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ULTRAVIOLET B RADIATION MODIFIES CIRCADIAN TIME IN EPIDERMAL SKIN AND IN SUBCUTANEOUS ADIPOSE TISSUE

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Short title: UVB modifies circadian time in human skin

The research was conducted in Päijät-Häme Social and Health Care Group, Department of Dermatology and Allergology, Lahti, Finland.

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Background

Recent findings suggest that circadian time regulates cellular functions in the skin and may affect protection against ultraviolet radiation (UVR). It is not known, however, whether UVR through skin directly affects the expression of circadian genes. We investigated the effect of ultraviolet-B (UVB) exposure on cryptochrome circadian clock 1 (*CRY1*), cryptochrome circadian clock 2 (*CRY2*), and circadian associated repressor of transcription (*CIART*) genes.

Methods

Healthy volunteers (n=12) were exposed to narrow-band UVB radiation of 4 standard erythemal dose (SED). Epidermal/dermal and subcutaneous adipose tissue samples were obtained by punch biopsies from irradiated and non irradiated skin 10 cm away from the irradiated site 24 hours after UVB exposure. Gene expression of *CRY1*, *CRY2*, and *CIART* was measured using RT-PCR (TaqMan).

Results

UVB radiation affected mRNA expression in the epidermal/dermal skin and in the subcutaneous adipose tissue. It down-regulated expression of *CRY2* gene in the epidermal/dermal skin, whereas it up-regulated expression of *CRY1* and *CIART* genes in the subcutaneous adipose tissue.

Conclusion

We showed for the first time that UVB radiation affects expression of circadian genes in the subcutaneous adipose tissue. Further studies are warranted to understand the mechanisms in detail.

Keywords: CRY1, CRY2, CIART, mRNA, gene

INTRODUCTION

Ultraviolet radiation (UVR) reaching the skin is a carcinogen, inducing immunosuppression and DNA mutations (1) and the wavelengths of ultraviolet light B (UVB) represent a biologically very active part of it (2). The chromophores transduct the electromagnetic energy of UVB into neural, chemical and hormonal signals to produce rapid (neural) or slow (humoral or immune) responses at the local and systemic levels (3,4). Skin irradiated by UVB can activate both the central and local hypothalamic-pituitary-adrenal (HPA) axis to turn on homeostatic responses and deactivate local and systemic damage (5,4). Locally UVB irradiation of the skin leads to activation of pro-opiomelanocortin (POMC) and corticotropin releasing hormone (CRH) in epidermal and dermal skin, produces cutaneous POMC peptides and activates the corresponding receptors that are important regulators on the pigmentary and the inflammatory reactions. (6,7,8) In addition keratinocytes stimulated by UVB can, as a rapid effect, secrete e.g., interleukin-1β (IL-1β), IL-6 and tumor necrosis factor (TNF) (26). The transmission of UV radiation to the skin depends decisively on the individual skin type and UV adaptation status. UV wavelegths below 305 nm are almost totally absorbed into the stratum corneum and epidermis. For UV wavelengths above 305 nm about 10% reach the dermis. No UV radiation penetrates through the dermis to the subcutaneous adipose tissue. (1,9,10).

The skin epithelium is one of the most actively proliferating tissues in the human body and characterized by an approximately 24-hour rhythm in almost all its functions (11,12) These circadian rhythms are regulated by an endogenous time-keeping system, the circadian clock. The central pacemaker of human circadian rhythms is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in the brain, while peripheral tissues follow the pace at transcriptional levels using a transcriptional-translational feedback loop (13). The

genes involved in the regulation and maintenance of the circadian rhythms are called clock genes. Canonical clock genes, such as cryptochrome circadian clocks (*CRY1*, *CRY2*), encode proteins which interact with each other to build up an autoregulatory feedback loop (14,15). The CRY1/CRY2 ratio alters the circadian period, an increasing ratio being associated with a longer circadian period (16). CRY1 or CRY2 as well as the period circadian clock (PER1 or PER2) as dimers are transcriptional repressors in the core transcription-translation feedback loop (17). Aryl hydrocarbon receptor nuclear translocator-like (ARNTL) protein is the key circadian clock protein and a transcription factor of *CRY1* and *CRY2* genes (18).

There is a daily rhythm in the expression of circadian associated repressor of transcription (*CIART*) gene, and it is also regulated by stress responses (19). CIART protein has been shown to have a role in the transcriptional regulation of clock-controlled genes, as it can repress circadian locomotor output cycles kaput (CLOCK) and ARNTL protein activity in different time-windows from CRY proteins and PER proteins (20,15). In a diurnal primate, the baboon, the most frequent cycling gene was *Ciart*, as it was cycling in 52 of the 64 tissues sampled every two hours over a period of 24 hours, and the expressions of the seven main clock genes in the diurnal baboon and in the nocturnal mouse were in antiphase to each other (21). Circadian clock proteins and genes have been shown to regulate cellular responses after UVB exposure: in mice, sensitivity to UVB-induced DNA damage in the epidermal skin is a process that is dependent on ARNTL as a function of the time of day (22,23). Earlier we showed the effect on circadian timing in UVB-induced erythema response in human epidermal/dermal skin (24). However, the effect of UVB radiation on clock genes is not fully understood.

Human skin cells are known to express circadian clock proteins (25), but very little is so far known about the regulation of their expression and activity. In adipose tissue *CIART* gene oscillates with a circadian frequency (26). In addition, human adipocytes express other clock genes (27,28), but as far as we know there is no earlier data on whether the clock genes are expressed in the subcutaneous adipose tissue after UVB exposure. The effects of UVR on subcutaneous adipose tissue are transmitted through cytokines such as IL-6, IL-8, monocyte chemoattractant protein–3 (MCP-3) and placenta growth factor (PlGF) (29) and signal transduction can occur through melanocortin receptors (MC1R and MC2R) (30).

Our aim was to explore the effects of UVB on mRNA levels of *CRY1*, *CRY2*, and *CIART* genes in the skin of healthy volunteers. We also investigated for the first time whether the effects of UVB on circadian gene expression extend from epidermal/dermal skin to the subcutaneous adipose tissue and measured amounts of mRNA of the *IL-1β, IL-6, TNF, POMC*, and melanocortin receptors (*MCR1/MCR2*) in the both tissues to confirm earlier findings and find possible link between UVB and clock proteins below the skin surface.

MATERIALS AND METHODS

Healthy adult volunteers (n=12) presenting with anamnestic skin phototypes II or III (31) were eligible to participate. The skin phototype was confirmed in a clinical interview. Subjects with photosensitizing, immune modulating or psychiatric medication, photosensitivity, pregnancy, lactation, history of skin cancer or extensive scarring, or marked UVR exposure in the three preceding months were not eligible to participate. During the study no medication was allowed. Three subjects had recently taken some medication and to meet the inclusion criteria this had to be discontinued for a minimum of 48 hours before the beginning of the study.

Ethics

This study was carried out in the Department of Dermatology, Päijät-Häme Central Hospital, Lahti, Finland, from January to April 2016. Written informed consent was obtained from the participants and the ethics committee of Hospital District of Pirkanmaa approved the study protocol (#R16001)

UVB radiation

One square cm area of the buttock skin was exposed to 4 standard erythemal dose (SED), while the rest of the skin was protected. A Waldmann UV 801KL phototherapy device equipped with 4 TL20W/01 narrowband UVB (NB-UVB) tubes was used for UVB irradiations. The bulbs were preheated for seven minutes before the exposure. The irradiance of the device was measured before the study using an Ocean Optics S2000 spectroradiometer by the Nuclear Safety Authority of Finland. The time to irradiate the test area was calculated on the basis of these measurements so that the 4 SED area was irradiated for 6 min 28 sec. One SED is defined as an erythemal effective radiant exposure of 10 mJ/cm^2, and is equivalent to a non-weighted physical UV dose of 172 mJ/cm^2 emitted from our narrowband UVB lamps. The 4 SED thus equals a non-weighted physical dose of 688 mJ/cm^2. Most of the radiation of the narrowband UVB lamp's radiation is in the 311 nm emission peak. The spectrum of the narrowband UVB lamps is presented in Figure 1. Uncertainty (2σ) of the measurement of the OceanOptics S2000 was estimated to be approximately 14% (32). The measurements are traceable to the National Institute of Standards and Technology (Gaithersburg, Maryland, USA).

Sample collection

While the narrowband UVB irradiation was administered in the morning between 7 a.m. and 9 a.m. on day one, the 3 mm skin punch biopsies, consisting of epidermis/dermis and subcutaneous adipose tissue, were sampled 24 hours after the exposure on day two. A non-irradiated skin control biopsy was obtained at least 10 cm away from the irradiated skin square, and had been protected against UVB with impermeable fabric. Lidocaine 1% without epinephrine was used for local anesthesia, and the punch biopsies were obtained thereafter. The subcutaneous adipose tissue and epidermis/dermis were cut separately from each other with a scalpel and incubated separately. All samples were incubated in RNAlater (Thermo Fischer Scientific, Massachusetts, USA) for 24 hours at +4°C, and thereafter deep-frozen and stored at from −80°C to −20°C until mRNA extraction.

Total-RNA extraction, cDNA synthesis and RT-PCR

Both epidermis/dermis and subcutaneous adipose tissue samples were mechanically homogenized by Ultra-turrax homogenizer (IKA-Analysentechnik) in RLT buffer (Qiagen, Hilden, Germany). To sediment the small tissue pieces and to layer possible fat, the homogenates were centrifuged twice at 12 000 × g for 10 min at +4°C. Total RNA from epidermal/dermal skin biopsies was isolated using AllPrep kit (Qiagen, Venlo, The Netherlands). Four hundred micrograms of RNA was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, U.S.A.) to cDNA as previously described (33). And from subcutaneous adipose tissue, 80 □g of RNA was reverse-transcribed with VILO kit (Thermo Fisher Scientific/Invitrogen, Carlsbad, CA, U.S.A.) according to manufacturer's instructions. The expression of the

cytokines and genes involved in the circadian rhythm were studied by TaqMan 7500 Fast System (Applied Biosystems) with PerfeCTa qPCR FastMix (Quanta Biosciences, Gaithersburg, MD, U.S.A.). PCR primers and probes for cytokines and chemokines were obtained as predeveloped assay reagents from Applied Biosystems. Ribosomal 18S gene was used as housekeeping gene in the TaqMan analyses.

Statistics

Gene expressions of selected circadian genes and cytokines were first compared using ordinary one-way ANOVA at different time points. Statistical analysis of target gene expression was performed with the nonparametric Mann–Whitney test before and after UVB treatment. A single apparent outlier from the null samples was excluded from the analysis. The association between the expression of *CRY1* and *CRY2* in the skin at different time points was examined using the Pearson's correlation test. All statistical analyses were performed with GraphPad Prism 7 Software (GraphPad Software Inc., San Diego, California).

RESULTS

Altogether 12 healthy subjects participated in the study, 2 men and 10 women, aged 41 (26 to 64) years on average and with BMI 23.5 (SD ±3.4) on average. Four presented with anamnestic skin phototype II and eight with phototype III (31). We found the epidermal/dermal skin mRNA of *CRY2* gene to be at a lower level of expression after UVB irradiation than skin without irradiation. We also found that in the null epidermal/dermal skin sample there was 6.5 times more and in the subcutaneous adipose tissue as much as 20 times more *CRY2* than *CRY1* mRNA.

Clock gene expressions after UVB

After the UVB exposure expression of *CRY2* mRNA in epidermal/dermal skin was significantly decreased (p=0.0492), and a similar tendency was seen in *CIART* mRNA (Figure 2a). UVB irradiation of the skin had no statistically significant impact on the expression of epidermal/dermal skin *CRY1* mRNA, but changes in gene expression were seen in the subcutaneous adipose tissue; UVR significantly enhanced the expression of *CRY1* (p=0.0263) (Figure 2b). The expression of *CIART* also enhanced (p=0.0411) and a small increase was seen in *CRY2*. We found a positive linear correlation between *CRY2* and *CRY1* mRNA amounts in epidermal/dermal skin, especially after irradiation (r=0.84) (Figure 3a) and the *CRY1/CRY2* ratio was significantly different after irradiation (p=0.0011). In subcutaneous adipose tissue the correlation was weak (Figure 3b).

UVB modifies cytokine expressions in skin but not in adipose tissue

UVB exposure significantly enhanced the expression of interleukin-1β *(IL-1β)* mRNA in the epidermal/dermal skin (p=0.0011), and a similar tendency was also observed in the expression of *TNF* (Figure 4a). UVB exposure did not induce clear changes in *IL-6* after 24 hours. No significant changes in the subcutaneous adipose tissue were seen in the cytokine expressions studied (Figure 4b). A small increase was also seen after UVB exposure in *POMC* and melanocortin 1 receptor (*MC1R)*, both in the epidermal/dermal skin and subcutaneous adipose tissue. melanocortin 2 receptor (*MC2R*) level showed no significant change (Figure 4a-b).

DISCUSSION

In addition to epidermal/dermal skin UVB radiation seems also to affect mRNA expression in the subcutaneous adipose tissue. We found *CRY2* expression to be down-regulated in epidermal/dermal skin following UVB radiation, and in subcutaneous adipose tissue *CRY1* and *CIART* expression appeared to be up-regulated. The differences in trend in the two tissues with CRY2 and CIART genes may be due to the fact that no NB-UVB penetrates into the subcutaneous adipose tissue.

In humans and other mammals visible light is capable of interacting with the master circadian clock through retinal rods, cones, and ganglion cells that project information from the eyes to the SCN (34-38). Both CRY1 and CRY2 have been detected in the human retina by immunohistochemistry (39) and can function as blue-light photoreceptors, but may even be more sensitive to shorter (from 300 nm to 450 nm) UV- wavelengths (40,41). While the SCN is a pacemaker and the main link between the circadian clock and light exposure, in plants and animals peripheral clocks play a role in perceiving changes in environmental radiation (42), which, based on CRY1 and CRY2 signaling mechanisms, may also be possible in humans (43). In the human retina *CRY2* mRNA is 11 times more abundant than *CRY1* and expected to capture the photons necessary to initiate a light-sensitive response (39). We found that in untreated control skin there was 6.5 times and in the subcutaneous adipose tissue as much as 20 times more *CRY2* than *CRY1* mRNA, which corroborates the putative role of CRY2 protein interactions with UVB. Although UVB induced mRNA expressions of the clock genes have not been studied earlier *in vivo*, in cultured human keratinocytes UVB downregulated *ARNTL*, *CLOCK* and *PER1* gene expressions. (44).

The observed change in clock genes due to UVB may affect the repair of UVR- induced damage. In mice inflammatory cytokine induction, sunburn apoptosis, and erythema are controlled by circadian rhythm (22). Huber et al. suggested that CRY2 can limit tumor formation by promoting the turnover of c-MYC, a critical regulator of cell proliferation (45). This observation is in line with our earlier finding that human skin with a neglible amount of CRY2 showed more erythema (24). While we now found *CRY2* expression to be down-regulated in epidermal/dermal skin following UVB radiation, is possible that UVB exposure undermines the protective mechanisms in epidermal/dermal skin.

Since after UV exposure proinflammatory cytokines IL-1, IL-6, and TNF are expressed in various skin cell types (30), we expected to detect a significant increase in their mRNA levels. Based on cytokine mRNA levels a slight inflammation occurred in the epidermal/dermal skin but not in the subcutaneous adipose tissue. As UVB induces POMC, we expected to see a significant increase in the expression of *POMC* mRNA when comparing the irradiated and non-irradiated skin specimens. The UVB dose used in our study was large enough to induce visible erythema at 24h, whereas the increase in pigmentation takes more time (46). It is thus possible that later timing of biopsy, e.g. some days later, would have revealed a more pronounced increase in *POMC* mRNA. However, we already detected a small increase in *POMC* mRNA expression both in the epidermal/dermal skin and subcutaneous adipose tissue at 24h after exposure, which may indicate that later sampling might have worked better for detecting *POMC* mRNA. As we found no proof of the involvement of melanocortin receptors in signaling between UVB and chryptocromes in adipose tissue at 24h, these could be expressed later at 3 to 6 days, when pigmentation reaches its maximum (46).

As we detected an increase in the *CRY1/CRY2* ratio after irradiation, it is possible, in light of earlier findings, that UVB extends the circadian period (16). In addition melatonin produced in epidermal/dermal skin may play a role in the UVB damage responses and mRNA synthesis of clock proteins, but this needs to be clarified in future clinical studies (47). Due to the important role that circadian functions play in the human body, it is essential to understand the mechanisms behind the circadian clock of the metabolic active tissue, the subcutaneous adipose tissue, as part of the skin. As clock genes in adipose tissue have been linked to the metabolic syndrome (48) our data opens up a new perspective when considering the implications of UVR exposure for human health. Unfortunately a limitation of our study is the relatively small number of participants and the fact that we only measured mRNA but not proteins, which is due to the invasive nature. Larger cohorts are needed to confirm these links from UVB to adipose tissue disturbances.

CONCLUSION

We investigated the effect of UVB radiation on the expression of key clock genes in epidermal/dermal skin and subcutaneous adipose tissue, showing for the first time that UVB radiation affects expression of circadian genes in the subcutaneous adipose tissue. Variation in the expression of *CIART* gene suggests that it is a possible oscillator of skin functions. More studies are warranted to understand further actions and mechanisms of clock genes in detail. Our findings suggest that UVB may influence peripheral clock genes in the skin to modulate e.g. cellular responses against to UVR induced damage.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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LEGENDS FOR ILLUSTRATION

Figure 1. **The spectrum of the narrowband UVB lamp TL01.** The spectrum has an emission maximum at 311 nm. 63% of the lamps UV and visible (280 -780 nm) radiation is in the narrow 305 -315 nm band. The UVB (280 – 315 nm) range contains 64%, UVA (315 – 400 nm) 16% and visible light (400 -780 nm) 19% of the lamps UV and visible light radiation.

Figure 2. **Circadian gene expression in a) epidermal/derman skin and in b) subcutaneous adipose tissue** 24h after four SED UVB exposure. The total number of samples in group is 11-12. Nonparametric Mann–Whitney test was used to represent the column of group means, and standard errors of the means (SEM). Ctrl, untreated control skin; UV, UV-treated skin; RU, relative units. *p<0.05

Figure 3. **Correlation between *CRY1* (y-axis) and *CRY2* (x-axis) in UV-exposed and control tissues in a) epidermal/dermal skin and in b) subcutaneous adipose tissue** 24h after four SED UVR exposure. The total number of samples in a group is 11. Linear regression was used to model the data per CRY2, and Pearson's correlation was used to compute the correlation coefficients (r). Ctrl, untreated control skin; UV, UV-treated skin. **p<0.005

Figure 4**. Cytokine, *POMC* and melanocortin receptor gene expression in a) skin and in b) subcutaneous adipose tissue** 24h after four SED UVR exposure. The total number of samples in group is 11-12. Nonparametric Mann–Whitney test was used to represent the group means and standard errors of the means (SEM). Ctrl, untreated control skin; UV, UV-treated skin. *p<0.05, **p<0.005

Figure 1

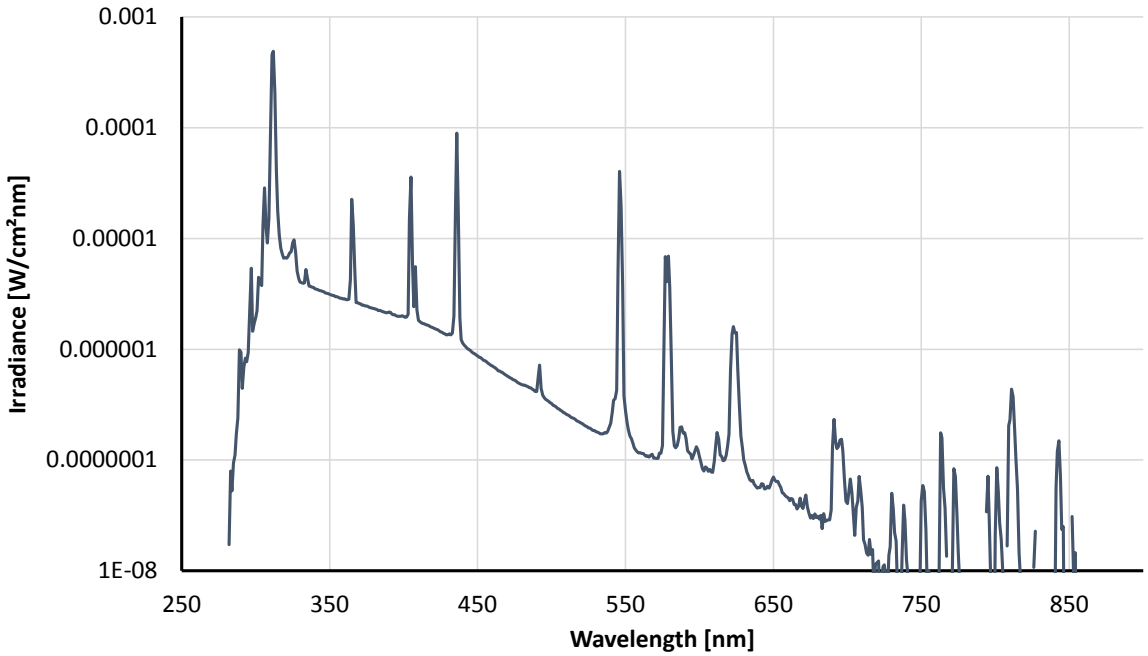
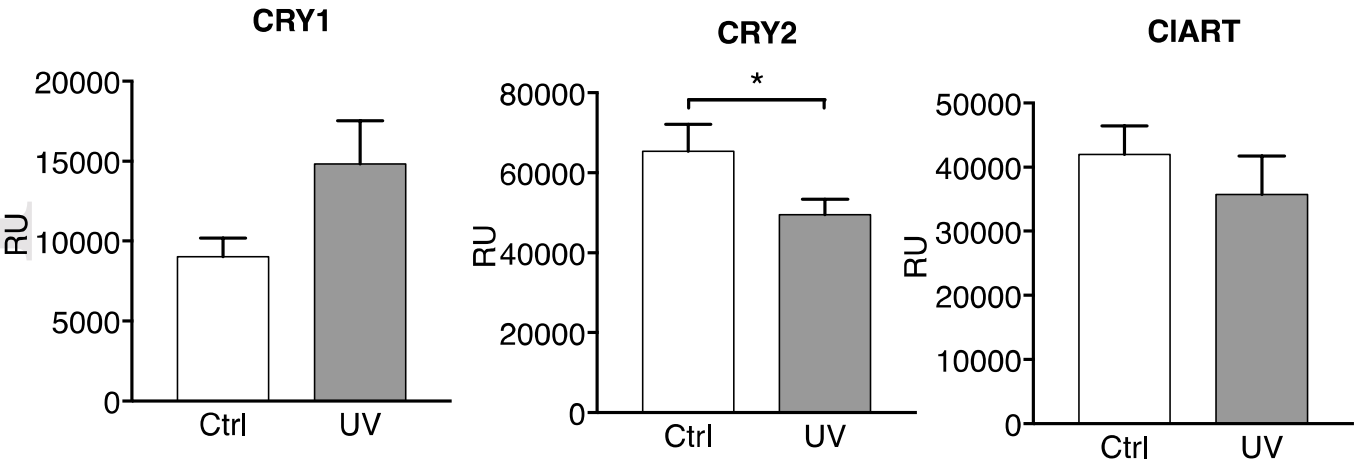


Figure 2.

a



b

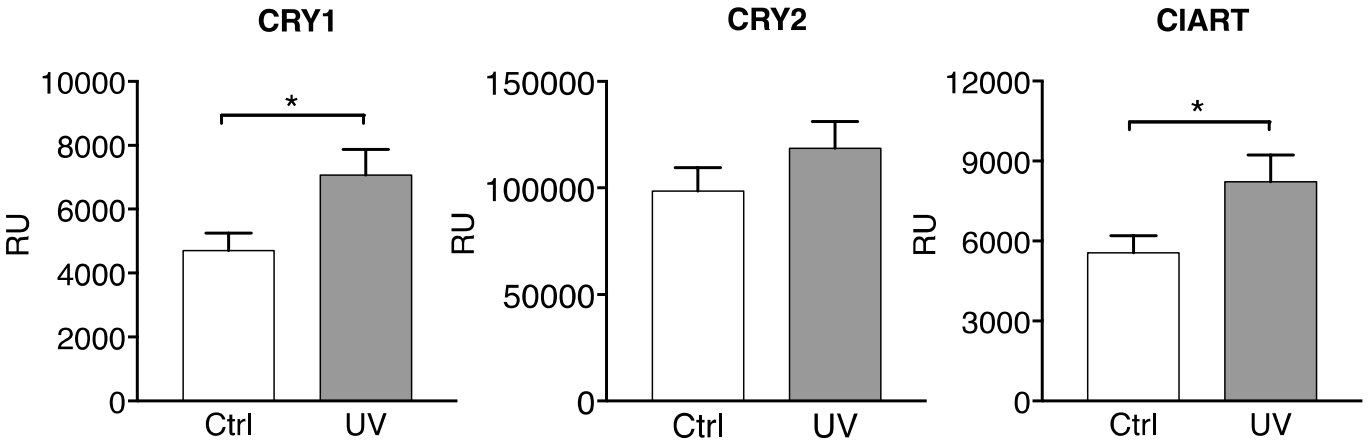


Figure 3

a

b

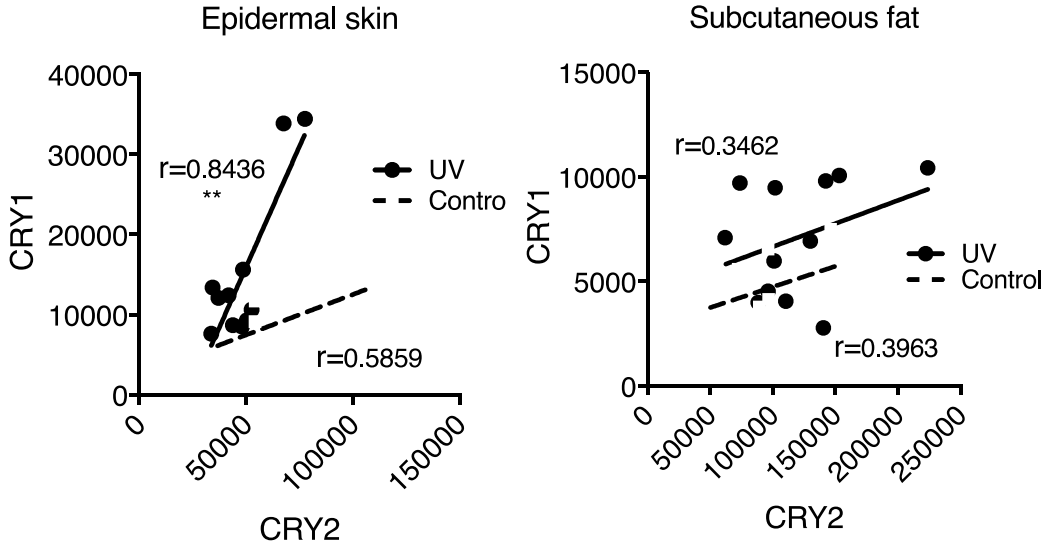


Figure 4