacity (Figure 1b), reduced levels of oxidants (Figure 1c), and decreased expression levels of senescence-associated molecular markers, p21 and p16 (Figure 1d), compared with untreated senescent HDFs.

Next, we investigated the uncoupling effect triggered by FCCP on collagen homeostasis in premature senescent HDFs. Aberrant collagen homeostasis, a prominent feature of aged human skin,

Abbreviations: FCCP, carbonyl cyanide (p-trifluoromethoxy)-phenylhydrazone; HDF, human dermal fibroblasts; JNK, c-Jun N-terminal kinases; MMP-1, Matrix metalloproteinase-1; SA-βgal, senescence-associated β-galactosidase

Accepted article preview online 20 August 2013; published online 19 September 2013

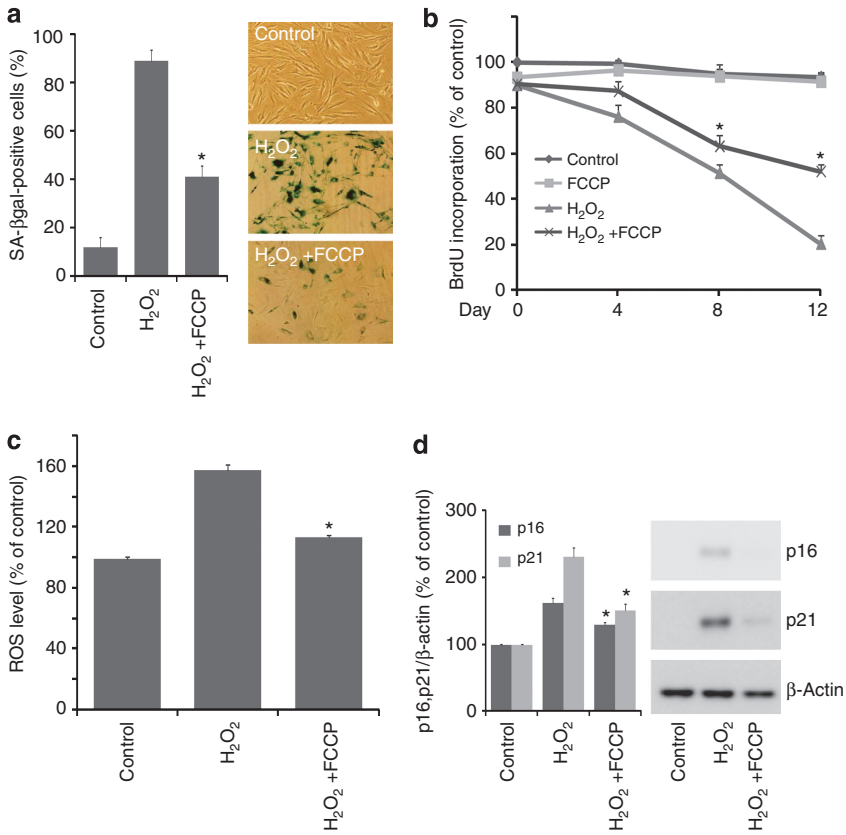

Figure 1. Carbonyl cyanide (*p*-trifluoromethoxy)-phenylhydrazone (FCCP) inhibits oxidative stress–induced premature senescence in human dermal fibroblasts (HDFs). After 30 min of pre-treatment with or without FCCP (60 nM), cells were exposed to H_2O_2 and continuously subcultivated for 12 days. (**a**) SA-βgal activities in HDFs exposed to H_2O_2 and pretreated with or without FCCP. (**b**) Cell proliferation was examined using BrdU incorporation analysis. (**c**) Cellular ROS levels were analyzed by CM-H_2DCFDA staining followed by flow cytometry analysis. (**d**) Levels of senescence-associated molecular markers p21 and p16INK4A proteins were examined by western blot. Actin levels are shown as loading controls. All results are representative or means ± SD of nine independent experiments using HDF cell line established from a 43-year-old adult male Caucasian. Differences were determined by *t*-test or a two-way analysis of variance. *$P<0.05$ vs. H_2O_2-treated senescent HDFs.

is due to reduced collagen production and increased collagen degradation, driving the wrinkling and thinning of skin (Fisher *et al.*, 2009). Matrix metalloproteinase-1 (MMP-1) is the major enzyme for the collagen degradation, and its expression is elevated in senescent HDFs and aged human skin (Quan *et al.*, 2012). As shown in Figure 2, we observed that H_2O_2-induced premature senescence increased expression levels of both MMP-1 mRNA and protein and decreased the amount of extracellular secreted collagen. Pretreatment of HDFs with FCCP alleviated the increase in the intracellular production and extracellular secretion of MMP-1 and the decrease in the extracellular generation of collagen (Figure 2a–e). MMP-1 expression is predominantly regulated at the transcriptional level by the activation of activator protein-1 (AP-1) complex and its upstream the mitogen-activated protein kinase (MAPK) signaling networks (Dasgupta *et al.*, 2010). Among MAPKs, c-Jun N-terminal kinase (JNK) signaling cascade has a critical role in responding to cellular stress, particularly oxidative stress (Chambers and LoGrasso, 2011). Indeed, JNK activity is increased in intrinsically aged human skin *in vivo* versus young skin (Shin *et al.*, 2005), and its activation is essential for the age or senescence-associated increases in MMP-1 expression (Dasgupta *et al.*, 2010). During aging, redox unbalance induces oxidative stress, causing JNK to amplify mitochondrial ROS generation and induce the expression of the redox-dependent MMP-1 protein, which could provide a mechanistic link between the involvement of free radicals in cellular senescence and age-associated aberrant collagen homeostasis (Fisher *et al.*, 2009; Chambers and LoGrasso, 2011). To evaluate the contribution of ROS–JNK pathway on oxidative stress–induced premature senescence and MMP-1 production, HDFs were pretreated with JNK-specific inhibitor, SP600125, JNK siRNA, or a well-accepted ROS scavenger *N*-acetyl-L-cysteine (NAC). JNK inhibition reduced the H_2O_2-induced increase of SA-βgal positivity and of MMP-1 mRNA levels (Supplementary Figure S3a–c online). Similarly, we found that NAC treatment had a similar effect to JNK inhibition (Supplementary Figure S3a–c online). These findings indicate that oxidative stress – induced premature senescence and its associated increases in MMP-1

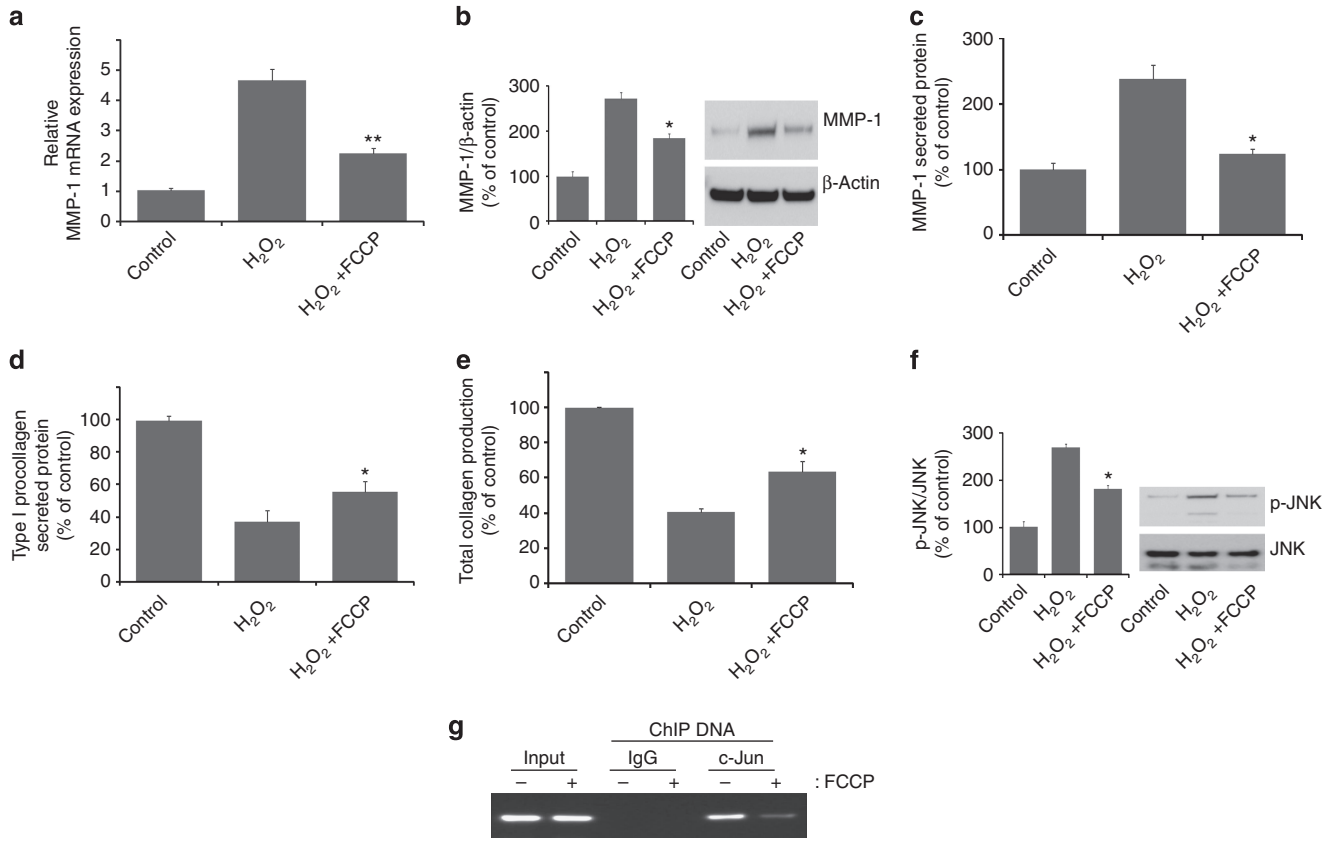

Figure 2. Carbonyl cyanide (*p*-trifluoromethoxy)-phenylhydrazone (FCCP) prevents senescence-associated increases in Matrix metalloproteinase-1 (MMP-1) expression via suppressed activation of the c-Jun N-terminal kinases (JNK) pathway. (**a**) Intracellular mRNA, (**b**) protein, and (**c**) secreted active MMP-1 levels from FCCP-treated or untreated H_2O_2-exposed premature senescent HDFs were determined by real-time reverse transcriptase–PCR, western blot, and ELISA, respectively. The levels were quantified and normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for RNA levels or actin for protein levels, respectively. (**d**) Total amount of soluble collagen type I–V and (**e**) secreted type I procollagen protein levels in culture media were determined by the Sircol collagen assay and ELISA. (**f**) Levels of phosphorylation of JNK were examined by western blotting to determine the activation of JNK. p-JNK levels were normalized to total JNK. (**g**) ChIP analysis of c-Jun binding to the MMP-1 promoter. FCCP-treated or untreated H_2O_2-exposed premature senescent HDFs were subjected to ChIP assay using an anti-c-Jun antiserum with MMP-1-specific primers (sense 5′-CCTCTTGCTGCTCCAATATC-3′ and antisense 5′-TCTGCTAGGAGTCACCATTTC-3′). All results are representative or means ± SD of nine independent experiments. Differences were determined by *t*-test. $^{**}P<0.01$, $^{*}P<0.05$ vs. H_2O_2-treated senescent HDFs.

expression are primarily JNK-dependent. We then evaluated whether the inhibitory effects of mitochondrial uncoupling on senescence-associated MMP-1 expression involve the inhibition of the JNK pathway. H_2O_2-induced premature senescence increased the phosphorylation of JNK, whereas FCCP treatment decreased the phosphorylation of JNK in senescent HDFs (Figure 2f). We further investigated the effects of mitochondrial uncoupling on the recruitment of c-Jun, a key subunit of AP-1, to the MMP-1 promoter by the chromatin immunoprecipitation assay. The recruitment of c-Jun to the MMP-1 promoter was significantly decreased in FCCP-treated HDFs compared with that in the untreated senescent HDFs (Figure 2g). The effects of FCCP on the cellular senescence of HDFs are obviously dependent on the mitochondrial uncoupling as they can be mimicked and reproduced by the presence of another mitochondrial uncoupler, 2,4-dinitrophenol (Supplementary Figures S4–S6 online). Collectively, our data suggest that a mild mitochondrial uncoupling could prevent or reduce oxidative stress–induced premature senescence by attenuating the alteration in redox state and suppressing redox-dependent JNK-mediated MMP-1 expression, which may offer a strategy for improving the collagen loss of chronologically aged human skin.

Indeed, Caldeira da Silva *et al.* (2008) demonstrated that chronic mitochondrial uncoupling, similar to caloric restriction, effectively prevents mitochondrial generation of ROS and is a highly effective *in vivo* antioxidant strategy.

Our findings provide an insight that mild mitochondrial uncoupling has the potential to prevent, delay, or treat age-related skin changes as well as age-associated diseases. Further studies, such as the evaluation of the ability of mild systemic uncoupling in the prevention of collagen destruction and skin damage in animal models are needed to confirm the anti-skin aging effects of mild uncoupling.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Valter D Longo (University of Southern California) for helpful discussion and extensive review.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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AKT1 Gene Mutation Levels Are Correlated with the Type of Dermatologic Lesions in Patients with Proteus Syndrome

Journal of Investigative Dermatology (2014) **134,** 543–546; doi:10.1038/jid.2013.312; published online 15 August 2013

TO THE EDITOR

Proteus syndrome (PS) is characterized by progressive, mosaic, segmental overgrowth and occurs sporadically (Biesecker, 2001; 2006). The mosaic nature and sporadic occurrence with the lack of familial transmission led to the hypothesis that PS is caused by a post-zygotic somatic mutation, which was confirmed with the discovery of a mosaic activating c.49G>A, p.Glu17Lys *AKT1* mutation (Lindhurst *et al.*, 2011). To date, all patients who meet the clinical diagnostic criteria for PS and have been tested have this mutation (MJ Lindhurst and LG Biesecker, unpublished results).

Although any organ or tissue can be affected, skeletal overgrowth and dermatologic lesions are the most common manifestations of PS (Turner *et al.*, 2004; Beachkofsky *et al.*, 2010). Cerebriform connective tissue nevi (CCTN) are a highly specific and common lesion in patients with PS (Biesecker, 2001; Nguyen *et al.*, 2004). These lesions are very firm and contain deep sulci that resemble the brain, thus giving the lesion its name. Histology sections of CCTN show massively expanded dermis filled with thick collagen bundles (Figure 1d–f) (McCuaig *et al.*, 2012). Epidermal nevi (EN) can be non-syndromic or occur as part of several syndromes including PS (Happle, 2010). The keratinocytic EN found in PS have a rough surface, are dark in color, usually follow the lines of Blaschko, and exhibit epidermal hyperkeratosis, papillomatosis, and acanthosis (Figure 1a–c; Nguyen *et al.*, 2004). EN are generally noticed in the first year of life and are stable in extent, whereas CCTN grow progressively after first appearing later in the first or second year (Twede *et al.*, 2005).

It is unknown which cells determine the formation of these lesions. On the basis of the histology, we hypothesized that CCTN were generated by *AKT1* p.Glu17Lys in the dermis and that EN were generated by this mutation in the epidermis. To test this hypothesis, we isolated fibroblasts and keratinocytes from lesional (CCTN or EN) and non-lesional (''normal'') skin samples and measured the level of the mutant allele in each cell type.

Skin samples were collected during surgical procedures or by punch biopsies from patients with PS under an institutional review board-approved protocol. The epidermis was separated from the dermis by treatment with

Abbreviations: CCTN, cerebriform connective tissue nevi; EN, epidermal nevi; PS, proteus syndrome

Accepted article preview online 24 July 2013; published online 15 August 2013