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Complete List of Authors:	Vidali, Silvia; University of Lübeck, Department of Dermatology Knuever, Jana; University of Lübeck, Department of Dermatology Lerchner, Johannes; TU Bergakademie, Institute of Physical Chemistry Giesen, Melanie; Henkel AG & Co. KGaA, Skin and Hair Research Unit Bíró, Tamás; University of Debrecen, Department of Physiology Klinger, Matthias; University of Luebeck, Department of Anatomy Kofler, Barbara; Paracelsus Medical University, Research Programm for Receptor Biochemistry and Tumor Metabolism, Department of Pediatrics Funk, Wolfgang; Klinik Dr Koslowski, - Poeggeler, Burkhard; University of Lübeck, Department of Dermatology Paus, Ralf; University of Lübeck, Department of Dermatology
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Hypothalamic-pituitary-thyroid (HPT) axis hormones stimulate mitochondrial function and biogenesis in human hair follicles

Silvia Vidali[1,*], Jana Knuever[1,*,$], Johannes Lerchner[2,#], Melanie Giesen[3,#], Tamás Bíró[4], Matthias Klinger[5], Barbara Kofler[6], Wolfgang Funk[7], Burkhard Poeggeler[1,$$] and Ralf Paus[1,8]

[1]Department of Dermatology, University of Luebeck, Luebeck, Germany; [2]Institute of Physical Chemistry, TU Bergakademie, Freiberg, Germany; [3]Henkel AG & Co. KGaA [4]Department of Physiology, University of Debrecen, Debrecen, Hungary; [5]Department of Anatomy, University of Luebeck, Luebeck, Germany; [6]Research Programm for Receptor Biochemistry and Tumor Metabolism, Department of Pediatrics, Paracelsus Medical University, Salzburg, Austria; [7]Klinik Dr Koslowski, Munich, Germany; [8]Institute of Inflammation and Repair, University of Manchester, Manchester, UK

*, # *These authors contributed equally.*

$ *Current address: Department of Dermatology, University of Cologne, Cologne, Germany*

$$ *Current address: QUIRIS Healthcare, Guetersloh, Germany*

Correspondence: Ralf Paus, MD, Department of Dematology, University of Luebeck, Ratzeburger Allee 160, 23538 Luebeck, Germany. E-mail: ralf.paus@uksh.de

Short title: HPT axis and mitochondria in hair follicles

Abbreviations: TRH, Thyrotropin-realising hormones; TSH, thyrotropin; HF, hair follicle; T_3, triiodothyronine; T_4, thyroxine; ORS, outer root sheath; HPT-axis, hypothalamic-pituitary-thyroid axis; MTCO1, mitochondrial-encoded subunit 1 of cytochrome c oxidase; TFAM, mitochondrial transcription factor 1; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; HSP60, heat shock protein 60; BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1; PGC1α, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha.

ABSTRACT

Thyroid hormones regulate both, hair growth and mitochondrial functions. Since the other hypothalamic-pituitary-thyroid (HPT) axis hormones, i.e. thyrotropin-releasing hormone (TRH) and thyrotropin (TSH), are expressed in human hair follicles (HFs) and regulate mitochondrial functions in human epidermis, we have investigated in organ-cultured human scalp HFs whether TRH (30 nM), TSH (10 mU/ml), thyroxine (T_4) (100 nM) and triiodothyronine (T_3) (100 pM) alter intrafollicular mitochondrial biology and energy metabolism. All HPT-axis members increased gene and protein expression of MTCO1, a key subunit of complex IV of the electron transport chain, and of TFAM, a key transcription factor that controls mitochondrial DNA synthesis. T_3, T_4, TSH, and TRH also stimulated intrafollicular complex I and IV activity and promoted intrafollicular mitochondrial biogenesis. Notably, T_3 also increased follicular heat production. HPT-axis hormones did not increase ROS production in cultured human HF keratinocytes, T_3 and T_4 even reduced ROS formation, and all HPT-axis hormones increased transcription of ROS-scavenging key enzymes (catalase and/or SOD2) in these cells. Thus, mitochondrial biology, energy metabolism, and redox state of human HFs are subject to profound (neuro-)endocrine regulation by HPT-axis hormones. The novel neuroendocrine controls of mitochondrial biology in a complex human mini-organ identified here may be therapeutically exploitable.

INTRODUCTION

The hypothalamic-pituitary-thyroid (HPT)-axis controls multiple metabolic processes in vertebrate organisms (*Bassett et al., 2008; Costa-E-Sousa et al., 2012*), including human skin (*Paus et al., 2010, 2011*). Stimulation of the pituitary gland by hypothalamus-derived TRH leads to TSH secretion. TSH then stimulates thyroid gland activity, namely synthesis and secretion the thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3) (*Zoeller et al., 2007*). Circulating T_4 is thought to largely act after deiodination into T_3 in peripheral organs, including human hair follicles (HFs) (*Kaplan et al., 1988; Safer et al., 2009; van Beek et al., 2008*).

Several elements of the HPT-axis are also expressed in human skin *in situ* and/or some of its constituent cell populations *in vitro* (*Slominski et al., 2002; Paus, 2010*). For example, TRH mRNA has been identified in cultured human dermal and HF fibroblasts (*Slominski et al., 2002*), while TSH mRNA and protein is found in normal human epidermis (*Bodó et al., 2010*), and TSH receptor (TSH-R) transcripts and protein in human skin and HF mesenchyme (*Bodó et al., 2009*; *Cianfarani et al., 2010*). Both TRH and its receptor (TRH-R) are expressed in the epithelium of normal human scalp HFs (*Gáspár et al., 2010, 2011*). Human

skin may therefore possess a peripheral equivalent of the central HPT-axis (*Slominski et al., 2002*, *Bodó et al., 2010; Paus, 2010*).

THs directly alter important human HF functions, e.g. THs prolong anagen, stimulate hair matrix keratinocyte proliferation and pigmentation, and modulate intrafollicular keratin expression (*van Beek et al., 2008*). Instead, the intrafollicular functions of TSH, which regulates the expression of selected keratins in human HFs (*Ramot et al., 2011*), remain unclear (*Bodó et al., 2009; Paus, 2010*). However, TRH has surfaced as a potent stimulator of human hair growth and pigmentation (*Gáspár et al., 2010, 2011*) and regulates intrafollicular prolactin and prolactin-receptor expression *in situ* (*Langan et al., 2010*).

THs greatly impact on mitochondria (*Harper et al., 2008, Weitzel et al., 2003, 2011*), whose activities control human energy metabolism and homeostasis, (*Scheffler, 2008*, *Portman, 2008; Kharlip et al., 2009)*. Mitochondria process products of glycolysis and fatty acid oxidation to generate ATP, contain enzymes critical for multiple biosynthetic processes, contribute to the amino acid metabolism and ion homeostasis, regulate cell death pathways by ROS and Ca^{2+} signalling, and are involved in aging, cancer development and the pathogenesis of an ever-increasing list of human diseases (*Larsson, 2010; Birch-Machin, 2006; Scheffler, 2008; Wallace and Fan, 2009; Wallace, 2010*). Therefore, fully understanding the hormonal controls of mitochondrial biology is of major biological and clinical interest.

Though mainstream mitochondrial research has largely ignored human skin as a research model, human skin actually is a most instructive model system for identifying novel, clinically relevant hormonal controls of mitochondrial function and biogenesis. Namely, both TRH and TSH are previously unknown, yet powerful neuroendocrine regulators of mitochondrial biology in human epidermis (*Poeggeler et al., 2010a; Knuever et al., 2012*). However, whether THs, TRH and/or TSH exert a similar function in human HFs was unknown as HF mitochondrial biology has remained almost completely unexplored. Since hair growth is a highly energy-consuming process (*Kealey et al., 1994; Stenn and Paus, 2001*), we hypothesized that these HPT-axis hormones may also regulate human HF energy metabolism and mitochondrial function.

Therefore, using serum-free organ culture of healthy human scalp HFs (*Philpott et al., 1990; Kloepper et al., 2010*), we asked whether and how TRH, TSH and THs influence intrafollicular mitochondrial biology in the absence of all neural, vascular, and extrafollicular hormonal inputs. The list of mitochondrial read-out parameters that we had previously established and reported for organ-cultured human epidermis (*Poeggeler et al., 2010a; Knuever et al., 2012*) was further extended, notably including porin expression as an additional indicator of mitochondrial biogenesis (*Feichtinger et al., 2011*) and calorimetry as an indicator of mitochondrial energy metabolism-dependent heat production (*Lerchner et al.,*

2008a, b). Furthermore, in cultured human ORS (outer root sheath) keratinocytes, mitochondrial data were correlated with ROS production and the expression of important ROS scavenging enzymes (catalase, SOD2).

Collectively, these studies provide the first evidence that all examined key players of the HPT-axis profoundly stimulate mitochondrial biology and regulate the redox state of human HFs.

RESULTS

HPT-axis elements increase MTCO1 and TFAM protein expression *in situ* while T_3/T_4 stimulate MTCO1 and TFAM transcription

Previously, we had shown that TSH upregulates MTCO1 gene and protein expression in human HFs (*Bodó et al., 2009)* and human epidermis, where TSH also increases the expression of TFAM (*Poeggeler et al., 2010a*). TFAM controls mitochondrial DNA synthesis (*Scarpulla, 2008*) by initiating mtDNA replication and mitochondrial protein transcription (*Maniura-Weber et al., 2004; Viña et al., 2009; Hallberg et al., 2011*) and is thus indispensable for a functional electron transport chain (*Baris et al., 2011*). TFAM also appears to be important for the maintenance and stabilization of mitochondrial DNA (*Larsson et al., 1998*). Therefore, we assessed the impact of HPT-axis hormones on MTCO1 and TFAM expression in human scalp HFs.

We found that T_3, T_4 and TSH significantly increase MTCO1-IR in the proximal ORS *in situ* wether TRH only showed a slight increase in IR (**Figure 1a-f**). Moreover, TH treatment increased intrafollicular MTCO1 mRNA transcription (**Figure 1g**). Furthermore, TFAM protein expression *in situ* was significantly increased after 24 hrs of treatment with TRH, TSH and THs (**Figure 2a-f**), T_3 and T_4 also stimulated TFAM mRNA expression (**Figure 2g**).

T_3/T_4, TRH and TSH treatment increases mitochondrial biogenesis

These TFAM data raised the question whether these HPT-axis hormones can actually stimulate mitochondrial biogenesis in the HF, just as we had already shown for TSH and TRH in human epidermis (*Poeggeler et al., 2010a; Knuever et al., 2012*). TEM showed that the number of ultrastructurally detectable mitochondria was upregulated by all HPT-axis players, particularly in the perinuclear region of human ORS keratinocytes (**Figure 3a-f**).

To confirm this, qualitative and semi-quantitative ultrastructural evidence on the level of fluorescence microscopy, porin-IR was assessed by quantitative immunohistomorphometry. Porin is located in the mitochondrial membrane and acts as a channel that enables small molecule diffusion and it is often used as a marker for mitochondrial mass (*Feichtinger et al.,*

2011). Indeed, 24 hrs of treatment with T_3 and TRH significantly increased ORS porin-IR *in situ*, whether T_4 and TSH shown just a trend (**Figure 3g-l**). Thus, all tested HPA-axis elements stimulate mitochondrial biogenesis within the epithelium of healthy human HFs.

Thyroid hormones enhance gene expression of HSP60, BMAL and PGC1α in HFs

Next, we checked the transcription of three additional key players in mitochondrial biology: HSP60 (**Figure 4a**), which protects mitochondria from stress damage (*Cappello et al., 2008*), BMAL1 (**Figure 4b**), a component of the biological clock with a central role in cell aging (*Jung-Hynes et al., 2010*), and PGC-1α (**Figure 4c**), the "master regulator of mitochondriogenesis" (*Safdar et al., 2011*). Treatment with T_4 (100 nM, 24 hrs) significantly increased the mRNA steady-state levels of PGC-1α and HSP90, while T_3 (100 pM) stimulated HSP60 and BMAL transcription. This underscores that THs potently stimulate mitochondrial function in human HFs *in situ.*

Mitochondrial activity is enhanced by treatment with HPT-axis hormones

We then asked whether mitochondrial activity itself is also enhanced by THs, using follicular heat production as a physical indicator of enhanced mitochondrial energy metabolism. In contrast to glycolysis and glutaminolysis, intramitochondrial redox processes strongly contribute to the overall heat production (*Gnaiger et al., 1990; Jastroch et al., 2010*). Therefore, changes in the measured heat rate indicate an increase or decrease of the electron transfer rate in the electron transport chain (*Gnaiger et al., 1990*).

Using chip-calorimetry (*Lerchner et al., 2008a, b*), we present here the first heat measurement of an intact human (mini-)organ *in vitro*: As shown in **Fig. 5**, HF treatment with T_3 (100 pM) resulted in a significantly enhanced heat release compared to vehicle controls. Interestingly, HF heat production already increased after only 2 hrs of T_3 stimulation. **Figure 5b** shows two examples of the heat rate development of sets of five HFs each are depicted. After stabilization of the metabolism, a rather constant signal of about 9 µV could be observed over several hours. The corresponding heat rate of about 0.3 µW per follicle is in line with previously reported data for metabolic flows of human HFs (*Kealy et al., 1994*).

The scarcity of human HFs for study excluded the calorimetric examination of heat production effects by T_4, TRH or TSH. Instead, we performed classical biochemical enzyme activity measurements of key components of the respiratory chain. These showed that T_3, T_4, TRH and TSH all stimulate the activity of both complex I and IV after 24 hrs of treatment (**Figure 5c-d**). Together, these calorimetric and enzymatic activity data demonstrate that the tested HPT-axis players not only stimulate mitochondrial gene expression and biogenesis, but also mitochondrial activity within normal human scalp HFs.

TRH, TSH and TH treatment does not alter the redox state of ORS keratinocytes

As increased mitochondrial activity can lead to enhanced ROS production and consequently to increased oxidation and ROS-mediated cell damage (*Gemma et al., 2007; Vendelbo et al., 2011; Wallace, 2010*), enhancing mitochondrial function is not necessarily a beneficial event. Therefore, we checked ROS production in cultured ORS keratinocytes, using the ROS-sensitive CM-H_2DCFDA probe (*Eruslanov and Kusmartsev, 2010*). This showed that, after 24 hrs of treatment, ROS production did not significantly differ in HF keratinocytes treated with any of the HPT-axis hormones compared to vehicle. Rather, T_3 and T_4 seemed to exert a slightly (yet not significant) ROS-protective effect after a 10 minutes H_2O_2 challenge: while there was a moderately increased ROS production in vehicle-, TRH- and TSH-treated cells, this was lacking in the TH-treated HF keratinocytes (**Figure 6a**).

HPT-axis hormones up-regulate the expression of ROS scavenging enzymes by human HF keratinocytes

Finally, we investigated the transcription of two crucial ROS scavenging enzymes of human skin in cultured human ORS keratinocytes, catalase and SOD2 (*Chelikani et al., 2004; Halliwell and Gutteridge, 2007; Marionnet et al., 2011; Silva et al., 2005; Vafaee et al., 2010*) which are expressed in human HFs and whose activity likely impacts on HF aging and greying (*Slominski et al., 2005; Wood et al., 2009*). Interestingly, catalase transcription was significantly upregulated in all test groups treated with HPT-axis hormones, and TSH and T_4 also enhanced SOD2 mRNA steady-state levels in ORS keratinocytes (**Figure 6b and c**). These cell culture data raise the possibility that HPT-axis players may even boost intrafollicular ROS scavenging systems.

DISCUSSION

Taken together, the biochemical, gene expression, ultrastructural, calorimetric and quantitative immunohistomorphometric data presented here provide unequivocal evidence that the tested HPT-axis hormones rapidly stimulate mitochondrial gene expression and even mitochondrial biogenesis within normal human scalp HFs. Moreover, TRH, TSH and THs also stimulate mitochondrial activity. These findings are well in line with the long-recognized stimulatory role of THs on mitochondrial biology and with the recent discovery of TRH and TSH as potent, novel neuroendocrine regulators of human epidermal mitochondrial biogenesis and function *in situ* (*Poeggeler et al., 2010a; Knuever et al., 2012*).

Given that more mitochondria or an increased mitochondrial protein expression does not necessarily imply increased mitochondrial energy production, it was important to investigate the functionality of mitochondrial energy metabolism. This was done by chip-calorimetry and

classical enzyme activity assays. These clearly demonstrate that T_3 enhances HF heat production and that TRH, TSH and THs stimulate mitochondrial chain complex I and IV activity in human HFs. This shows that our relatd prior findings in epidermis and brain tissue (*Poeggeler et al., 2010a, b; Knuever et al., 2012*) also apply to human HFs.

The current study represents the first systematic characterization of HF mitochondrial biology in any mammalian species, and encourages one to further explore the as yet under-investigated energy metabolism of human HFs (*Kealey et al., 1994; Williams et al., 1993*). They also underscore that, contrary to conventional wisdom in mainstream mitochondrial research, not only human epidermis (*Birch-Machin et al., 2006; Knuever et al., 2012; Poeggeler et al., 2010a*), but also the metabolically highly active HFs of human skin offer excellent model systems for dissecting the activities, biogenesis, and controls of human mitochondria *in situ*. Human HF chip-calorimetry even permits one to directly measure heat production of a living human (mini-)organ *in vitro*. Moreover, our data render it likely that, besides THs, the intrafollicularly produced HPT axis neurohormones TSH and TRH play a role in regulating HF energy metabolism.

Our findings emphasize the importance of neuroendocrine controls not only in general mitochondrial biology (*Poeggeler et al., 2010 a,b; Knuever et al., 2012),* but also in human skin and HF biology (*Arck et al., 2006; Slominski et al., 2002, 2007; Paus, 2010, 2011; Zmijewski and Slominski, 2011; Slominski and Wortsman, 2000*). The corresponding lessons learned from studying organ-cultured human HFs, i.e. complex mini-organs, may well be transferrable to other less accessible human tissues. The current data also provide clinically relevant pointers to how HF aging and HF disease related to declining mitochondrial function might be effectively counteracted in the future by endogenous neurohormones produced in the human HF itself, i.e. TRH (*Gáspár et al., 2010*) and TSH (*Bodo et al., 2009*). This also applies to T_3 and T_4, which have long been known to modulate human HF growth, hair shaft quality, and/or pigmentation (*Messenger, 2000; van Beek et al., 2008*). Both TRH and T_4 are administered routinely in thyroid medicine and are FDA-approved agents with a well-known toxicity profile. Therefore, regulatory hurdles to repositioning these hormones for novel "mitochondrial hair medicine" approaches are relatively low.

Current "mitochondrial medicine" concepts (e.g., Wallace et al., 2010; Wallace, 2011) operate under the assumption that, in systems with declining mitochondrial function, the therapeutic increase of mitochondrial activity generally has beneficial effects. However, increased mitochondrial activity could also have undesirable effects, such as increased ROS production (*Gemma et al., 2007; Vendelbo et al., 2011*). While we could not analyze this in human HFs *in situ* due to insufficient HF availability, our HF keratinocyte data (employed as surrogate system) suggest that HPT-axis hormones stimulate HF mitochondrial biogenesis and activity without increasing ROS production. These hormones may even promote

intrafollicular ROS scavenging by upregulating catalase and SOD2 transcription. Of course, only long-term stimulation experiments and in-depth analyses of ROS production, ROS scavenging and ROS damage indicators, can dissipate remaining concerns that long-term administration of HPT-axis hormones might eventually tip the balance of desired towards deleterious effects that could arise from chronically increased intrafollicular mitochondrial energy metabolism. Insights won from such long-term human HF studies are likely to provide important pointers to future application of HPT-axis hormones in general "mitochondrial medicine" and gerontobiology.

In summary, mitochondrial functions, energy metabolism, and biogenesis of human HFs are subject to profound (neuro-)endocrine regulation by TRH, TSH and THs. This underscores the importance of further exploring the HPT-axis equivalent system of human skin and its appendages, and of dissecting how this system can be therapeutically targeted.

MATERIALS AND METHODS

Skin samples, human HF organ culture and ORS keratinocytes culture

Human scalp skin samples were obtained after written informed consent and institutional research board ethics license (University of Luebeck) from 8 healthy middle-aged females undergoing routine cosmetic plastic surgery, adhering to Helsinki guidelines and with informed patient consent. The varying numbers of HFs and subjects are stated separately in each figure legend.

HF organ culture was performed in serum-free Williams´ E medium supplemented with glutamine, insulin, hydrocortisone and an antibiotic/antimycotic mixture as described before (*Philpott et al., 1990; Bodó et al., 2009; Kloepper et al., 2010; Gáspár et al., 2011)*. After pre-culture of microdissected HFs for 24 hrs to minimize dissection-associated disturbances of mitochondrial function during this calibration period, vehicle (WE medium only), T_3, T_4, TRH or TSH (Sigma-Aldrich) were added for an additional 24 hrs. After treatment the HF samples were shock frozen in liquid nitrogen and stored at -80°C. 6 µm thick sections were cut and used for immunostainings. Since only a very limited number of human HFs was available for study, one carefully selected concentration was assessed for each hormone, based on prior published results: T_3 (100 pM), T_4 (100 nM) (*van Beek et al., 2008*), TRH (30 nM) (*Gaspar et al., 2010, 2011*), TSH (10mU/mL) (*Bodó et al., 2009; Poeggeler et al., 2010*).

Human ORS keratinocyte cultures were obtained from face-lift surgery samples of healthy subjects. Primary cultures of ORS keratinocytes were established from plucked anagen HFs as described (*Philpott and Kealey, 1994*)**.** Humans ORS keratinocytes (passage 3) were

seeded into 96-well plates. After reaching 70% confluency cells were treated with bioactives for 24 hrs.

Immunohistochemistry, immunofluoresescence, and quantitative immunohistomorphometry

MTCO1 immunoreactivity (IR) was determined as previously described (*Poeggeler et al., 2010; Knuever et al., 2012*). TFAM- and porin-IR were investigated by fluorescence microscopy. For staining of the mitochondrial DNA-binding transcription factor TFAM, a rabbit polyclonal antiserum (kindly provided by Rudolf Wiesner, University of Cologne, Germany) was used 1:200, with +4°C over night (O/N) incubation. As secondary antibody we used a goat anti-rabbit Rhodamine (Jackson ImmunoResearch), 1:200, incubated for 2 hrs at room temperature.

VDAC1/Porin was used to depict mitochondrial biogenesis (*Feichtinger et al., 2011*), utilizing a rabbit polyclonal antibody (1:600, O/N at +4°C). As secondary antibody we used a 1:200 goat anti-rabbit biotinylated antibody (Jackson Immuno Research), incubated for 45 minutes at RT. The TSA method was used for detection (Perkin Elmer TSA kit, FITC). Omission of the primary antibody served as negative control. Cell nuclei were demarcated by DAPI and images were obtained by digital microscope (Keyence, Germany).

Quantitative immunohistomorphometry was performed by assessing the relative intensity of immunoreactivity in precisely defined and standardized HF reference areas with ImageJ (NIH), as indicated in the corresponding illustrations.

Transmission electron microscopy (TEM)

TEM was performed as previously described (*Kramer et al., 2005; Knuever et al., 2012*). Briefly, following a fixation with 2.5% glutaraldehyde for 2 hrs, human dissected HFs were washed in 0.1 M cacodylate buffer, pH 7.2. Using 1% osmiumtetroxide samples were postfixated, dehydrated in ethanol and embedded in araldite (Fluka, Buchs, Switzerland). Ultrathin sections were stained with uranyl acetate and lead citrate and then viewed with a Philips electron microscope 400 at 6000x magnification.

Chip calorimetry

Heat measurements as a reliable indicator of follicular mitochondrial heat production and thus activity/energy metabolism were performed using a newly developed prototype chip calorimeter (*Lerchner et al., 2008a, b*)

The main component of heat measurement by chip calorimetry is a silicon chip with an integrated thin film thermopile which consists of 472 BiSb/Sb thermocouples that convert the heat rate, produced by the sample located inside the flow channel, into a voltage signal . The

complete chip module is enclosed in a high precision thermostat. The fluidic system used for the injection of medium consists of two units comprising a stepping motor driven piston pump (280 µL) and several micro-valves each (*Lerchner et al., 2008a, b*). Five dissected HFs were inserted into the flow channel and fixed in one segment (**Figure 5a***).* After mounting of the flow channel atop the thermopile chip inside the calorimeter and sufficient thermal equilibration, the generation of heat signals is stimulated by periodic injections of 280 µL WE medium at a flow rate of 50 $\mu L min^{-1}$. After relaxation of an endothermal injection effect caused by incomplete thermal equilibration of the injected liquid, an exothermal signal shift Δu indicates metabolic heat production by the HFs. The baseline is recovered after about 30 minutes because of oxygen depletion. After about two hours of periodic injection of WE medium the injection was switched to WE medium containing 100 pM T_3, delivered by a second fluid unit.

Quantitative real-time PCR (qRT-PCR)

Steady-state mRNA levels from organ-cultured human HFs were studied by qRT-PCR after 24 hrs of incubation with the HPT-axis hormones for the following key genes of mitochondrial biogenesis and/or energy metabolism: MTCO1, TFAM, heat shock protein 60 (HSP60), peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) and brain and muscle ARNT (aryl hydrocarbon receptor nuclear translocator)-like protein 1 (BMAL1) (*Cappello et al., 2008*; *Safdar et al., 2011; Jung-Hynes et al., 2010*). Gene expression was assessed by quantitative real-time PCR performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) following the published protocol, and normalized to one of three tested housekeeping genes that proved to be least-regulated by the test hormone (*Knuever et al., 2012).*

The qRT-PCR in ORS keratinocytes for SOD2 and catalase was performed as previously described in Giesen et al 2011 *(further details, see figure legend)*.

Biochemical assays

Complex I and complex IV activity were analyzed in HF homogenates as described by Poeggeler et al. (2010), following the protocols of Mazzio et al. (2004), Dabbeni-Sala et al. (2001) and Rustin et al. (1994), respectively. Both experiments were performed with 8 HFs each and were repeated multiple times, i.e. with HFs from 6 different subjects.

ROS production in ORS keratinocytes

Production of reactive oxygen species (ROS) was detected using the specific dye CM-H_2DCFDA (Life Technologies), which is oxidized to green fluorescent DCF

(dichlorofluorescein) in the presence of ROS according to the manufacturer protocol and as indicated in the corresponding figure legend.

Statistical analysis

If not stated otherwise, all data were analyzed using ANOVA one way analysis of variance (Tukey's test) by employing the Graph Pad Prism software (Graph Pad Prism, USA).

CONFLICT OF INTEREST

None declared.

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Figure 1. MTCO1 expression. HFs were treated with (**a**) vehicle, (**b**) T_3 (100 pM), (**c**) T_4 (100 nM), (**d**) TSH (10 mU/mL) or (**e**) TRH (30 nM) for 24 hrs and immunostained for MTCO1 (nuclear counterstain: haematoxylin). DP = dermal papilla, HM = hair matrix, ORS = outer root sheath. Magnification: 200x. Quantitative immunohistomorphometry in defined reference areas was performed by ImageJ software (NIH). (**f**) IMTCO1 immunoreactivity compared to vehicle. Treatment groups were normalized to the vehicle arbitrarily set at 100. n = 6-11 HFs (2 subjects). (**g**) MTCO1 mRNA steady-state levels (qRT-PCR) in HFs treated with T_3 (100 pM) or T_4 (100 nM) for 24 hrs. Data from 15 HFs (1 subject).
Mean ± SEM (one-way ANOVA), *p < 0.05, **p < 0.01, ***p<0.001.

Figure 2. TFAM expression. HFs treated with (**a**) vehicle, (**b**) T_3 (100 pM), (**c**) T_4 (100 nM), (**d**) TSH (10 mU/mL) or (**e**) TRH (30nM) for 24 hrs and immunostained for TFAM (nuclear counterstain: DAPI) and evaluated by quantitative immunohistomorphometry. DP = dermal papilla, HM = hair matrix, ORS = outer root sheath. Magnification: 200x. (**f**) Increased immunoreactivity compared to vehicle (arbitrarily set to 100). n = 5-10 HFs (2 subjects). (**g**) TFAM mRNA mRNA steady-state levels (qRT-PCR) in HFs treated with T_3 (100 pM) or T_4 (100 nM) for 24 hrs. n = 15 HFs (1 subject).
Mean ± SEM (One-way Anova), **p < 0.01, ***p < 0.001.

Figure 3. Mitochondrial biogenesis. HFs treated with (**a**, **g**) vehicle, (**b, h**) T_3 (100 pM), (**c**, **h**) T_4 (100 nM), (**d, j**) TSH (10 mU/mL) or (**e, k**) TRH (30nM) for 24 hrs. (**a**-**f**) Number of ultrastructurally detectable mitochondria (TEM), particularly in the perinuclear region of human ORS keratinocytes. n = 10-12 keratinocytes (6 subjects). N=nucleus, red arrows indicate exemplary mitochondria. Magnification: 6000x. (**g-k**) Porin-IR DP = dermal papilla, HM = hair matrix, ORS = outer root sheath. Magnification: 200x. (**l**) Quantitative immunohistomorphometry of Porin-IR was assessed by quantitative immunohistomorphometry . Data are reported in percentages, normalized to the vehicle. n = 5-10 HFs (2 subjects).
Mean ± SEM (one-way ANOVA), * p<0.05, *** p < 0.001.

Figure 4. Mitochondrial gene expression: qRT-PCR. Graphs show mRNA steady-state levels for (**a**) PGC1α, (**b**) BMAL1 and (**c**) HSP60 in human HFs treated with T_3 (100 pM) or T_4 (100 nM) for 24 hrs.
Mean ± SEM (One-way Anova), *** p < 0,001, ** p < 0,01. n=15 HFs (1 subject).

Figure 5. HF heat production: Calorimetry. (**a**) Representation of the calorimetric chip, the flow channel and the position of the HFs. (**b**) Difference of heat production quantified in µV. n

= 8 HFs (2 subjects). The arrow indicate start of the treatment with T_3 (100 pM) in the green line (■) and red line (▼). The grey line (●) represent subject 2 without treatment. (**c**, **d**) Human dissected HFs treated with T_3 (100pM), T_4 (100 nM), TRH (30 nM) or (TSH 10 mU/mL) for 24 hrs. The graphs show (**c**) complex I activity (NBT reduction) (n = 6 subjects, 8 HFs/subjects, mean ± SEM, p<0.001) and (**d**) complex IV activity in human HF mitochondria (n = 6 subjects, 8 HFs/subject, mean ± SEM, p<0.001).

Figure 6. ORS keratinocytes (**a-c**) treated with T_3 (100 pM), T_4 (100 nM), TRH (30 nM) or TSH (10 mU/mL) for 24 hrs. (**a**) after incubation with the substances, the medium was replaced by 100 µl/well culture medium containing DCFDA and incubated for 30 min. To further stimulate oxidative stress in the cell cultures, cells were treated with 30 µM H_2O_2 for 30 min. at 37°C. Subsequently, cells were incubated for further 90 min in fresh media and fluorescence was measured using a 96-well plate reader (Tecan) with excitation/emission wavelengths of 485/527. Graph shows the relative fluorescence unit (RFU) detected by DCF measurement reported in percentages where the vehicle (basal level) is set at 100%. Mean ± SEM (one-way ANOVA). * $p < 0.05$, ***$p < 0.001$, n= 6-24 wells.
(**c**,**d**) qRT-PCR mRNA extracts were analyzed for catalase (NM_001752) and SOD2 (NM_000636), steady-state levels. After total RNA isolation according to the manufacturer's instructions using the RNeasy MinElute Spin Column system (Qiagen), qRT-PCR was performed using a MX3000Pro RT-PCR System (Stratagene). Expression of each gene was first normalized against human reference total RNA (Stratagene), which worked as an internal standard, and subsequently against the expression of the house keeping gene GAPDH. Mean ± SEM (One-way Anova), * $p < 0.05$ ** $p < 0.01$, n = 3 wells.

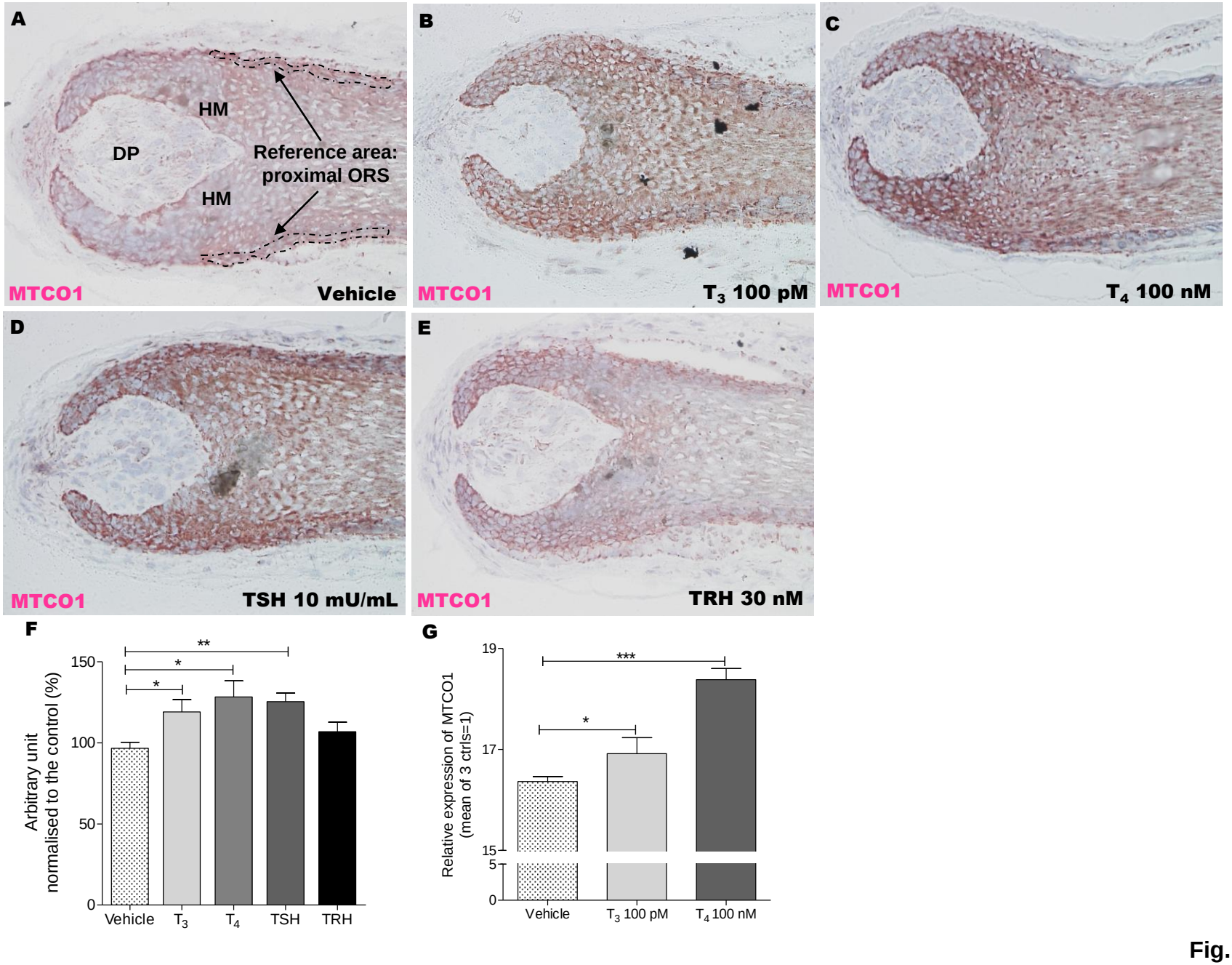
A
HM
DP
Reference area:
proximal ORS
HM
MTCO1
Vehicle
B
MTCO1
T3 100 pM
C
MTCO1
T4 100 nM
D
MTCO1
TSH 10 mU/mL
E
MTCO1
TRH 30 nM
F
Arbitrary unit
normalised to the control (%)
150
100
50
0
**
*
*
Vehicle
T3
T4
TSH
TRH
G
Relative expression of MTCO1
(mean of 3 ctrls=1)
19
17
15
5
0
***
*
Vehicle
T3 100 pM
T4 100 nM

Fig.1

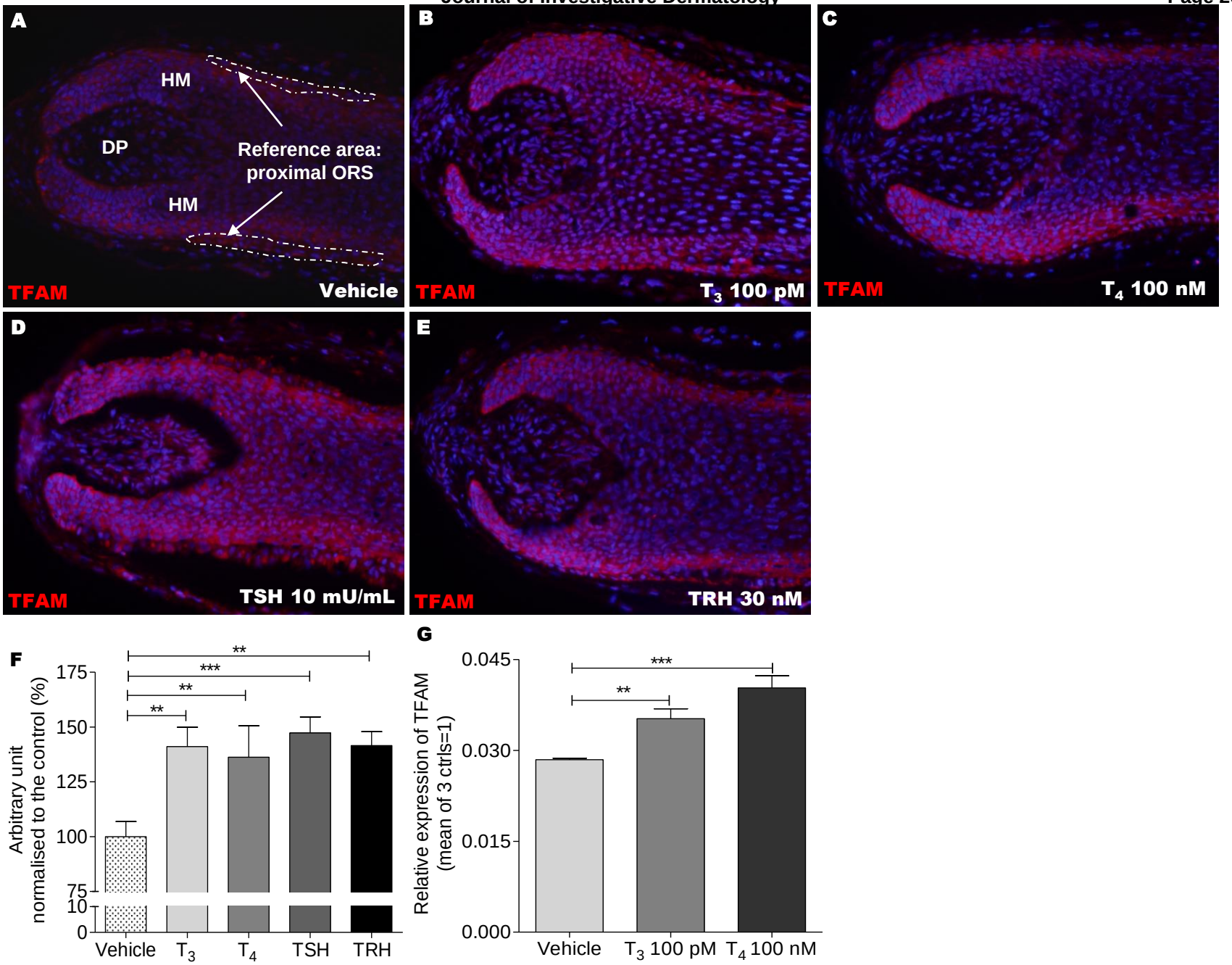
A
HM
DP
Reference area:
proximal ORS
HM
TFAM
Vehicle
B
TFAM
T3 100 pM
C
TFAM
T4 100 nM
D
TFAM
TSH 10 mU/mL
E
TFAM
TRH 30 nM
F
Arbitrary unit
normalised to the control (%)
175
150
125
100
75
10
0
**
***
**
**
Vehicle
T3
T4
TSH
TRH
G
Relative expression of TFAM
(mean of 3 ctrls=1)
0.045
0.030
0.015
0.000
***
**
Vehicle
T3 100 pM
T4 100 nM

Fig.2

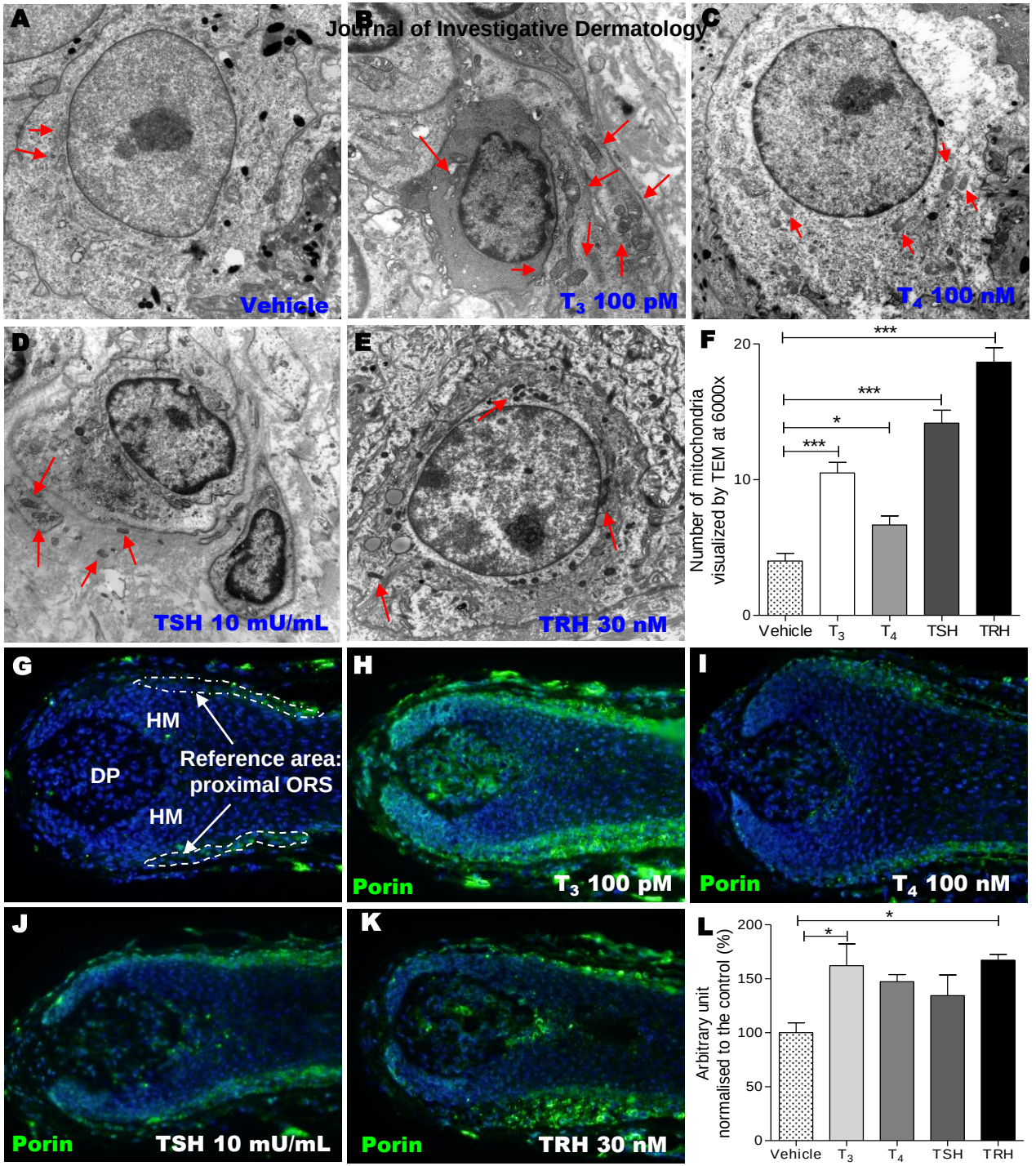
A
Vehicle
B
T3 100 pM
C
T4 100 nM
D
TSH 10 mU/mL
E
TRH 30 nM
F
Number of mitochondria visualized by TEM at 6000x
20
10
0
***
***
*
***
Vehicle
T3
T4
TSH
TRH
G
HM
Reference area: proximal ORS
DP
HM
H
Porin
T3 100 pM
I
Porin
T4 100 nM
J
Porin
TSH 10 mU/mL
K
Porin
TRH 30 nM
L
Arbitrary unit normalised to the control (%)
200
150
100
50
0
*
*
Vehicle
T3
T4
TSH
TRH

Fig.3

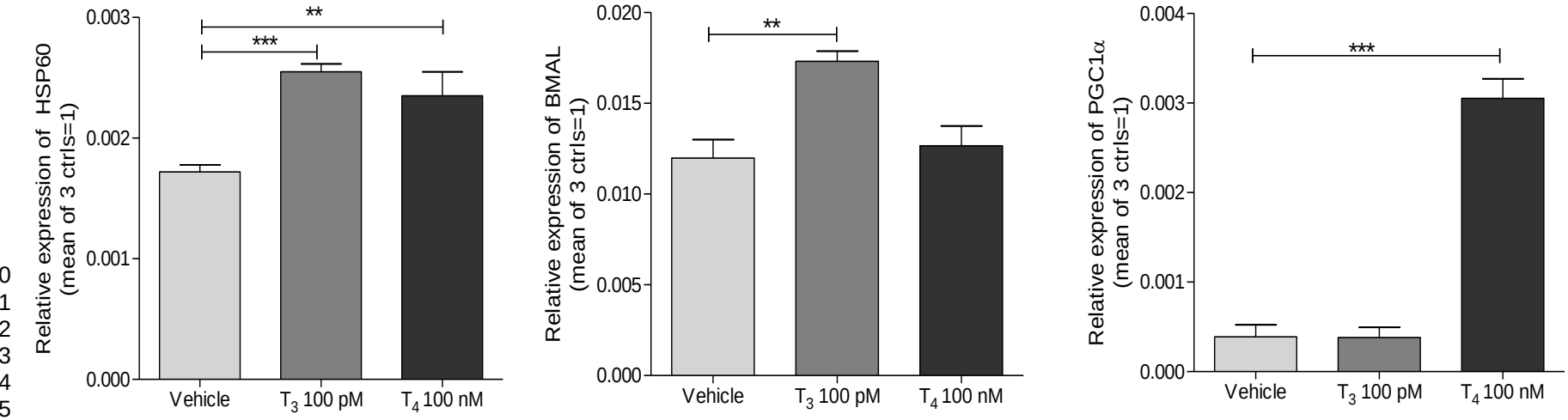
A
Relative expression of HSP60
(mean of 3 ctrls=1)
0.003
0.002
0.001
0.000
**
***
Vehicle
T3 100 pM
T4 100 nM
B
Relative expression of BMAL
(mean of 3 ctrls=1)
0.020
0.015
0.010
0.005
0.000
**
Vehicle
T3 100 pM
T4 100 nM
C
Relative expression of PGC1α
(mean of 3 ctrls=1)
0.004
0.003
0.002
0.001
0.000
***
Vehicle
T3 100 pM
T4 100 nM

Fig.4

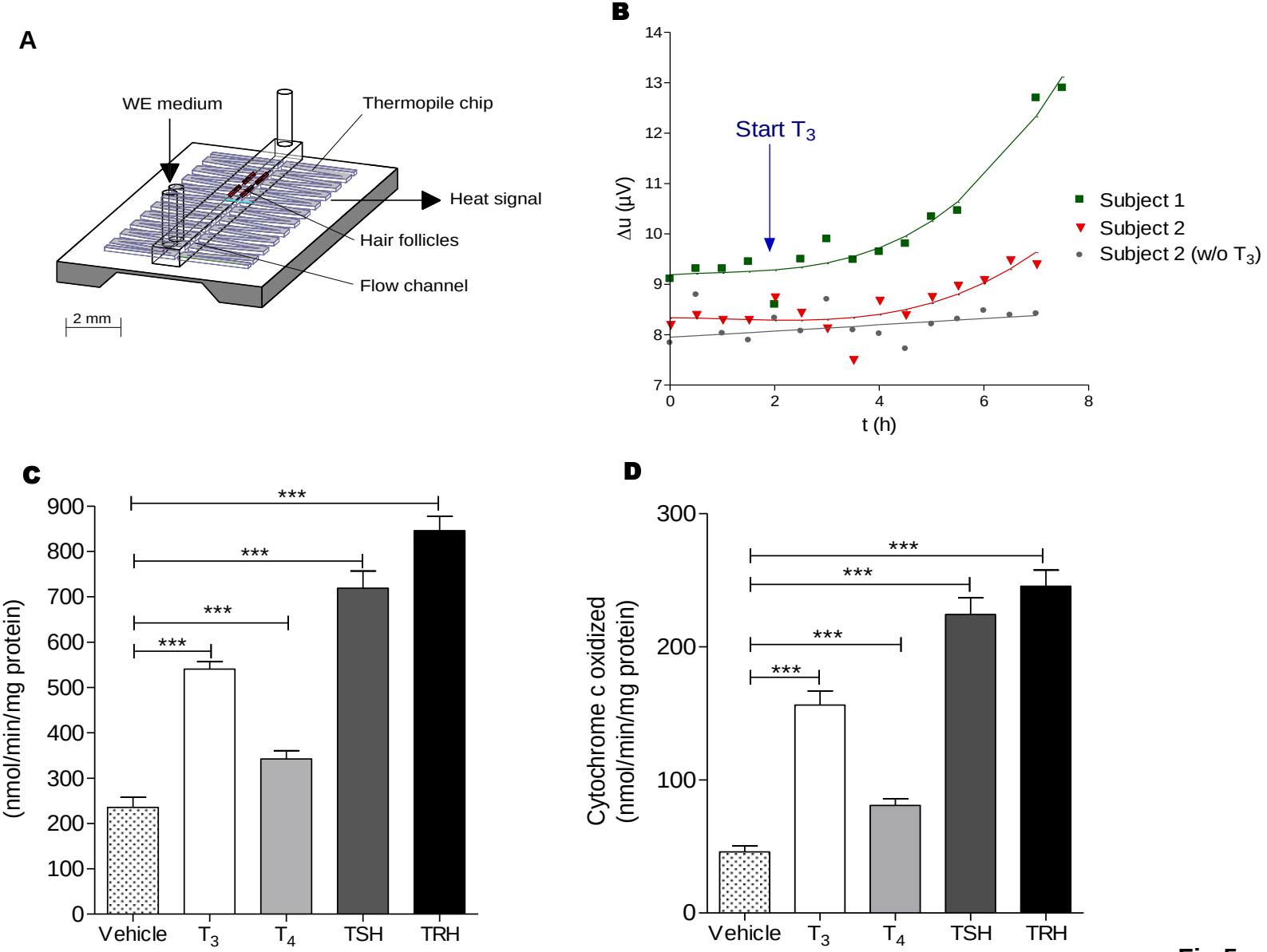
A
WE medium
Thermopile chip
Heat signal
Hair follicles
Flow channel
2 mm
B
Start T_3
Δu (μV)
t (h)
Subject 1
Subject 2
Subject 2 (w/o T_3)
C
NBT reduction (nmol/min/mg protein)
***
Vehicle
T_3
T_4
TSH
TRH
D
Cytochrome c oxidized (nmol/min/mg protein)

Fig.5

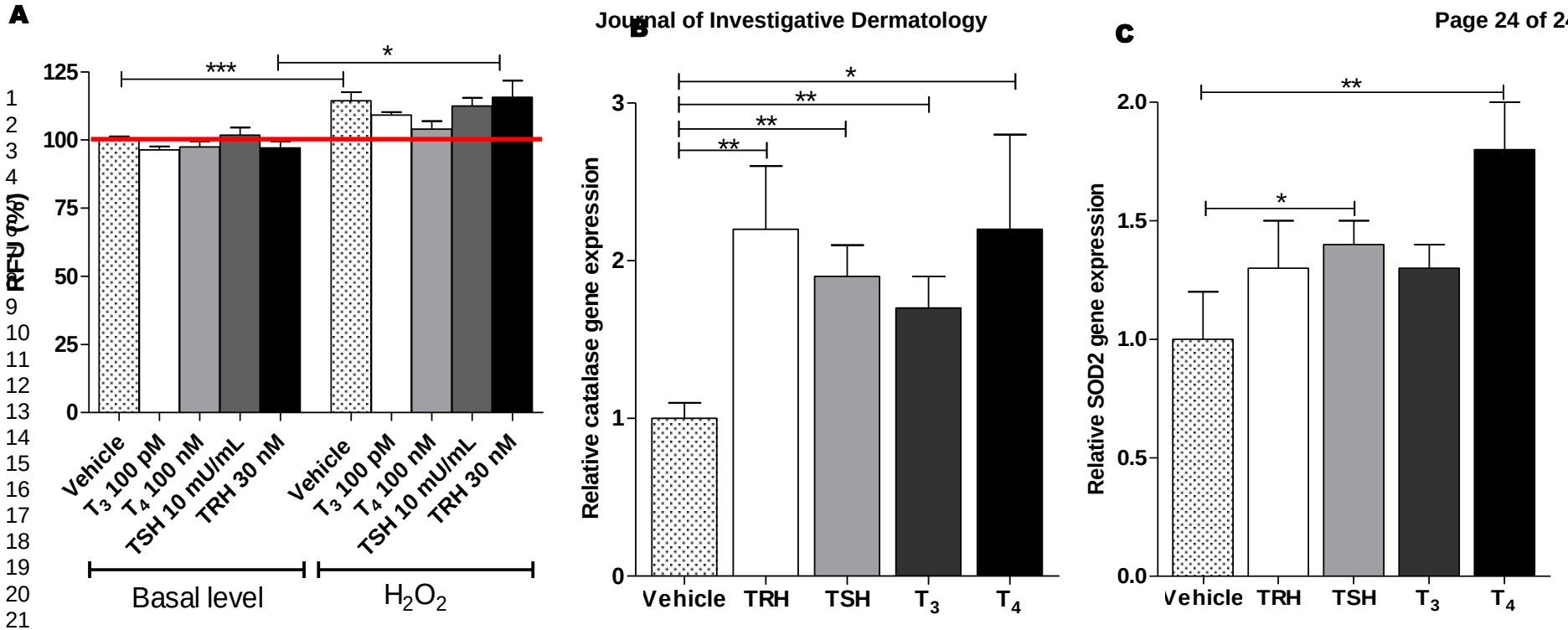
A
RFU (%)
125
100
75
50
25
0
***
*
Vehicle
T3 100 pM
T4 100 nM
TSH 10 mU/mL
TRH 30 nM
Basal level
H2O2
B
Relative catalase gene expression
3
2
1
0
*
**
**
**
Vehicle
TRH
TSH
T3
T4
C
Relative SOD2 gene expression
2.0
1.5
1.0
0.5
0.0
**
*
Vehicle
TRH
TSH
T3
T4

Fig.6