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Temporal variations in sirtuin expression under normal and ultraviolet B-induced conditions and their correlation to energy levels in normal human epidermal keratinocytes

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Synopsis

Sirtuins are post-translational modifiers that affect transcriptional signaling, metabolism, and DNA repair. Although originally identified as gene silencers capable of extending cell lifespan, the involvement of sirtuins in many different areas of cell biology has now become widespread. Our approach has been to study the temporal variation and also the effect of environmental stressors, such as ultraviolet B (UVB) and ozone, on sirtuin expression in human epidermal keratinocytes. In this report, we measured the variation in expression of several sirtuins over time and also show how a low dose of UVB can affect this pattern of expression. Moreover, we correlated these changes to variations in hydrogen peroxide (H_2O_2) and ATP levels. Our data show significant variations in normal sirtuin expression, which may indicate a generalized response by sirtuins to cell cycle kinetics. These results also demonstrate that sirtuins as a family of molecules are sensitive to UVB-induced disruption and may suggest a new paradigm for determining environmental stress on aging and provide direction for the development of new cosmetic products.

INTRODUCTION

Post-translational modifications exert control across a diverse array of cellular functions (1). One area that has generated much recent interest has been the enzymatic activity of sirtuins on various proteins affecting aging (2), metabolism (3), and response to environmental trauma (4,5). Although sirtuins have several different modes of action, they are generally classified as Class III nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases that remove an acetyl group from a lysine side chain of its protein substrate (6). In mammalian cells, seven sirtuin (SIRT1-7) homologs have been characterized that are distributed in several sub-cellular compartments (7).

Human skin is constantly exposed to environmental hazards, such as ultraviolet B (UVB) radiation, leading to photo aging and skin cancer (8). Although it is well established that

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sirtuins can contribute to the longevity of many organisms by silencing genes during times of nutritional deprivation (9), they may also increase cell survival after UV-induced stress using the same mechanisms. In a previous report (4), we described the effects of low levels of UVB radiation on *sirt3* expression and its inverse relationship to *sirt4* expression in normal human epidermal keratinocytes (NHEK). We further demonstrated how UVB can disrupt the normal activity and cycle of expression of sirtuins, both of which appear necessary for maintaining cutaneous health. In this report, we expand these findings to include *sirt1* and *sirt6* and correlate these results with the effects of UVB on H_2O_2 and energy metabolism.

Our objective was to determine the relative levels of expression of *sirt1*, *sirt3*, and *sirt6* over time in NHEK synchronized by starvation. Additionally, we determined sirtuin expression after UVB irradiation. If the patterns of sirtuin expression were similar, this might imply a generalized response by sirtuins to environmental challenge that might also be related to ATP levels and reactive oxygen species (ROS).

MATERIALS AND METHODS

CELL CULTURE

NHEK were obtained from a commercial supplier (Cascade Biologics, Portland, OR) and cultured according to the manufacturer's recommendations at 37°C in a 5% CO_2 humidified incubator. Cells were incubated in EpiLife medium (Cascade) without any human keratinocyte growth supplement (HKGS; Cascade) during starvation. The supplement included bovine pituitary extract, human epidermal growth factor, hydrocortizone, insulin, and transferrin. Cells were released from starvation by incubating cells in full EpiLife medium with HKGS.

CELL VIABILITY

Cell toxicity was determined with an Alamar Blue solution (Invitrogen, Carlsbad, CA), which was prepared at 10% in cell media and added to cells for 2 hours. After incubation, cellular fluorescence (Ex_{535nm}/Em_{612nm}) was measured in a SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). A decrease in fluorescence is indicative of cytotoxicity.

UV RADIATION

Cells were irradiated before being released from starvation. Cells were washed with Dulbecco's modified phosphate-buffered saline, pH 7.4 (D-PBS), covered with a thin layer of D-PBS (3 ml in a 100 mm plate) and then irradiated with FS40 UVB bulbs (Philips; Fisher Scientific, Pittsburgh, PA) through a Kodacel filter (Kodak, Rochester, NY). UVB fluences were measured with an IL1400A radiometer (International Light, Newburyport, MA).

RNA EXTRACTION AND QUANTIFICATION

NHEK were plated in 100 mm plates at 5×10^5 cells per dish. After treatments were completed, RNA was extracted from cells using a QIAshredder tube (Qiagen, Valencia, CA) in conjunction with the RNeasy mini kit (Qiagen) as per the manufacturer's instructions. RNA was then quantified by measuring its absorbance at 260 nm with a DU7500 spectrophotometer (Beckman, Fullerton, CA).

REVERSE TRANSCRIPTION AND REAL-TIME POLYMERASE CHAIN REACTION

RNA was reverse transcribed using a High Capacity cDNA Archive Kit from Applied Biosystems, Inc. (ABI, Foster City, CA), as per the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was employed using the 2X TaqMan Fast Universal PCR Master Mix (ABI) in conjunction with a 20X TaqMan Gene Expression Assay Mix (ABI), which consists of human primers and probes for Sirt1 (ABI P/V Hs01009005_m1), Sirt3 (ABI P/N Hs00202030_m1), Sirt4 (ABI P/N Hs01015516_g1), Sirt6 (ABI P/V Hs00966002_m1), and the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ABI P/N Hs99999905_m1). Primers and probes were designed to span exon–exon junctions to ensure amplification of only the transcribed gene of interest and not any residual genomic DNA that might have been present. Relative gene expression (change in expression of target gene normalized to an endogenous control (GAPDH) and relative to a reference group (untreated cells)) of real-time RT-PCR data was calculated using the $2^{-\Delta\Delta CT}$ method.

ATP/H_2O_2 DETERMINATION

NHEK were grown in 96-well black clear bottom plates at 3×10^5 cells per plate. Cells were starved, released from starvation, and irradiated as described above. ATP was measured with a luminescence-based assay, ATP Lite (Perkin Elmer, Waltham, MA), according to the manufacturer's instructions. Luminescence was measured on an Lmax luminometer (Molecular Devices). H_2O_2 was measured with a method using CM-H2DCFDA (Invitrogen, Eugene, OR). Fifty micrograms of CM-H2DCFDA was dissolved in 100 µl ethanol and then a 1:100 working dilution of the CM-H2DCFDA/ethanol solution was prepared in D-PBS. Cells were incubated with the working dilution at 37°C for 20 min. Then an equal amount of 25 m*M* NaN_3 in D-PBS was added directly to the working dilution and incubated for another 2 h at 37°C. Fluorescence was measured at 485 nm/530 nm (excitation/emission) with a Spectra Max Gemini EM (Molecular Devices) (10).

RESULTS

NHEK were synchronized by serum supplement deprivation for 15 hours followed by repletion in full media to follow the cycle of sirtuins under normal conditions. Samples were then harvested at 0, 2, 4, 6, and 8 h and RNA extracted and analyzed by real-time RT-PCR. These results are illustrated in Figure 1a and show that *sirt1*, *3*, and *6* follow the

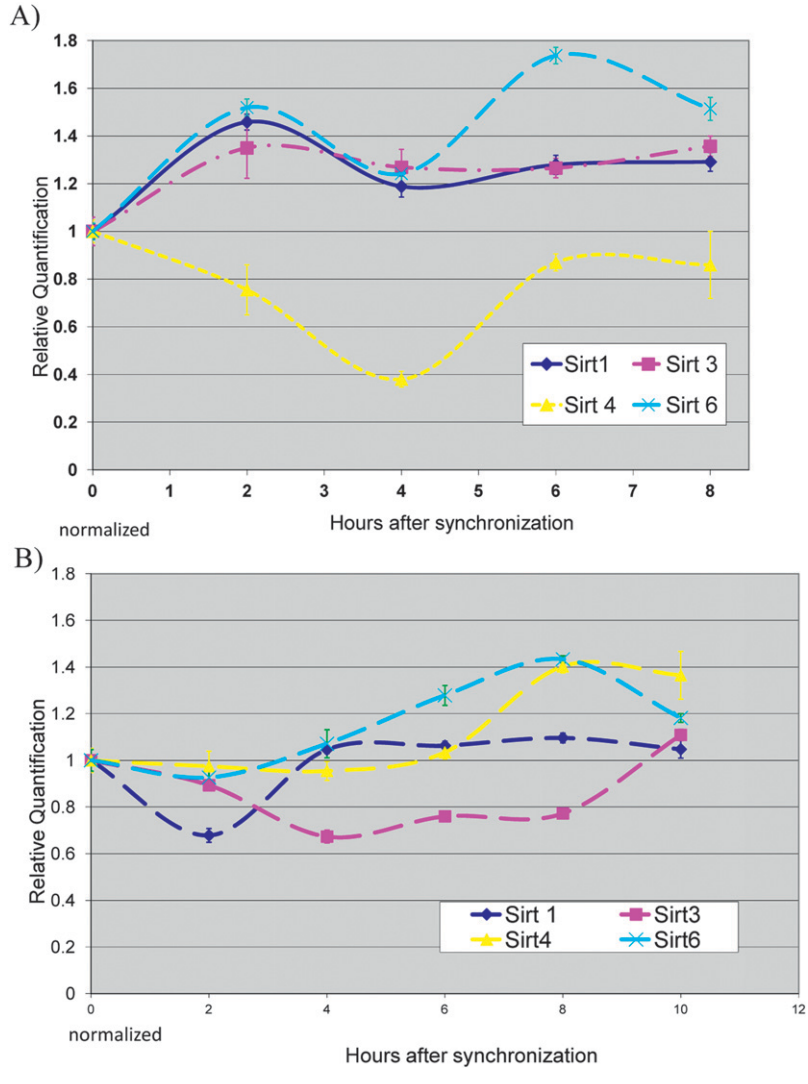

Figure 1. (A) Real-time RT-PCR analysis of sirtuin expression in synchronized NHEK 0, 2, 4, 6, and 8 h after repletion in complete media. Values were normalized to GAPDH housekeeping genes and expressed as 1 at the zero time point using the calculation method: $2^{-\Delta\Delta CT}$. *Sirt1, 3,* and *6* followed a similar temporal pattern of expression whereas *sirt6* increased at the 6-h time point. (B) UVB (10 mJ/cm^2) irradiation of NHEK, followed by repletion in complete media, disrupted sirtuin expression over time although *sirt6* appeared to recover and increase at the 6-hour time point. Error bars were calculated for both graphs using S.E. measurement (n = 3).

same kinetics for the first 4 h unlike *sirt4*, which responds inversely. Interestingly, a subsequent rise in transcription was observed for *sirt6* after 6 h that was not detected in either *sirt1* or *sirt3* and may reflect an increased need for DNA base repair related to cell cycle kinetics and replication.

When NHEK were then either exposed to 10 mJ/cm^2 UVB or sham-irradiated under the same conditions, a different pattern of sirtuin expression emerged. As shown in

Figure 1b, there is an abrogation of expression as a result of UVB irradiation indicating that, at a non-cytotoxic dose of UVB, as measured by the Alamar Blue assay for cell viability (data not shown), sirtuin expression is significantly reduced. Thus, in the first hours after irradiation, *sirt 1, 3,* and *6* are all reduced in contrast to their unirradiated controls. *Sirt4* activity also changed after UVB exposure and instead of a reduced amount of activity over the first 4 hours, as seen in the controls, an overall increase that peaked at 8 h was also noted. Since SIRT4 inhibits the ability of glutamate dehydrogenase to convert glutamate to α-ketoglutarate, leading to ATP synthesis, an increase in SIRT4 should contribute to reduced ATP levels. Inversely, impairment of SIRT3 activity by UVB exposure should also lead to reduced ATP levels due to a reduction in acetyl CoA synthetase activation. Moreover, despite these changes, *sirt6* recovered the fastest after 8 h; again possibly due to its importance in DNA repair, genome maintenance, and inflammation control.

To determine the impact on energy production, the effects of 10 mJ/cm^2 UVB on ATP and ROS were also measured in NHEK over time and correlated to sirtuin expression. Since sirtuins are closely related to metabolism, we expected a strong effect on these after UVB exposure due to the loss of sirtuin expression. In Figure 2(A), a reduction in ATP was observed after UVB irradiation. Unexposed samples followed a temporal pattern similar to *sirtuins 1*, *3*, and *6* expression and inversely to *sirtuin 4* expression and demonstrate a correlation to metabolism. Alternatively, Figure 2(B) shows an increase in H_2O_2 in response to UVB compared to unexposed controls. Thus, after irradiation, an increase in H_2O_2 and a concomitant reduction in ATP were observed, which closely resembled the UVB-exposed expression profiles for the sirtuins except for *sirt6* at the 8 h time point. Significant reduction in ATP as a result of UV exposure clearly correlates to changes in sirtuin transcription profiles.

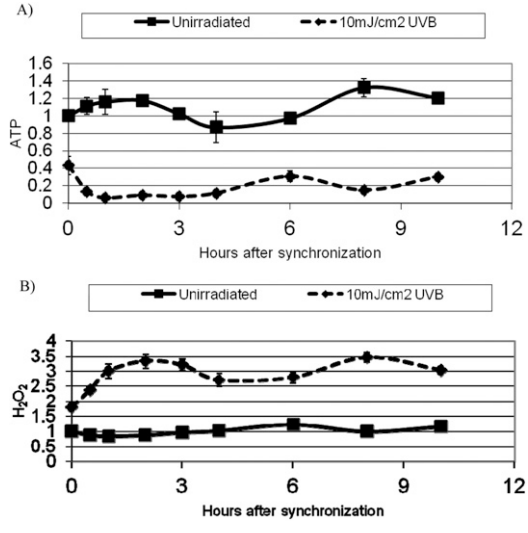

Figure 2. (A) ATP levels were plotted at 0, 2, 4, 6, 8, and 10 h after repletion with complete media and either exposed to UVB radiation or sham-irradiated. A temporal pattern similar to sirtuin expression was observed. However, UVB irradiation significantly disrupted this pattern. (B) H_2O_2 levels in NHEK were plotted at 0, 2, 4, 6, 8, and 10 h after repletion with complete media and either exposed to UVB irradiation or sham-irradiated. UVB exposure significantly increased H_2O_2 levels which were observed to modulate over time. Error bars were calculated for both graphs using S.E. measurement (n = 3).ABAB

DISCUSSION

Although most sirtuin research has focused on their effects on longevity, our approach has been to measure temporal changes in sirtuins in skin cells and also as a result of environmental challenge because the skin is the first line of defense against the environment. Deleterious changes sustained over time in skin due to environmental insult can lead to photoaging and increased visible signs of aging. We report here that *sirt1* and sirt6 in NHEK follow a definite temporal pattern that is similar to the pattern that we had previously observed for *sirt3.* These data support the findings of Asher *et al.* (11) who showed that SIRT1 follows a circadian pattern. Further, decreases in sirtuin expression after exposure to a non-cytotoxic dose of UVB were also measured which disrupted their temporal pattern. Since *sirt6*, unlike *sirt1*and *3*, increased after 6 h under normal conditions and then was the first to recover after UVB exposure, may indicate its importance for DNA repair (12).

In parallel to these changes in sirtuin expression, we also observed a similar temporal pattern of ATP levels in NHEK. However, after irradiation, this pattern was significantly altered, as in the sirtuins. Part of the reason for this may be attributable to a reduction in SIRT3 and the inactivation of acetyl CoA synthetase, which would normally lead to increased ATP synthesis (13). Additionally, if SIRT3 is unable to deacetylate SOD2, there will be a concomitant increase in ROS (14) that we also observed.

These results show how less obvious targets of sun exposure, such as sirtuins, may have a more subtle and long-term effect on skin. Our data present for the first time a unified modality for sirtuin expression and the effects that UVB has on their expression. Intriguingly, the disruption of energy levels also points to the role that sirtuins play in metabolism and how low levels of UVB may quickly affect metabolic regulation. Taken together, these results further emphasize the importance of protection against sun exposure and may suggest a new paradigm for determining environmentally induced aging. Finally, our results show the importance of sustaining sirtuin levels and developing new cosmetic products in support of repair and metabolic mechanisms for a healthy skin.

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