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Thyroid hormones enhance mitochondrial function in human epidermis

Silvia Vidali^{1,2}, Jérémy Chéret³, Melanie Giesen⁴, Swantje Haeger¹, Majid Alam³, Rachel E. B. Watson⁵, Abigail K. Langton⁵, Matthias Klinger⁶, Jana Knuever^{1*}, Wolfgang Funk⁷, Barbara Kofler² and Ralf Paus^{3,5}

¹Department of Dermatology, University of Luebeck, Luebeck, Germany; ²Research Program for Receptor Biochemistry and Tumor Metabolism, Laura Bassi Centre of Expertise-THERAPEP, Department of Pediatrics, Paracelsus Medical University, Salzburg, Austria; ³Department of Dermatology, University of Münster, Münster; Germany; ⁴Henkel Beauty Care, Henkel AG and Co. KgaA, Düsseldorf, Germany; ⁵Center for Dermatology Research, University of Manchester, Manchester, UK; ⁶Department of Anatomy, University of Luebeck, Luebeck, Germany; ⁷Klinik Dr Funk, Munich, Germany.

* : current address: Department of Dermatology, University of Cologne, Cologne, Germany

Correspondence: Ralf Paus, MD, Centre for Dermatology Research, School of Biological Sciences, University of Manchester, Stopford Bldg., Oxford Rd, Manchester M13 9PR, UK , ralf.paus@manchester.ac.uk

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Keywords: collagen, fibrillin, matrix metalloproteinase, MTCO1, TFAM, thyroxine, triiodothyronine.

Abbreviations: IHC, immunohistochemistry; IF, immunofluorescence; IR, immunoreactivity; MMP, matrix metalloproteinase; MTCO1, mitochondrially encoded cytochrome c oxidase I; mTORC1/2, mammalian target of rapamycin complex 1/2; NBT, nitro blue tetrazolium; P16^{ink4}, cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species; sirt1, sirtuin1; TH, thyroid hormones; TFAM, mitochondrial transcription factor; T₃, triiodothyronine; T₄, thyroxine.

ABSTRACT

Since it is unknown whether thyroid hormones (THs) regulate mitochondrial function in human epidermis, we treated organ-cultured human skin with triiodothyronine (T₃, 100 pM) or thyroxine (T₄, 100 nM). Both THs significantly increased protein expression of the mitochondrially-encoded cytochrome C oxidase I (MTCO1), complex I activity, and the number of perinuclear mitochondria. T₃ also increased mitochondrial transcription factor A (TFAM) protein expression, while T₄ stimulated complex II/IV activity. Increased mitochondrial function can correlate with increased reactive oxygen species (ROS) production, DNA damage and accelerated tissue aging. However, in isolated cultured human epidermal keratinocytes, THs neither raised ROS production nor matrix metalloproteinase (MMP)-1, -2 and -9 activity, nor decreased Sirt1 immunoreactivity. Instead, T₃ increased sirtuin-1, fibrillin-1, proliferator-activated receptor-gamma 1-alpha (PGC1 α), collagen I and III transcription, while T₄ decreased p16^{ink4} expression in organ-cultured human skin. Moreover, TH treatment increased intracutaneous fibrillin-rich microfibril and collagen III deposition and decreased mammalian target of rapamycin (mTORC1/2) expression *ex vivo*. This identifies THs as potent endocrine stimulators of mitochondrial function in human epidermis, which down-regulate, rather than enhance, the expression of skin aging-related biomarkers *ex vivo*. Therefore, topically applied THs deserve further exploration as candidate agents for treating skin conditions characterized by reduced mitochondrial function.

INTRODUCTION

Thyroxine (T₄) and triiodothyronine (T₃) are amino acid derivate hormones synthesized and secreted by the thyroid gland. They operate as key regulators of human energy metabolism and stimulate mitochondrial activity and biogenesis in many different organs (Cioffi et al., 2013; Johannsen et al., 2012; Norman and Henry, 2015; Weitzel et al., 2003; Yehuda-Shnaidman et al., 2014). Yet, although mammalian skin is a prominent target organ of thyroid hormones (THs) in health and disease (Antonini et al., 2013; Contreras-Jurado et al., 2011; Ramot et al., 2009; Safer, 2012; van Beek et al., 2008), it remains unknown whether and how THs impact on mitochondrial function in human epidermis.

Since TH can stimulate mitochondrial energy metabolism in human scalp hair follicle, the main aim of our study was to clarify whether mitochondrial activity is also stimulated by THs in human epidermal keratinocytes *in situ*, i.e. in organ-cultured human skin under serum- and TH-free conditions.

First, we investigated the expression of mitochondrially encoded cytochrome c oxidase I (MTCO1), the mitochondrial transcription factor A (TFAM), which controls mitochondrial biogenesis and mitochondrial DNA synthesis (Baris et al., 2011; Campbell et al., 2012; Kloepper et al., 2015; Shutt et al., 2011), and the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α), a key transcription factor that regulates mitochondrial biogenesis and numerous other cell functions including aging, whose reduction in activity has an aging-promoting effect (Dabrowska et al., 2015; Gilbert, 2013; Riera and Dillin, 2015). To assess whether any expression changes in these parameters altered also the intraepidermal mitochondrial oxidative phosphorylation, we asked whether THs modified the activity of complex I, the initial step of the mitochondrial respiratory chain (Chandel and Jeffs, 2015; Hirst, 2013; Scheffler, 2008). Finally, since THs stimulate mitochondrial biogenesis in several human cell types (Cioffi et al., 2013; Lee et al., 2012; Marin-Garcia, 2010) and human hair follicles (Vidali et al., 2014), we asked by transmission electron microscopy whether this also occurred in human epidermis *ex vivo*.

Increased mitochondrial activity may exert both beneficial and deleterious effects; the latter include the promotion of aging-associated pathways by enhancing ROS production and subsequent DNA damage (Bratic and Larsson, 2013; Koziel et al., 2011; Swerdlow, 2016). Therefore, a secondary aim of our study was to explore whether THs promote the expression of oxidative-stress and/or skin aging-related biomarkers routinely used in human skin aging research (Berneburg, 2008; Gilchrist, 2013; Jeong et al., 2015; Langton et al., 2012; Yoon et al., 2014).

For this purpose, matrix-metalloproteinases (MMPs) -1 (collagenase), -2 and -9 (gelatinases-A and -B), were investigated since these are typically up-regulated in aged skin where they degrade dermal collagens, elastic fibers and fibrillin-rich microfibrils (Ashworth et al., 1999; Kim et al., 2010; Sherratt

et al., 2010; Veidal et al., 2010). The major structural component of fibrillin-rich microfibrils, fibrillin-1, was also analysed, since it is a sensitive indicator of intrinsic and extrinsic skin aging (Naylor et al., 2011; Watson et al., 1999; 2008; 2009; 2014), along with mature collagen type I and III, since their expression decreases progressively during skin aging (Attia-Vigneau et al., 2014; Baroni Edo et al., 2012; Berneburg, 2008; Gilchrest, 2013; Langton et al., 2012).

Moreover, we investigated the effect of THs on the transcription of sirtuin-1 (*sirt1*), which is associated with lifespan expansion in common laboratory species, regulates senescence, aging, genomic stability and mitochondrial homeostasis (Fang et al., 2016; Hayakawa et al., 2015; Poulou and Raju, 2015), and the *sirt1* target, *PGC1 α* , a central regulator of aging, which enhances mitochondrial biogenesis and controls oxidative stress by up-regulating important ROS scavengers (Austin and St-Pierre, 2012; Gilbert, 2013; Riera and Dillin, 2015; Wenz, 2011). Finally, we quantified the cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1 (*p16^{ink4a}*), a key cell cycle senescence biomarker (Baker et al., 2011) and the mammalian target of rapamycin (*mTORC1/2*), another key regulator of ageing (Chiao et al., 2016; Evangelisti et al., 2016; Johnson et al., 2013).

Taken together, these experiments identify THs as potent endocrine stimulators of mitochondrial function in human epidermis, which down-regulate, rather than enhance, the expression of skin aging-related biomarkers *ex vivo*.

RESULTS

T₃ and/or T₄ increase MTCO1 and TFAM immunoreactivity in human epidermis *in situ*

Quantitative immunohistomorphometry revealed that both T₃ and T₄ significantly increased epidermal immunoreactivity (IR) of MTCO1 in human keratinocytes *in situ* (Figure 1a-d). T₃ also significantly increased the mitochondrial transcription factor A (TFAM) IR (Figure 1e-h), showing that THs up-regulate the expression of a key mitochondrial enzyme and a key transcription factor in human epidermis *in situ*. *PGC1 α* protein immunoreactivity was not significantly affected by THs, although treatment with T₃ showed a tendency towards an increased intraepidermal *PGC1 α* protein expression (Figure 1i-l).

THs enhance intraepidermal oxidative phosphorylation activity

Nitro blue tetrazolium (NBT) reduction assay (Knuever *et al.*, 2012; Poeggeler *et al.*, 2010) showed that T₃ and T₄ significantly enhanced complex I activity in human epidermis *ex vivo* (Figure 1m-p). Increased NBT reduction by THs was due to complex I oxidation, as demonstrated via complex I inhibition by rotenone (Supplementary Figure S1a-b). T₄ treatment also significantly increased

intraepidermal complex II and IV activity (Figure 1q-t); skin treatment with potassium cyanide, a potent inhibitor of complex IV, served as a negative control (Supplementary Figure S1c-d). This shows that THs enhance oxidative phosphorylation activity in human epidermis *ex vivo*.

Since actual ATP production in human epidermis *in situ* could not be measured, we investigated ATP production in cultured human primary epidermal keratinocytes. However, neither T₃ nor T₄ treatment significantly altered ATP formation in our experimental system (Supplementary Figure S2). Therefore, it remains to be determined whether TH stimulation impacts on intraepidermal ATP synthesis.

T₃ and T₄ treatment increases the number of mitochondria

By transmission electron microscopy, we found that T₃ and T₄ significantly increased the number of ultrastructurally detectable mitochondria in the perinuclear area of individually examined human epidermal keratinocytes *in situ* by 30% and 33%, respectively (Supplementary Figure S3). Although we cannot exclude that THs only shifted the intracellular position of mitochondria to a perinuclear location, our data, together with the increased TFAM and MTCO1 expression, suggest that THs might stimulate mitochondrial biogenesis in healthy human epidermis.

THs modulate intraepidermal expression of standard aging-related read out parameters in human skin

Quantitative immunohistomorphometry showed that both THs significantly down-regulated MMP-1 IR (Figure 2a-h) after 24 hours, but not after 6 days. As antibody-based methods identify both the pro- and active forms of these enzymes, intraepidermal MMP-1 activity (Figure 2i-p) was also investigated by *in situ* zymography. This showed that MMP-1 activity remained unaffected after both 24 hours and 6 days of TH treatment. Furthermore, we found that T₃ significantly decreased the intracutaneous activity of MMP-2 and -9 after 6 days *ex vivo* (Figure 3a-h).

Quantitative immunohistomorphometry for mature collagen type III revealed that its IR was strongly up-regulated after 6 days of treatment with T₃ (Figure 3i-p). IR for mature (i.e. processed) collagen type I was not significantly altered by TH treatment under assay conditions (Supplementary Figure S4a-h). Moreover, 24 hours of T₃ treatment increased deposition of fibrillin-rich microfibrils in the papillary dermis, with microfibrils appearing noticeably longer and less fragmented compared to vehicle-treated skin (Supplementary Figure S4i-l). This suggests the partial restoration of the fibrillin-rich microfibrillar network by THs, reminiscent of phenotypically “younger” skin (Naylor et al., 2011; Watson et al., 2014). Moreover, qRT-PCR showed that T₃ treatment significantly stimulated fibrillin 1, collagen I and III gene expression already after 24 hours (Supplementary Figure S5).

Although TH-treatment did not alter epidermal sirt1 IR (Figure 4a-h), T₃ significantly up-regulated sirt1 transcription (Supplementary Figure S5). Interestingly, both THs slightly, but significantly decreased mTORC1/2 IR in human epidermis *in situ* (Figure 4i-l), indicating a down-regulation of this key aging-promoting pathway. In contrast, PGC1 α mRNA levels were significantly increased after 24 hours of treatment with T₃, (Supplementary Figure S5) similar to PGC1 α protein expression (Figure 1i-l), p16^{ink4a} transcription was significantly down-regulated by T₄ (Supplementary Figure S5), again suggesting skin anti-aging effects of TH treatment.

Interestingly, T₄ decreased the mRNA levels of collagen I and III, fibrillin 1, sirt1 and PGC1 α , but this did not affect the protein expression, as shown by the immunoreactivity results.

THs do not up-regulate ROS production in human epidermal keratinocytes

Finally, we probed whether TH-stimulated keratinocyte mitochondrial activity is associated with increased ROS production. As the latter is difficult to assess in tissue sections, this was examined in cultured primary human epidermal keratinocytes (NHEK), using a ROS-specific dye (CM-H2DCFDA) (Vidali et al., 2014). This showed that treatment with different concentrations of either T₃ (50, 100 and 200 pM) or T₄ (50, 100 and 200 nM) for 24 hours failed to increase ROS production (Supplementary Figure S6).

DISCUSSION

Here, we present, that physiological concentrations of exogenous THs (Molina and Ashman, 2010; van Beek et al., 2008) up-regulate mitochondrial function and the number of mitochondria in normal human epidermis in serum- and TH-free skin organ culture (see Supplementary Table 1 for an overview of the results). As declining mitochondrial function and energy metabolism are characteristics of aged and aging tissues, the restoration of mitochondrial bioenergetics has been advocated as an effective approach for the treatment of numerous aging-associated pathologies (Fang et al., 2016; Poulouse and Raju, 2015; Szeto, 2014; Wallace, 2011; Wallace et al., 2010). Moreover, increasing evidence suggests that numerous skin diseases are linked to mitochondrial dysfunction and that restoration of mitochondrial function may offer an attractive auxiliary therapy for managing these skin disorders (Boulton et al., 2015; Feichtinger et al., 2014). Therefore, promoting mitochondrial function in human skin, in theory, may also exert anti-aging properties.

However, a pragmatic and effective pharmacological strategy for achieving this in human skin remains to be developed. The current study suggests that T₄, an inexpensive, toxicologically very well-defined hormone that has been systemically administered in clinical medicine for decades (Biondi and Wartofsky, 2014; Topliss and Soh, 2013) and is intracutaneously deiodinated to T₃ (van Beek et al., 2008), could serve in the treatment of skin conditions and pathologies connected with a decline in mitochondrial function (Boulton et al., 2015), intrinsic and extrinsic skin aging (Anderson et al., 2014;

Berneburg, 2008; Gilchrist, 2013; Glass et al., 2013; Grillon et al., 2012; Kaneko et al., 2012; Oyewole et al., 2014). Topical application of T₄ is expected to reduce the well-known adverse effects associated with elevated systemic TH levels (Kharlip and Cooper, 2009; Taylor et al., 2013; Walrand et al., 2014). Interestingly, topical T₃ has already been shown to promote murine skin wound healing and hair growth *in vivo* (Safer, 2012; Safer et al., 2005), while a topically applied TH analogue counteracted glucocorticoid-induced human skin atrophy (Yazdanparast et al., 2006).

Whilst the current study was not designed to prove or disprove anti-aging effects of THs in human skin, which requires additional systematic research and longer-term analyses, we aimed to probe whether THs also promote undesired collateral effects associated with increased mitochondrial ROS production which could potentially promote tissue damage and skin aging. While the data reported herein cannot yet conclusively rule out this possibility, they render this possibility unlikely. Instead, our current data suggest that THs rather exhibit anti-aging properties in human skin *ex vivo*, at least during the relatively short experimental window investigated here.

Firstly, MMP-1 protein expression and MMPs-2/-9 activity are both up-regulated in aged skin (Geng et al., 2010; Kim et al., 2010; Sherratt et al., 2010), yet are down-regulated by T₃ or T₄ treatment (Figure 2 and 3). Secondly, deposition of fibrillin-1 protein is enhanced by THs, along with a marked improvement in the overall structure of fibrillin-rich microfibrils in the papillary dermis (Supplementary Figure S4), similar to the effects reported for other candidate human skin anti-aging agents (Watson et al., 2001; 2008; 2009; 2014). Thirdly, T₄ down-regulated the senescence marker p16^{ink4}, while T₃ up-regulated the gene expression of both, sirt1 and PGC1 α on the mRNA and protein level, two key regulators of cellular oxidative stress, aging and mitochondrial function (Austin and St-Pierre, 2012; Baker et al., 2011; Dabrowska et al., 2015; Geng et al., 2010).

The mitochondrial data here presented suggest that THs stimulate intraepidermal mitochondrial biogenesis (Supplementary Figure S3) as they do in many other tissues, including human hair follicles (Cioffi et al., 2013; Lee et al., 2012; Marin-Garcia, 2010; Santillo et al., 2013; Vakitus et al., 2015; Vidali et al., 2014), via a sirt1-PGC1 α -mediated pathway. Also, both THs decreased mTORC1/2, whose inhibition is involved in mitochondrial biogenesis and reportedly restores a more juvenile phenotype and increased life span (Chiao et al., 2016; Evangelisti et al., 2016; Johnson et al., 2013). Finally, THs did not increase ROS production, at least in cultured primary human keratinocytes. Of course, this system cannot predict how long-term administration of THs might eventually upset the balance between beneficial and detrimental properties of these hormones with respect to intracutaneous oxidative stress.

Unexpectedly, 24 hours of treatment with T₄ decreased the mRNA levels of collagen I and III, fibrillin 1, sirt1 and PGC1 α , in contrast with the protein expression data, where T₄ did not change their immunoreactivity after 24 hours and, for sirt1, collagen I and III, also after 6 days. This suggests that,

as first response to T_4 treatment, the cells might down regulate the mRNA levels of these parameters, but their expression will then stabilize at the same level of the untreated skin in the longer run. However, to have a better view on how T_4 acts on a genomic level it would be necessary to investigate the mRNA expression at different time points, but due to the scarce amount of available human skin, this was not possible.

Another interesting point is that since T_3 is the active form of T_4 , one would expect that T_3 is indeed more active than T_4 . Here, this was not always the case in line with previous observations for the response of human hair follicles to T_3 versus T_4 (van Beek et al., 2008). This might relate to the finding that T_4 can also act directly through distinct pathways that do not require prior conversion to T_3 (Cheng et al., 2010; Davis et al., 2016; Oyanagi *et al.*, 2015). Given that we only examined skin from middle-aged/older women in order to circumvent confounding gender influences, it will be interesting to study, next, whether mitochondrial activity-promoting and/or skin aging inhibitory effects of THs are also seen in male skin (unfortunately, much less male skin is available from cosmetic surgery, making this considerably more difficult to study).

Since THs induce a plethora of gene expression changes and functional effects through genomic and non-genomic pathways (Chen et al., 2013; Davis et al., 2016; Iordanidou et al., 2010; Senese et al., 2014), it will be a major challenge to dissect the exact mechanisms by which THs impact on keratinocyte mitochondrial biology and human skin aging. However, given that genomic TH actions are mediated by TH nuclear receptors, c-erb A α and A β (Tata, 2013; Verga Falzacappa et al., 2009; Weitzel and Iwen, 2011), it is relevant that human keratinocytes express both TH nuclear receptors (Billoni et al., 2000; Contreras-Jurado et al., 2011). Intriguingly, isoforms of the two receptors, like p43, have also been found in mitochondria (Cioffi et al., 2013; Lin et al., 2011; Tata, 2013; Weitzel et al., 2003). Thus, it is conceivable that these isoforms could mediate some direct, non-genomic effects of THs on human keratinocyte mitochondria.

Finally, some of the mechanisms and read-out parameters described here are shared between wound healing and aging. Therefore, one might wonder whether THs only exert pseudo-anti-aging effect by inhibiting wound healing in our *ex vivo* assay, which operates with (mechanically wounded) human skin fragments. However, it is unlikely that the putative anti-aging effects of THs can be explained by wound healing *inhibition*. THs actually *promote* wound healing, a highly energy-consuming process, in murine skin *in vivo* (Kassem et al., 2012; Safer, 2012; Safer et al., 2005) and promotes re-epithelialization in experimentally wounded organ-cultured human skin (Meier *et al.*, 2013) *ex vivo* (Paus lab, unpublished observation), while mice devoid of the TR-alpha and -beta show retarded wound-healing (Contreras-Jurado *et al.*, 2014; 2015; Safer *et al.*, 2005). Moreover, all *in situ* analyses reported here were purposely performed distant from the periphery of the organ-cultured skin fragments, thus lowering the risk to confound aging-related phenomena with superimposed wound

healing processes (for detailed discussion of wound healing-related skin responses as a potential confounding element in the current study, see Supplementary Text S1).

In summary, our study identifies T₃ and T₄ as endocrine stimulators of mitochondrial activity and possible biogenesis in normal human skin and raises the intriguing prospect that topical T₄ may be exploited as future therapeutic for skin presenting a decreased bioenergetic status as it is found in various dermatoses and during skin aging (Boulton et al., 2015). The next challenge is to clarify whether endogenous THs and synthetic TR ligands can actually retard or even reverse mitochondrial function-dependent human skin aging, namely after long-term topical administration.

MATERIALS AND METHODS

Human skin organ culture

Human skin samples (scalp, breast, forearm) were obtained from 7 healthy females (age range: 38-71; mean age: 58) undergoing routine cosmetic plastic surgery after written informed consent and Institutional Research Ethics Committee permission (University of Luebeck), adhering to Helsinki guidelines.

Full-thickness human skin organ culture was performed as previously described (Bodo et al., 2010; Knuever et al., 2012; Lu et al., 2007). Biopsy punches (4 mm diameter) were generated and cultured at the air-liquid interface. After a 24 hour pre-incubation period, vehicle (William's E Medium), T₃ (100 pM), T₄ (100 nM) (Sigma-Aldrich; Steinheim, Germany) were added to the media for 24 hours or 6 days. For the 6 days incubation, medium and treatments were changed every second day; TH concentrations were selected based on their documented stimulatory effects on human hair growth (van Beek et al., 2008) and mitochondrial function within human hair follicle epithelium (Vidali et al., 2014). Human skin largely remains morphologically intact and shows continued proliferative activity under these long-term organ culture conditions (Lu et al., 2007).

Immunohistochemistry and immunofluorescence

Following cultivation, skin samples were embedded into cryomatrix (THERMO Fisher Scientific; Schwerte, Germany), frozen in liquid nitrogen and stored at -80°C. Sections (6 µm) were prepared for histological analysis.

Mitochondrially encoded cytochrome c oxidase I (MTCO1) and fibrillin-1 IR were investigated by light microscopy, using mouse monoclonal anti -MTCO1 (1D6E1A8, 1:50; Mitoscience; Eugene, OR, USA) and -fibrillin-1 (11c1.3, 1:200; AbCam; Cambridge, UK). As secondary antibody a biotinylated goat anti-mouse antibody (1:200; Jackson Immuno Research; Suffolk, UK) was used. The AEC method (Perkin Elmer; Rodgau, Germany) was used for immunodetection. Nuclei were demarcated with haematoxylin (Knuever *et al.*, 2012; Watson *et al.*, 2001).

Mitochondrial transcription factor A (TFAM), PGC1 α , collagen-I and -III, MMP-1 and mTORC1/2 IR were investigated by fluorescence microscopy, using a mouse polyclonal antibody anti-TFAM (1:200; kindly provided by Professor Rudolf Wiesner, University of Cologne, Cologne, Germany), a rabbit polyclonal anti-PGC1 α (1:100; AbCam; Cambridge, UK), and anti-mTORC1/C2 (1:200; AbCam; Cambridge, UK), a mouse monoclonal anti-collagen-I (5D8-G9, 1:500; AbCam; Cambridge, UK), anti-collagen-III (1E7-D7/Col3, 1:500; AbCam; Cambridge, UK) anti-MMP-1 (F15P3B6, 1:200; Biologend, San Diego, CA, USA).

As secondary antibodies a goat anti-rabbit FITC (1:200; Jackson Immuno Research; Suffolk, UK), a goat anti-mouse rhodamine (1:200; Jackson Immuno Research; Suffolk, UK), or a rabbit anti-mouse AlexaFluor 488 (1:200; Invitrogen; Paisley, UK) (Langton *et al.*, 2014; Vidali *et al.*, 2014) were used. The nuclei were demarcated by 4',6-diamidin-2-fenilindolo (DAPI). Omission of the first antibody and absence of IR in skin compartments known to be negative for tested antigens served as negative controls, while confirmation of the skin IR patterns expected from the literature served as positive control.

Quantitative immunohistomorphometry

Images were obtained with a digital microscope (Keyence, Neu-Isenburg, Germany). Quantitative immunohistomorphometry was performed as described before (Knuever *et al.*, 2012; Poeggeler *et al.*, 2010; Vidali *et al.*, 2014) (Supplementary Text S2).

All mitochondrial parameters investigated in the current study were exclusively evaluated in the epidermis, as it is the metabolically most active constituent of the skin, second only to hair follicle epithelium, whose response to THs we had already reported (van Beek *et al.*, 2008; Vidali *et al.*, 2014). Also, given that organ-cultured skin represents a traumatized tissue, which prominently exhibits wound healing phenomena such as re-epithelisation at the tissue edges (Meier *et al.*, 2013), all read-out parameters reported here were purposely evaluated in the center of the skin punches, i.e. as far away from the wound edge as possible. Therefore all data were harvested in the least-wounded skin environment.

Complex I and complex II /IV activity assay

Respiratory chain complex I activity was visualized by the nitro blue tetrazolium (NBT) reduction assay *in situ* by incubating skin sections for 3 hours at 37° C with a solution 0.2% of NBT (Merck; Darmstadt Germany) in phosphate buffer saline (PBS), a negative control with rotenone 3 μ M was used (see Supplementary Text S3). Complex II and IV activity was assessed by incubating the slides in a solution containing 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich; Steinheim, Germany),

followed by incubation with a solution containing NBT (Merck; Darmstadt, Germany) (see Supplementary Text S4). Staining analysis were performed as for MTCO1 (see above).

Transmission electron microscopy (TEM)

TEM was performed as previously described (Vidali et al., 2014) (see Supplementary Text S5).

***In situ* zymography**

In situ zymography was performed utilizing DQTM gelatine (Invitrogen; Paisley, UK) (to detect MMPs-2/-9) and DQTM collagen (Invitrogen; Paisley, UK) (to detect MMP-1) (see Supplementary Text S6).

qRT-PCR

Human scalp skin from a 61 years old female was obtained as for the organ culture (see above). Skin was kept in RNAlater (Qiagen, Hilden, Germany) to preserve tissue and kept at -20°C before RNA was extracted. Total RNA was extracted from 4 mm skin punch biopsies with fat removed, using Qiagen RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions (see Supplementary Text S7).

ROS and ATP production in cultured human keratinocytes

ROS and ATP production in normal human epidermal keratinocytes (NHEK) were assessed as previously described (Vidali et al., 2014) (see supplementary text S8)

Statistical analysis

All data were analyzed using One-way-ANOVA or Student's t-test for unpaired samples by employing GraphPad Prism software (GraphPad Prism, La Jolla, CA, USA) (Vidali et al., 2014) (see figure legends for details).

CONFLICT OF INTEREST:

None declared.

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Figures**Figure 1. MTCO1, TFAM and PGC1 α IR; complex I, II and IV activity**

Full thickness human skin organ culture biopsies were treated with (a, e, i, m, q) vehicle; (b, f, j, n, r) T₃ (100 pM); and (c, g, k, o, s) T₄ (100 nM) for 24 hours. (a-d) MTCO1 IHC, (e-h) TFAM IF, (i-l) PGC1 α IF, (m-p) complex I and (q-t) complex II and IV activity were performed.

(d) MTCO1 IR, (h) TFAM IR, (l) PGC1 α IR, (p) complex I, (t) complex II and IV activity were measured with ImageJ in precise and standardized epidermal areas. Ep=epidermis, bar: 100 μ m. n=8-9 samples (3 subjects analyzed). Mean \pm SEM (One-way analysis of variance (ANOVA)), * P <0.05, ** P <0.01, *** P <0.001.

Figure 2. MMP-1 IR and activity

Full-thickness human skin organ culture treated with (a, e, i, m) vehicle; (b, f, j, n) T₃ (100 pM) and (c, g, k, o) T₄ (100 nM) for (a-c, i-k) 24 hours or (e-g, m-o) 6 days, during which time the skin remains vital (Lu et al., 2007). (a-h) MMP-1 IF was performed to detect pro- and active- MMP-1, (i-p) *in situ* zymography (ISZ) to measure MMP-1 activity. Ep=epidermis, bar: 100 μ m. (d, h) MMP-1 IR and (l, p) MMP-1 activity were measured in precise and standardized epidermal and/or dermal areas. n=6-12 samples (3 subjects for MMP-1 IR, 24 hours; 4 subjects for MMP-1 ISZ, 24 hours; 2 subjects for MMP-1 IR and ISZ, 6 days). Mean \pm SEM (One-way analyses of variance (ANOVA)), ** P <0.01. It is normally believed that MMPs activity resides in the dermis; however, there is increasing evidence that MMPs (and LOX/LOXLs) have additional activity in the epidermis (Ågren et al., 2015; Fisher et al., 2009). It is possible that MMPs are released epidermally and then diffuse to the papillary dermis to act on collagens and elastic fiber components.

Figure 3. MMPs-2/-9 activity, collagen-III IR

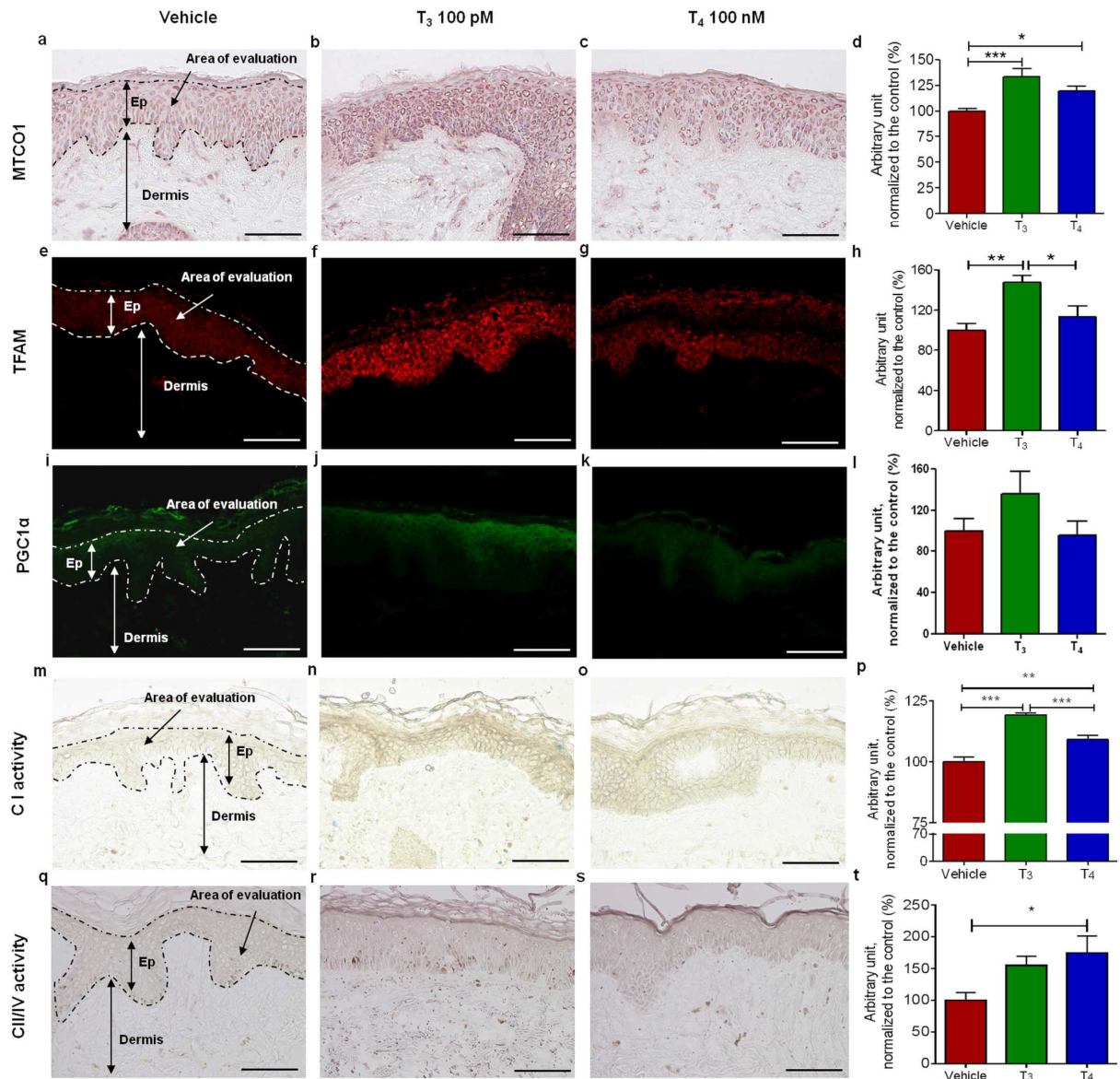
Full-thickness human skin biopsies treated with: (a, e, i, m) vehicle, (b, f, j, n) T₃ (100 pM) and (c, g, k, o) T₄ (100 nM) for (a-c, i-k) 24 hours or (e-g, m-o) 6 days. (a-h) *in situ* zymography assay was performed to detect the activity of MMPs-2/-9. (i-p) Collagen-III IF was performed. Ep=epidermis, bar: 100 μ m. (d, h) MMPs-2/-9 activity and (l, p) collagen-III IR measured in precise and standardized epidermal and/or dermal areas. n=6-12 samples (4 subjects for the 24 hours treatment, 2 subjects for the 6 days treatment). Mean \pm SEM (One-way analysis of variance (ANOVA)), * P <0.05, ** P <0.01, *** P <0.001.

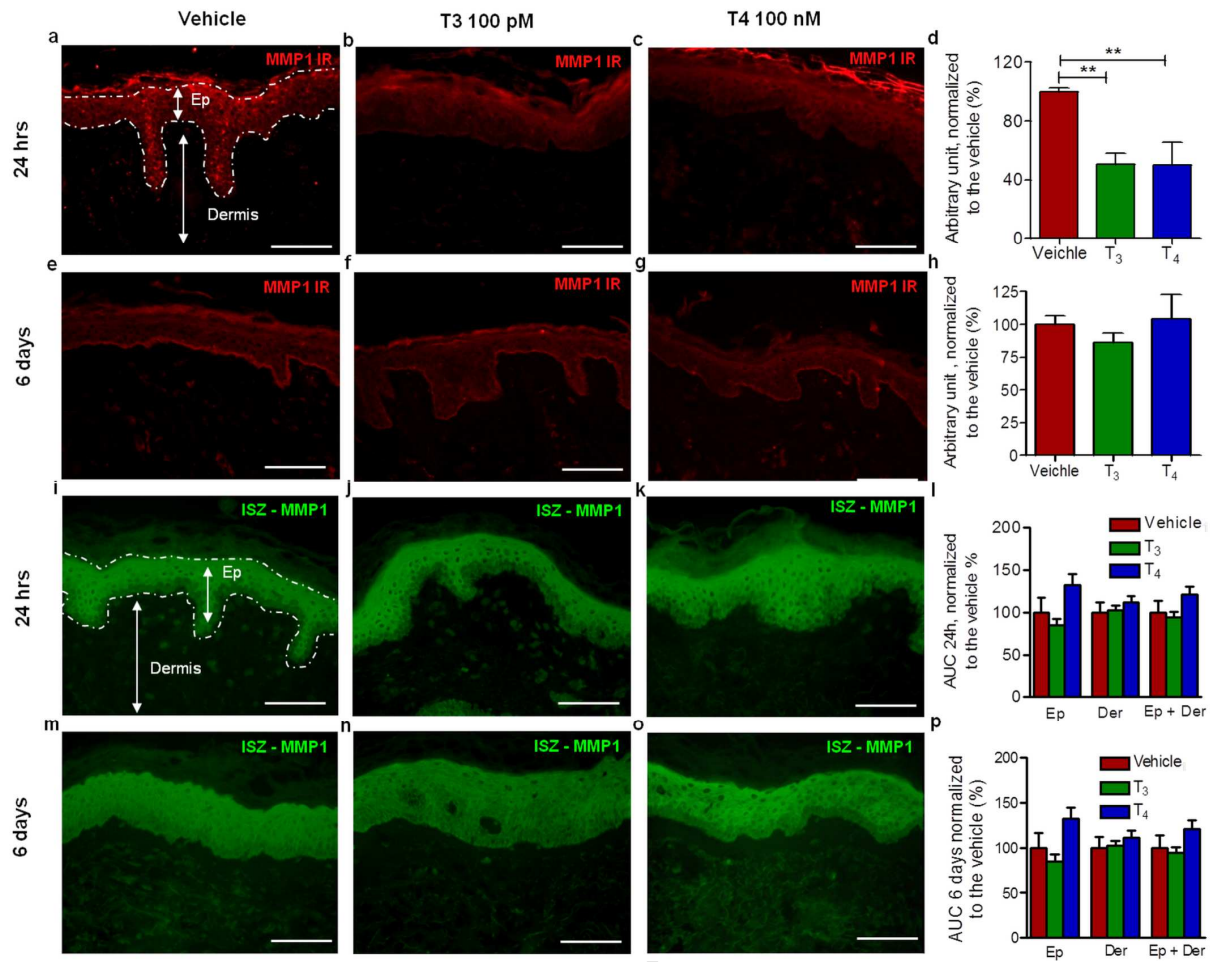
Figure 4. Sirt1 IR and mTORC1/2 IR

Full-thickness human skin organ culture treated with (a, e, i) vehicle; (b, f, j) T₃ (100 pM) and (c, g, k) T₄ (100 nM) for (a-c, i-l) 24 hours or (e-g) 6 days. (a-h) Sirt-1 IHC. Ep=epidermis, bar: 100 μ m. (d, h) Sirt-1 IR was measured in precise and standardized epidermal area. n=6-9 samples (3 subjects for the 24 hours incubation; 2 subjects for the 6 days). Mean \pm SEM (One-way analyses of variance

(ANOVA)). (i-l) mTORC1/2 immunofluorescence. Ep=epidermis, bar: 100 μ m. (l) mTORC1/2 IR was measured in precise and standardized epidermal area. n=9-11 samples (4 subjects). Mean \pm SEM (One-way analyses of variance (ANOVA)), * P <0.05.

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