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Title page

Aging is associated with impaired triggering of TRPV3-mediated cutaneous vasodilation: a crucial process for local heat exposure

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Abstract

Sensing temperature is vitally important to adapt our body to environmental changes. Local warm detection is required to initiate regulation of cutaneous blood flow, which is part of the peripheral thermoregulatory mechanisms, and thus avoid damage to surrounding tissues. The mechanisms mediating cutaneous vasodilation during local heat stress are impaired with aging. However, the impact of aging on the ability of the skin to detect subtle thermal changes is unknown. Among heat-activated cation channels, transient receptor potential vanilloid 3 (TRPV3) is a thermosensor predominantly expressed on keratinocytes and involved in local vascular thermoregulatory mechanisms of the skin in young mice. In the present study, using a murine *in vivo* model of local heat exposure of the skin, we showed that heat-induced vasodilation was reduced in old mice associated with reduced expression of TRPV3 channels. We also found a decrease in expression and activity of TRPV3 channel, as well as reduced TRPV3-dependent adenosine tri-phosphate release in human primary keratinocytes from old donors. This study shows that aging alters the epidermal TRPV3 channels, which might delay the detection of changes in skin temperature, thereby limiting the mechanisms triggered for local vascular thermoregulation in the old skin.

Keywords: TRPV3, keratinocytes, heat-induced vasodilation, aging

Introduction

The skin is the largest organ of our body. This envelop is involved in various functions, including temperature detection required to initiate regulation of cutaneous blood flow [1,2]. During environmental thermal stress, changes in skin temperature occurs prior to changes in core temperature, and allow a rapid and appropriate response to heat (vasodilation) or cold (vasoconstriction) [3]. Adaptation of the skin blood flow thus plays an essential role in thermoregulation, and therefore prevents damages to nearby tissues. The physiological regulation of the skin blood flow relies on the activation of the autonomic and sensory nervous system through the release of vasoactive neuropeptides [4], but also on the release of vasoactive factors by the endothelial cells [5,6]. The mechanisms underpinning the cutaneous vasodilation during local heat stress have been extensively studied [3,6–8], and include an initial increase of skin blood flow (axonal reflex), followed by a plateau predominantly dependent of the nitric oxide (NO) pathway. The effects of aging are also well characterized with a reduction in the ability to raise skin blood flow during local heat stress, due to a decrease of sympathetic transduction and endothelial dysfunction [9–15], including decrease of endothelium NO synthase (eNOS) activity and NO bioavailability. Although sensing environmental temperature changes is vitally important for the body to properly adapt its vascular thermoregulatory responses to local heat stress, temperature detection process remains poorly studied. It is therefore crucial to further improve our understanding of the impact of age progression on the physiological mechanisms of thermoregulation and in particular on the trigger mechanisms (local detection of moderate skin temperature changes).

Historically, sensory nerve endings passing through the epidermis were thought to be the exclusive transducers for temperature detection and consequently, thermoregulation [3,16,17]. Since the discovery of thermo-sensors of Transient Potential Vanilloid Channels (TRPV) family [18–20], substantial advances have been made on thermal sensors biology. Interestingly, keratinocytes, the major cell population of the epidermis, also expressed several thermo-sensors such as TRPV1, TRPV3, TRPV4 [21]. Our knowledge on the relationship between the thermo-TRP channels and the heat-evoked cutaneous vasodilation response is just emerging and has yet mainly focused on TRPV1 [22,23]. Being able to accurately detect subtle 1°C temperature deviations from our thermoneutral skin temperature of around 33°C, TRPV3 and TRPV4 channels localized on the epidermal keratinocytes [19,24,25] are likely to be first activated by local warming of the skin, ultimately triggering cutaneous vasodilation. However, it has recently been shown that TRPV4 channels did not contribute to the regulation of cutaneous vasodilation in human skin during local thermal hyperemia [26]. In contrast, using a murine model we previously demonstrated that epidermal TRPV3 acts as a crucial cutaneous thermo-sensor for the local thermoregulatory control of skin blood flow. Indeed, the local heat-evoked vasodilation was impaired in *Trpv3*-KO mice associated with local heat loss disruption, but not in *Trpv1*-KO mice [27]. This phenomenon requires mainly peptidergic nerve endings through calcitonin gene-related peptide (CGRP) release and NO produced by endothelial cells. Moreover, TRPV3 can answer to a heat stimulus by releasing various mediators such as adenosine tri-phosphate (ATP), NO, prostaglandin E₂ (PGE₂), nerve growth factor (NGF) [28–31]. Deprivation of cutaneous heat-induced vasodilation in *Trpv3*-KO mice was associated with a faster increase of internal temperature in passive whole-body heating, exhibiting an attenuated physiological ability to dissipate heat due to a reduced cutaneous vasodilation [27]. *Trpv3*-KO mice also present a profound deficit in sensing warm

external temperature and have a preference for cooler temperature (25°C) [32]. Such deficit in response to thermal change was also observed in elderly people [12,33,34]. We thus hypothesized that a decrease of TRPV3 expression or activity in keratinocytes could be involved in the default of warm detection and thus cutaneous vascular thermoregulation during aging.

In the present study, we firstly compared the vascular response of the skin in young and old control mice, as well as young *Trpv3*-KO mice upon a local heat exposure. To explore the impact of aging on epidermal TRPV3 channels, we analyzed the expression of TRPV3 channel in biopsies from young and old humans, as well as in mouse skin. We also measured TRPV3 activity from young and old human primary keratinocytes using calcium imaging. To identify the impact of aging on the soluble mediators released by epidermal keratinocytes following TRPV3 activation, we evaluated cytokines and other factors such as ATP, a cell communication mediator, following TRPV3 activation in 2D culture of young and old human primary keratinocytes. Our data showed a default of heat-induced vasodilation in old mice, also observed in *Trpv3*-KO mice. Moreover, our results demonstrated a strong decline of both expression and activity of TRPV3 in old human primary keratinocytes, which could contribute to the impaired initial thermal detection (>33°C) and consequently thermoregulation in the elderly population.

Materials and Methods

Animals

Young (2 months) and old (22 months) C57BL6 mice and young (2 months) *Trpv3*-KO mice (raised on a C57BL6 background) were used in all experiments. Mice were housed with *ad libitum* access to food and water, in a temperature-controlled room (about 23°C) with a 12-hours light/dark cycle. All experiments were conducted in accordance with European Union recommendations for animal experimentation and approved by scientific and ethics committees (agreement, #33946 Rhone-Alpes ethics). Special effort was made to minimize the number as well as the stress and suffering of mice used in this study. As they age, mice can lose weight and lower their internal temperature. These two parameters were monitored at least once a week for aged mice (>15 months old).

Assessment of skin blood flow

For the skin blood flow experiments, mice were anesthetized with thiopental (i.p. injection at 75 mg.kg⁻¹ of body weight). Anesthesia was monitored to ensure an appropriate anesthetic depth to avoid the confounding effects of movement artifacts on laser Doppler flowmetry. Anesthetized mice were placed in an incubator maintained at 30 °C in order to better control cutaneous temperature throughout the experiment (35.5±0.1°C) and avoid anesthesia-mediated hypothermia. Cutaneous blood flow and cutaneous temperature were continuously recorded by a data acquisition system (Biopac) and subsequently analyzed (Acknowledge Biopac). In addition, systolic arterial blood pressure was monitored using a tail cuff system (IITC INC Life Science Inc.) before and after the experiments. At the end of each experiment, animals were killed and whole (epidermis and dermis) skin samples were taken in the plantar hindpaw and hairless back.

Heat-evoked response in the plantar hindpaw by laser Doppler flowmeter

Cutaneous blood flow was measured in the plantar hindpaw area using a laser Doppler probe (457, Perimed, Sweden) in anesthetized mice. The heating model consisted of recording 1-min baseline blood flow to ensure that the haemodynamic vascular responses had stabilized following anesthesia, as previously described [27]. Briefly, the hindpaw was exposed to local heating at a rate of 1°C/min for 10 min (from 33 to 40°C) using Temp Unit (PF 5020, Perimed, Sweden). The response to local heating consisted of an increase in cutaneous blood flow (vasodilation). Results are expressed as (1) arbitrary flux units during the entire recording period or (2) arbitrary flux units ($\times 10^3$ flux units) measured as area under the recorded flux versus time for the entire recording period for 10-min temperature exposure or (3) a measure of maximum % increase in blood flow from preheating baseline to the end of the heat exposure (maximum vasodilation observed at 40 °C).

Endothelium-independent and -dependent responses on the dorsal skin by laser Doppler flowmeter coupled to iontophoresis

Skin blood flow was measured on a hairless area of the back of mice anesthetized with thiopental (75mg.kg⁻¹ body weight) using a laser Doppler probe (481-1, Perimed Sweden) for transcutaneous iontophoresis (probe area \approx 1.08 cm²). Hair was removed using depilatory lotion two days prior to iontophoresis. Cutaneous blood flow was recorded during 1-min baseline period prior to iontophoresis. The endothelium-independent response was assessed by using iontophoretic delivery of sodium nitroprusside (SNP) (2%) with a cathodal current application of 100 mA for 20 s, while the endothelium-dependent responses were assessed by using iontophoretic delivery of acetylcholine (ACh) (2%) with an anodal current application of 100 mA for 20 s. The iontophoresis technique was chosen to assess *in vivo* skin microvascular function to avoid any systemic effects. Blood flow data were expressed as a measure of maximum % increase in blood flow from baseline over the entire recording period (20 min) following iontophoretic delivery.

Assessment of thermal function of skin sensory nerve endings

Tail-flick

The conscious mouse was maintained in a restrainer and a cloth was used to cover their head. The tail (about 1.5 cm from the tip) was placed under a radiant heat source produced by a halogen lamp of a device (2TC series 8 Model, ITT Inc. Life science, CA, USA) previously calibrated for 30 s to deliver 25 W of heat. The heating rate was 1.3 °C/s, and the delay in tail removal was measured. A cutoff time of 10 s was imposed to prevent tissue damage. Five measurements of the tail withdrawal latency were taken and averaged for each mouse to determine the nociceptive thermal threshold for the animal.

Hot plate

The conscious mouse was placed on a Bioseb hot plate (Bioseb, USA, #BIO-CHP) calibrate at 55 °C. The delay in mouse behavioral change (jump or paw licking) was manually measured using a chronometer. A cutoff time of 10 s was imposed to prevent tissue damage. Three measurements of latency were taken and averaged for each mouse to determine the nociceptive thermal threshold for the animal.

In situ hybridization

Human paraffin embedded tissue microarray (TMA: #SK244A and #SKN1001), were purchased from US Biomax (Derwood, MD, USA). Details on skin sample are presented on Supplementary Table 1. *In situ* hybridization of *TRPV3* was performed using RNAscope® Multiplex Fluorescent Reagent Kit v2 (bio-technie, France, 323100) according to the manufacturer's instructions. *TRPV3* probe were labeled with Opal 570 (1:1500) supplied with the kit. At the end of the amplification and signal development, tissue sections were washed 3 times with PBS and blocked with PBS containing 5 % goat serum, 2 % BSA and 0.1 % Tween20 for at least 1 h at RT. After washing steps, primary antibodies targeting KRT14 (Abcam, ab181595, 1:400) were incubated overnight at 4°C. Sections were washed and incubated with secondary antibodies (Thermo Fischer Scientific, #A-21245, 1:1000) 45 min at RT and nuclear staining was performed using ProLong™ Glass Antifade Mountant with NucBlue™ (Thermo Fischer Scientific, #P36981). Negative controls were performed using probe diluent supplied with RNAscope kit. Images were visualized using High Content Screening Yokogawa CQ1 microscope (Yokogawa, Tokyo, Japan), digitalized using sCMOS camera (Olympus, Hamburg, Germany) and analyzed using QuPath software (version 0.2.3).

Cell Culture

Human primary keratinocytes (HPK) were isolated in-house from skin biopsies as previously described [6], or purchased from Lonza (Basel, Switzerland, #00192627). Skin biopsies were obtained from the DermoBioTec tissue bank at Lyon (Tissue Transfer Agreement n°214854) with the informed consent of adult donors (non-pathological tissues from abdomen or breast), in accordance with the ethical guidelines (French Bioethics law of 2021). Donor specifications are indicated in Supplementary Table 2. HPK were cultured in KBM Gold medium (Lonza, #00192060) at 37 °C and 5 % CO₂. The culture medium was renewed three times a week and cells were maintained to no more than passage 4.

RNA extraction and Real-Time PCR

For tissue RNA extraction, plantar hindpaw samples were immersed in RNA-later for 24 h at 4 °C and further stored at -80 °C. Total RNA was isolated using RNeasy® Fibrous Tissue Mini Kit (Qiagen, France, #74704), according to the manufacturer's instructions. For cell RNA extraction, 6 h after treatment, cells were washed 3 times with PBS and RNA were extracted using NucleoSpin® RNA Plus (Macherey-Nagel, 740984.250). Quantity and purity of RNA were evaluated using NanoDrop™ 2000. cDNA synthesis was performed from at least 200 ng of RNA using PrimeScript™ RT reagent kit (Takara Bio Europe, #RR037A) and analyzed in real-time qPCR using SYBR® Premix ExTaqII (Takara Bio Europe, # RR820A) on an AriaMx Realtime PCR system (Agilent Genomics, Santa Clara, CA, USA). Results were normalized to *TBP* and *RPL13A* (human) or *Rpl13a* and *Eif4h* (mouse) housekeeping genes expression levels, using the $2^{-\Delta\Delta Ct}$ quantification method. RT-qPCR program and primers were listed in Supplementary Table 3.

Chemical compounds

The TRPV3 agonists carvacrol (Sigma-Aldrich, France, # W224511) and 2-Aminoethoxydiphenyl borate (Sigma-Aldrich, France, #100065-100MG), and TRPV3 antagonist isochlorogenic acid B (MedChemExpress, Sweden, #HY-N0057) were sequentially diluted in DMSO (stock solutions) and further diluted in KGM-gold just before use. All chemical treatments were performed when cells reached 70 % confluence, with, depending on the conditions, 50 μ M 2-APB, 300 μ M Carvacrol, 100 μ M IAB or an equivalent volume of DMSO diluted in medium.

Ca²⁺ measurement

Cells were cultivated in μ -Dish 35mm High (Ibidi GmbH, #81156). After 45 min incubation at 37 °C with 2 μ M of fura-2-acetoxymethyl ester (Fura2-AM) (Thermo Fischer Scientific, #F1221) in culture medium, cells were washed twice for 5 min with a Hank's Balanced Salt Solution (HBSS 1X) with 1,26mM Ca²⁺ and 0,49 mM Mg²⁺ (Gibco, UK, #14025-050), and placed at RT on a DMI6000 inverted wide-field microscope (Leica microsystem). Images were acquired with an Orca-Flash 4.0 Scientific CMOS camera (Hamamatsu) using a 40X oil-immersion objective. Using a Lambda DG-4+ filter (Sutter instruments), Fura-2 AM was excited at 340 and 380 nm and the fluorescent signal emitted measured at 510 nm. Images (1024 \times 1024 pixels) were taken with a 3 s interval. Fluorescence ratios (F340 nm/F380 nm) were analyzed with MetaFluor 6.3 (Universal Imaging) after removing background fluorescence[35]. After 1 min of baseline, cells were treated with or without IAB (100 μ M) for 10 min and then with Carvacrol (300 μ M) for 5 min, or 2-APB (50 μ M) 2 min and Carvacrol (300 μ M) 5 min. Results are expressed as ratio 340/380 for each cell (~30/field) of each donor.

Luminex multiplex assay

Procartaplex 17 Plex (Life Technologies, France, #PPX-17) were designed in order to identify cytokines in cell supernatant 16 hours after treatment: EGF, FGF-2, FGF-21, INF α , IFN β , INF γ , IL-1 α , IL-1 β , IL-6, IL-8, IL-17A, IL-23, IL-29, NGF- β , TGF- α , VEGF-A and VEGF-D. Measurement of cytokines were done according to the manufacturer's instruction with MAGPIX®. Cell culture supernatants were collected 16h after treatment.

Extracellular ATP assay

RealTime-Glo™ Extracellular ATP Assay (Promega, France, #GA5010) was used to measure ATP released after 10 min following TRPV3 activation. Doxorubicin (Sigma-Aldrich #D1515) 50 nM was used as positive control.

Statistics

Data are expressed as mean \pm SD or mean \pm SEM or median (IQR). According to the normality (Shapiro-Wilk test), statistical significance was calculated by student t.test or Mann-Withey, One-way analysis of variance (ANOVA1) or Kruskal-Wallis, Two-way analysis of variance (ANOVA2), and Pearson correlation using Prism software (version 7.0, GraphPad Software, San Diego, CA, USA). Mean differences were considered statistically significant when $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Cutaneous heat-induced vasodilation is reduced in a similar fashion in young *Trpv3*-KO and old mice

Using the murine *in vivo* model as previously [27], we measured cutaneous blood flow on hindpaw of young wild-type (WT; 2 months), old wild-type (22 months) and young *Trpv3*-KO (2 months) mice in response to a local heat exposure (from 33 to 40 °C) (Figure 1A-B). Young WT mice displayed a blood flow increase immediately after the onset of local heating. With exception of *Trpv3*-KO female mice (max temperature achieved: 36 °C), the cutaneous blood flow of old and *Trpv3*-KO mice did not increase significantly from baseline compared to young WT mice (34.2°C for both male and female) (Figure 1A-B). As expected, the heat-induced vasodilation was significantly lower in old mice compared to young WT mice (Figure 1C-D). In contrast, heat-induced vascular response was not different between young *Trpv3*-KO mice and old WT mice (Figure 1C-D). The cutaneous vascular function (on back skin) assessed with acetylcholine and sodium nitroprusside, the systolic arterial blood pressure and skin temperature at baseline were not different between all groups, strengthening that neither aging nor TRPV3 inactivation impaired endothelium or vascular smooth muscle integrity (Supplementary Figure 1 A-D). Thus, the delay of local heat-induced vasodilation in old WT mice was not due to a vascular defect. Using sensory tests (tail flick and hot plate) we showed that the nociceptive responses were well-preserved in old WT and *Trpv3*-KO mice compared to young WT mice (Supplementary Figure 1E-F). These data suggest that the default of local heat-induced vasodilation in old mice was due to a failure of the initial heat detection (likely via warm-sensitive TRPV3) and/or consequent triggering neurovascular mechanisms, rather than peripheral sensory nerve impairments.

Altogether, these results highly suggested that impairment of heat-induced vasodilation in old WT mice is related to a decreased ability to sense heat ($\geq 33^{\circ}\text{C}$), likely due to an altered TRPV3 function.

TRPV3 expression is decreased with aging in both mouse and human epidermis

As *Trpv3*-KO and old WT mice displayed a similar deficit in response to local heat, we explored the epidermal expression of *Trpv3* in young and old WT mice. Using RT-qPCR, we found a ~ 47% decrease of *Trpv3* transcripts in old mice as compared to young animals in hindpaw skin, the major skin area involved in thermoregulation with the tail (Figure 2A). In contrast, this decrease in *Trpv3* expression was not observed in the back skin, which is not involved as a thermoregulatory area (Supplementary Figure 2A). It is noteworthy to note that the expression of *Trpv3* is significantly higher in hindpaw skin than in back skin regardless of both age and sex (Supplementary Figure 2A). These results demonstrate a specific decrease of epidermal *Trpv3* expression with aging in a thermoregulatory area, which could contribute to the impaired vasodilation on exposure to local heat in old mice.

To further confirm the negative effect of aging on epidermal TRPV3, we looked at TRPV3 expression in human skin sections from both gender of several ages (Figure 2B-E). As TRPV3 localization is still controversial in the literature[36–38], we used fluorescent RNA *in-situ* hybridization of TRPV3 in combination with cytokeratin-14 (KRT14) immunostaining (Figure 2B-E). We showed that the percentage of TRPV3-positive cells (Figure 2F), as well as the number of TRPV3 transcripts per keratinocyte (Figure 2G) gradually decrease with aging in men and women (see Supplementary Table 1 for more details). Accordingly, a strong negative correlation was determined

between *TRPV3*-positive cells (r=0.7603) or estimated *TRPV3* transcripts per cell (r=0.7799) and the age. In addition, the KRT14 immunostaining allowed to discriminate basal cells (KRT14+) and suprabasal cells (KRT14-) (Figure 2H). Remarkably, when we compared the expression of *TRPV3* in old human biopsies (mean age 71.25 years) with young ones (mean age 33.5 years), we only observed a decrease in basal cells, but not in differentiated keratinocytes. These results strongly suggest that aging negatively affects the expression of TRPV3 in the proliferating basal layer of human epidermis.

TRPV3 activity is reduced with aging in human primary keratinocytes

Since we already demonstrated that primary skin cells retain some features of aging when they are cultured *in vitro* [39–41], we took advantage of this 2D model to measure the TRPV3 channel intrinsic activity. To do so, we used a cytosolic Ca²⁺ ratiometric dye (Fura-2AM) to follow intracellular Ca²⁺ flux at the single cell level in human primary keratinocytes from young (mean age 32.6 years) and old (mean age 81.8 years) individuals (Figure 3). We first confirmed the decrease of *TRPV3* expression in “old” keratinocytes (Supplementary Figure 3A). We next validated the specific activation of TRPV3 channel by its agonist carvacrol [42,43], which effectively induced the intracellular Ca²⁺ entry in keratinocytes from young skin (Supplementary Figure 3B-C). This effect was totally abolished by the co-treatment with the TRPV3 antagonist isochlorogenic acid B (IAB) [44], which confirm the specificity of TRPV3 activation (Supplementary Figure 3B-C). In addition, TRPV3 can be sensitized and increases its response upon repetitive stimuli [45,46]. Indeed, the sensitization with 2-APB followed by stimulation with carvacrol significantly increased the Ca²⁺ flux in keratinocytes compared to carvacrol only (Supplementary Figure 3D). Therefore, we used this dual treatment (2-APB sensitization + carvacrol activation) to get the maximal stimulation of TRPV3. With this treatment, we observed an increase of intracellular Ca²⁺ flux in both young and old primary keratinocytes (Figure 3). However, the maximal response of TRPV3 channel was ~31 % lower in old keratinocytes (Figure 3B). In addition, the TRPV3 antagonist IAB strongly reduced the intracellular Ca²⁺ flux in young keratinocytes but completely abolished the response in old ones (Figure 3A-B). These results indicate that the intrinsic activity of the TRPV3 channels is less effective in old keratinocytes, likely due to a decrease of its expression with aging.

TRPV3-dependent ATP release is altered with aging in human primary keratinocytes

After exploring the expression and the activity of the TRPV3 channel, we aimed at characterizing the effect of aging on the release of TRPV3-dependent mediators. We first decided to focus on a well-known downstream pathway of TRPV3, the EGFR/TGF- α /NF κ B pathway [36,47,48]. Using Luminex bead-based multiplex immunoassay, we found that TRPV3 stimulation (2-APB+carvacrol) induced an equivalent release of TGF α in young and old keratinocytes (Figure 4A). This TRPV3-dependent release of TGF α was blocked by the TRPV3 antagonist IAB (Figure 4A). The same tendency was observed by RT-qPCR for the following cytokines *IL-1 β* , *IL-6*, *IL-8*, and *NGF- β* 6h (Figure 4B-E), suggesting that the TRPV3/EGFR/TGF- α /NF κ B pathway was not affected with aging in human primary keratinocytes.

ATP is a key messenger involved in cell communication and various biological processes [49–52], including in temperature transmission from keratinocytes to sensory nerve endings [28]. In order to evaluate the impact of aging on the ability of keratinocytes to release ATP in response to TRPV3 stimulation, we quantified

extracellular ATP released by young and old keratinocytes using luminescence ATP assay. We found that ATP release after TRPV3 activation was strongly reduced in old compared to young keratinocytes (Figure 4F). Moreover, IAB significantly decreased ATP release only in young cells. These results indicate that aging impairs TRPV3-induced ATP release in human primary keratinocytes.

Discussion

In this study, we provide the first evidence that aging alters epidermal TRPV3, which is necessary for temperature detection by the keratinocytes to trigger heat-evoked cutaneous vasodilation. The major findings are as follows: (i) aging is associated to a decrease of *TRPV3* expression in mouse and human skin regardless of sex, (ii) the cutaneous vascular response upon local heat (from 33 to 40°C) is almost abolished in old mice, as previously reported in young *Trpv3*-KO mice (iii) TRPV3 activity and ATP release in human primary keratinocytes are greatly diminished with aging.

We previously demonstrated that vasodilation in response to local heating was reduced in non-neuropathic old subjects (60-75 years old) compared to young subjects (25-30 years old) [34], suggesting that aging alters ability of cutaneous microvessels to adapt to local heat. In the present study, we showed that 22-month-old mice presented a lack of vasodilation upon local heat exposure on paw skin (from 33 to 40°C), regardless of sex. This defect was similar to that observed in young male and female *Trpv3*-KO mice (2 months), as previously reported in males [27]. Although endothelial dysfunction has been described in very old mice (22-25 months) [10,53,54] and old individuals (~70 years old) [34,55,56], the old mice we used in the present study (22 months) displayed intact vasodilator function of the endothelium and smooth muscle in cutaneous microvessels, as assessed by the iontophoresis experiments using acetylcholine and sodium nitroprusside, respectively. As previously reported [27], we confirmed the intact vasodilator function of the endothelium and smooth muscle in cutaneous microvessels in *Trpv3*-KO mice. We also showed that old WT mice displayed intact sensory nerve integrity using tail flick and hot plate experiments. In a previous study, we already showed that motor and sensory nerve conduction velocities were not different between young adult C57BL6/J mice (6–7 months) and old mice (22–25 months) [53]. These data suggest that the impaired vasodilation on exposure to local heat in old mice is specifically due to a failure of the initial heat detection and/or consequent triggered neural mechanisms, rather than to downstream mechanisms required for heat-evoked vasodilation.

TRPV3 is an essential cutaneous sensor of warming, playing a primary role in the vascular response to heat exposure in young mice [27]. Accordingly, our findings demonstrated that *Trpv3* expression was significantly reduced in the hindpaw skin of old mice compared to young mice. In addition, the higher expression of TRPV3 in hindpaw compared to the back skin, regardless of the age, underlines the importance of thermo-sensors in thermoregulatory areas [57–59]. We thus suggest that the reduced *Trpv3* expression in the mouse epidermis alters the detection of moderate temperature in old mice and, in turn, triggers mechanisms necessary for the vascular response to local heat exposure. We also showed this decrease of *TRPV3* expression in elderly subjects, further strengthening the critical role of TRPV3 to adapt to local heat in mammalian homeotherms. Here, we showed a strong negative correlation between *TRPV3* expression and age for both women and men using *in situ* hybridization. We also observed that *TRPV3* expression was quite identical in photo-exposed (groin, scalp, face)

and photo-protected skin areas (back humeral, chest), suggesting that TRPV3 is homogeneously expressed in human skin regardless sun exposure. In contrast to TRPV3, TRPV1 channel expression has been shown to increase with aging especially in photo-exposed skin [60,61], implying that TRPV1 expression is sensitive to sun-exposure. The temperature activation of TRPV1 being $> 42^{\circ}\text{C}$, this may suggest that elderly are more able to detect painful/burning temperature, related to UV exposure, than moderate temperature of $33\text{--}39^{\circ}\text{C}$ (TRPV3 range) [62–64]. In addition to the decrease of *TRPV3* expression in aged keratinocytes, we also point out for the first time an impaired intrinsic activity of the TRPV3 channel. Alongside the decreased expression of TRPV3, others alterations may occur with aging. For example, it has been shown that some post-translational modifications, such as hydroxylation of TRPV3 on N242, altered TRPV3 current in HEK cells [65,66]. Indeed, hydroxylation at this site could destabilize TRPV3 multimer assembly or interactions with other protein partners that can modulate channel gating or addressing to the plasma membrane. However, changes in TRPV3 structure and post-translational modifications with aging remain unknown and would constitute a promising further study.

Activation of TRPV3 is associated with the release of various diffusible molecules, that act on neighboring cells. Stimulation of the channel is well known to trigger a proinflammatory response [36], but the signaling cascade associated with local heat-mediated vasodilation is still not fully depicted. It has been already described that keratinocytes can convey heat [28] and touch [67] stimuli through release of ATP. In this study, we confirmed that TRPV3 stimulation induced an extracellular ATP release from young keratinocytes, but to a less extent from old keratinocytes. As expected, the level of pro-inflammatory cytokines was not changed in the same context. In addition to its function of an energetic molecule, ATP plays various biological roles including sensory transduction via its neuroactive property [67] or regulation of blood vessels via its vasoactive property [49,68–70]. Since the discovery of synaptic-like contacts between keratinocytes and sensory neurons, it has been strongly suggested that keratinocytes could directly communicate with sensory nerve endings in the skin [71,72]. Thus, the ATP release from keratinocytes after TRPV3 activation could act on purinergic receptors from sensory nerves [49] and might regulate vascular tone in response to heat. Thus, the loss of ATP release with aging may compromise communication between sensory nerve endings and keratinocytes in the elderly. In addition, skin blood vessel also expressed purinergic receptor [50]. We cannot thus exclude that the TRPV3-dependent release of ATP by keratinocytes will directly act on skin blood vessel to regulate vascular tone. Finally, keratinocytes can release other neuroactive and/or vasoactive substances than ATP, such as CGRP, NO, PGI_2 [29,31,73], which could also contribute to local heat-evoked vasodilation either by acting on sensory nerve endings or dermal blood vessels. However, how keratinocytes can inform and modulate the vascular tone needs further exploration.

In conclusion, our study confirms that the initial detection of moderate temperature, through the warm-sensitive epidermal sensor TRPV3, is crucial for the skin to adapt to thermal stress such as heat. This offers a new function of the keratinocyte as a fundamental thermo-sensor and transducer of its thermal environment. With aging, decreased expression and activity of TRPV3 could reduce or delay the detection of changes in skin temperature, thereby limiting the mechanisms triggered for thermoregulation, including cutaneous vasodilation. Thus, TRPV3 may be a reliable target to counteract the thermoregulatory defect in the elderly. Aside TRPV3, decline of cold TRP channels with aging has been reported to influence temperature detection and may compromise vascular response to environmental cold in old individuals [74]. TRP channels are thus key actors in regulating the cutaneous vascular response to environmental temperature during aging, and need to be further considered.

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Statements and Declarations

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Informed Consent Statement: Skin biopsies were obtained from the DermoBioTec tissue bank at Lyon (Tissue Transfer Agreement n_214854) with the informed consent of adult donors undergoing surgical discard (non-pathological tissues from breast, face, or abdomen), in accordance with the ethical guidelines (French Bioethics law of 2021).

Institutional Review Broad Statement: The animal study protocol was approved by the Animal Experimentation Committee of the University Claude Bernard Lyon I (protocol agreement #33946 approved on 25 November 2021).

Conflicts of Interest: The authors declare no conflict of interest

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Figure Legends

Figure 1: Cutaneous heat-induced vasodilation is reduced in a similar fashion in young *Trpv3*-KO and old WT mice

Relative paw blood flow in response to local heating in anesthetized male (A) or female (B) of 2 months (n=12 ♂ and n=15 ♀), 22 months (n=11 ♂ and n=13 ♀) and 2 months *Trpv3*-KO (n=12 ♂ and n=11 ♀). Results are expressed as mean ± SEM. (C) AUC and (D) maximal skin blood flow from baseline of young wild type, old wild type, and young *Trpv3*-KO mice (median (IQR)). A Kruskal-Wallis was used to compare mean of each group. Two-way ANOVA (mixed-model) with Sidak's multiple comparison was used to compared blood flow from the baseline.

Figure 2: TRPV3 expression is decreased with aging in both mouse and human skin

(A) Relative expression of *Trpv3* normalized with mean *Rpl13a* and *Eif4h* in paw skin from young (5 months) and old (22-24 months) mice. *In situ* hybridization of *TRPV3* (red dots) in a (B) 29-year-old man's back humerus skin, (C) a 21-year-old woman's breast skin, (D) a 78-year-old man's back humerus skin and (E) a 71-year-old women's scalp skin, combined with KRT14 immunostaining (yellow) (scale bar 20µm). (F) Percentage of *TRPV3*-positive cells and (G) number of *TRPV3* transcripts per keratinocyte according to the age in 9 woman (circles) and 7 man (squares) epidermis. (H) *TRPV3* transcripts in basal cells (KRT14+) or differentiated cells (KRT14-) in young (19-52 years old) and old (66-78 years old) human skin. Results are expressed as median (IQR). A Mann-Whitney test was used to compare *Trpv3* in young (n=6 ♂ and n=4 ♀) and old (n=4 ♂ and n=7 ♀) mouse hindpaws. Pearson correlation was performed to evaluate *TRPV3* expression with aging (n=16). A Two-way ANOVA with Tukey's multiple comparison test was used to compared *TRPV3* transcripts in basal versus differentiated cells of young (n=8) and old (n=8) human epidermis.

Figure 3: Aging reduces TRPV3 activity in human primary keratinocytes

(A) Changes in the intracellular Ca²⁺ concentration (mean curve), expressed as the fluorescence intensity ratio at 340 and 380 nm (F340/F380), were monitored in response to 50µM 2-APB (2 min) and then 300µM carvacrol (5 min) with (dotted line) or without (solid line) 100µM IAB, in young (mean age 32.6 years) and old (mean age 81.8 years) human primary keratinocytes. (B) Measurement of the maximal Ca²⁺ response of keratinocytes from baseline (median (IQR)). A Two-way ANOVA Tukey's multiple comparison test was performed to determine effect of aging and TRPV3 antagonist. (n=4/group with at least 40 cells recorded in each biological replicate).

Figure 4: Aging reduces TRPV3-dependent release of ATP but not cytokines in human primary keratinocytes

(A) Relative TGF-α production following 16h TRPV3 stimulation using agonists (50 µM 2-APB and 300 µM carvacrol) with or without the TRPV3 antagonist 100 µM IAB in young (n=4, mean of age 34.3 years) and old (n=5, mean of age 81.1 years) keratinocytes. Relative expression of *IL-1β* (B), *IL-6* (C), *IL-8* (D) and *NGF-β* (E) normalized to the mean of *TBP* and *RPL13A* 6h following TRPV3 stimulation with or without TRPV3 blockade in young (n=5, mean of age 32.6 years) and old (n=5, mean of age 81.1 years) keratinocytes. (A-E) Results are expressed as a ratio treated/untreated. (F) Extracellular ATP release following TRPV3 stimulation with or without the TRPV3 antagonist IAB in young (n=5, mean age 32.6 years) and old (n=5, mean age 81.1 years) keratinocytes. Results are expressed as a ratio treated/untreated and then as percentage of variation from positive control (doxorubicin 50 mM) (n=5). A Two-way ANOVA (Mixed-model) with Sidak's multiple comparison was performed to determine effect of aging and TRPV3 antagonist groups. Results are expressed as mean ± SEM.

Figure 1

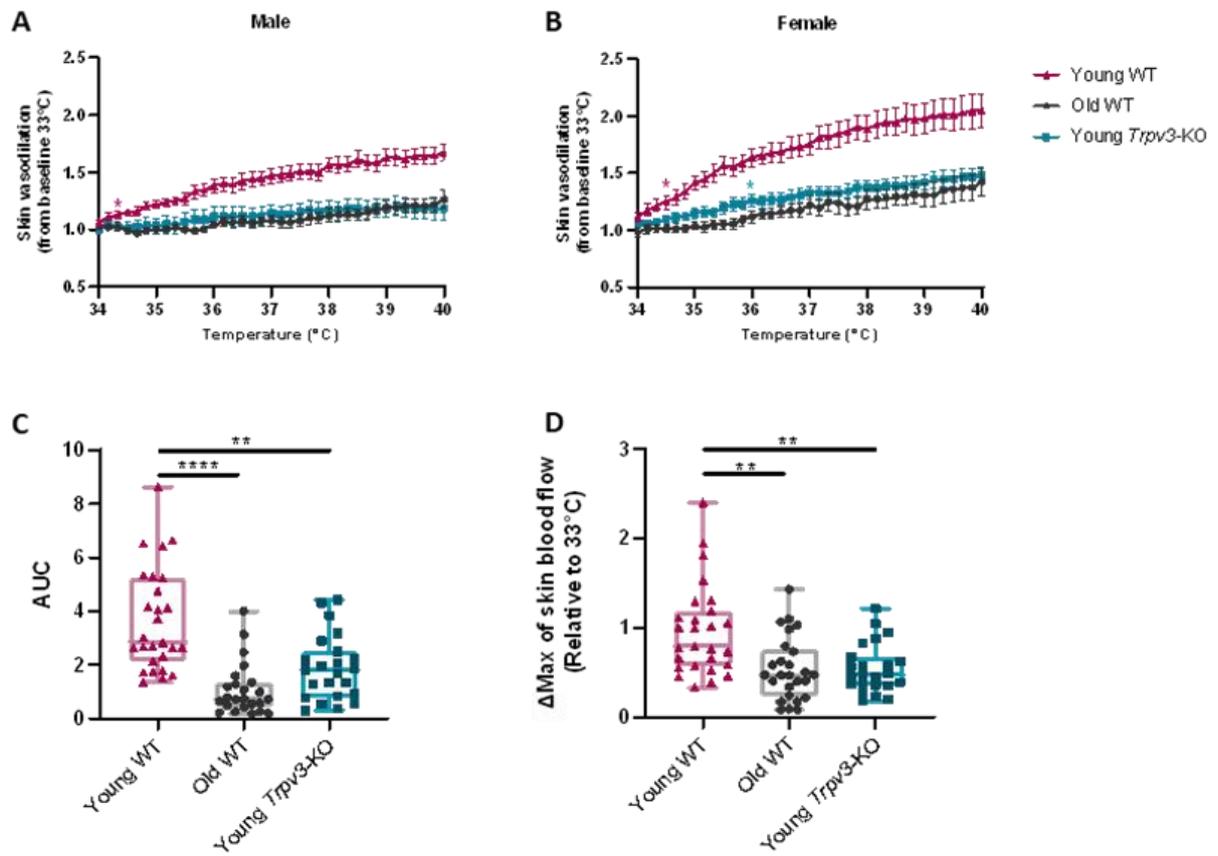


Figure 2

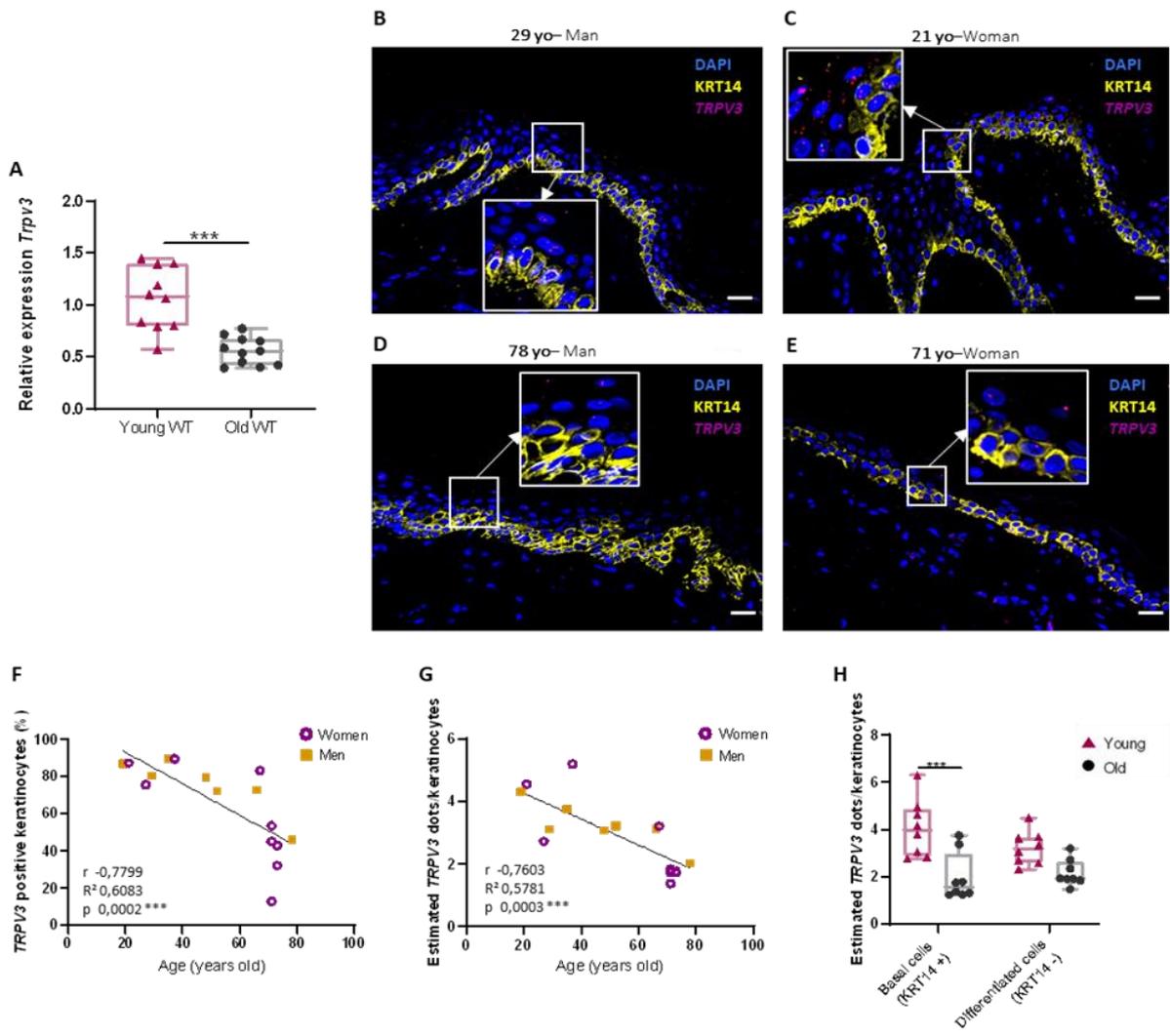


Figure 3

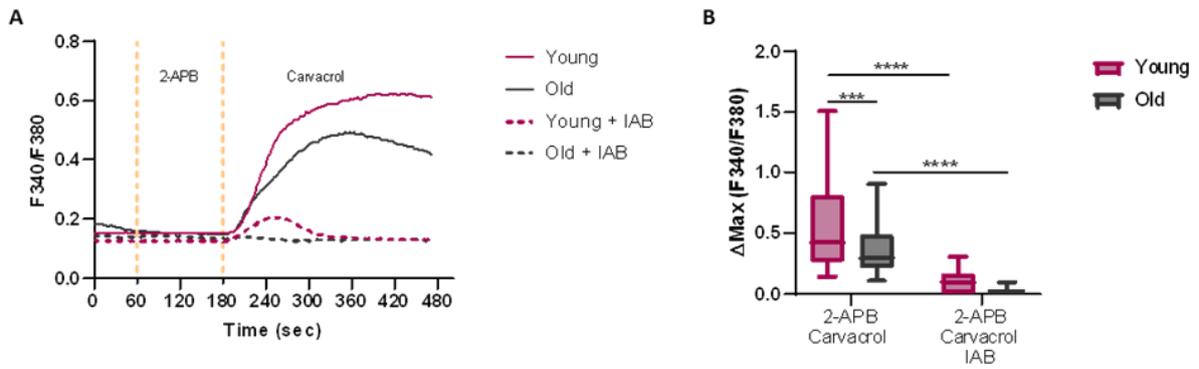
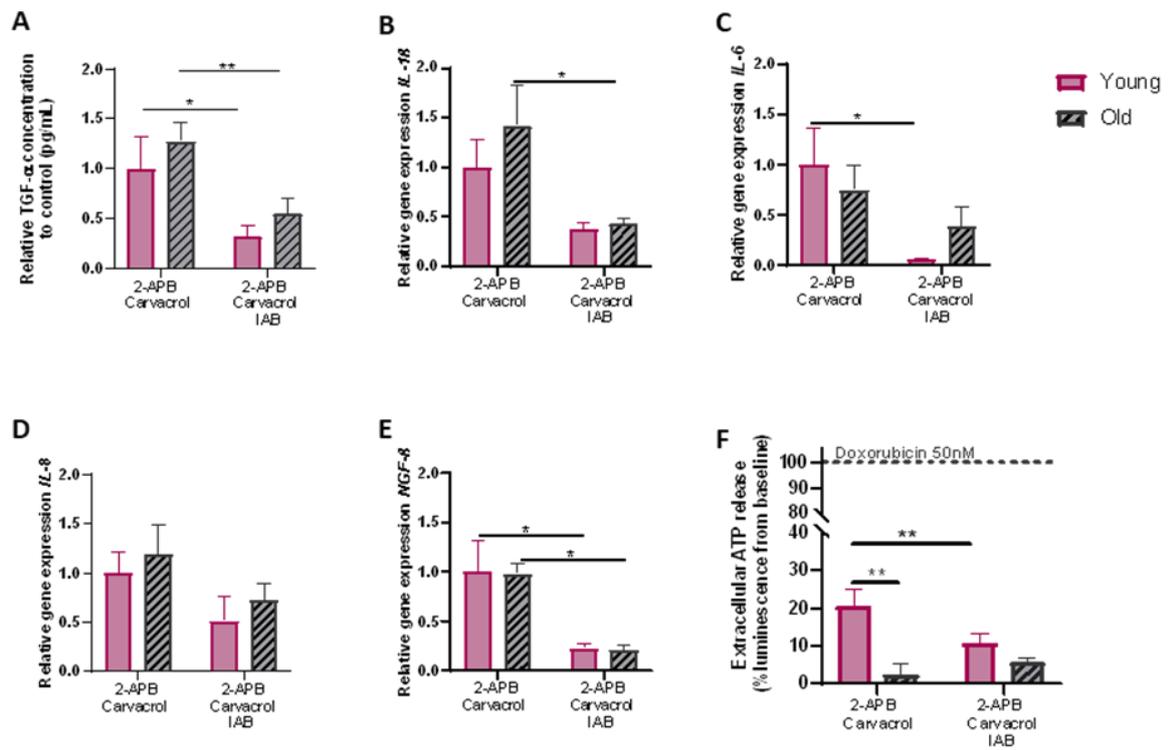


Figure 4



Supplementary Tables

Supplementary Table 1: Specification of human skin sections used for *in situ* hybridization selected from SK224a and SKN1001 from US Biomax

Tissus Microarray	Sex	Age	Anatomical localization	Group
SKN1001	Man	19	Groin	Young
SK224a	Woman	21	N/A	Young
	Woman	27	Scalp	Young
SKN1001	Man	29	Back humeral	Young
	Man	35	Chest	Young
	Woman	37	Chest	Young
	Man	48	Face	Young
	Man	52	Face	Young
	Man	66	Groin	Old
	Woman	67	Chest	Old
	Woman	71	Chest	Old
	Woman	71	Chest	Old
	Woman	71	Hip	Old
	Woman	73	Chest	Old
	Woman	73	Chest	Old
	Man	78	Back humeral	Old

Supplementary Table 2: Specifications of human primary keratinocyte donors

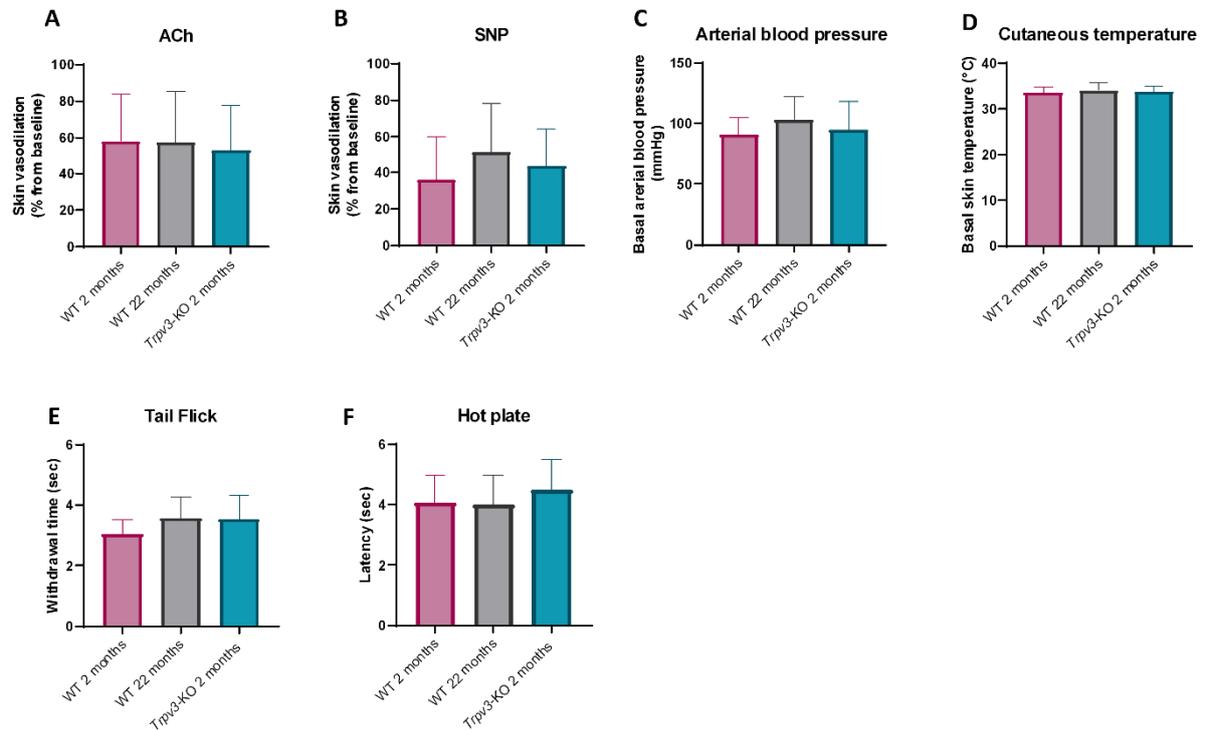
Donors n°	Anatomical Location	Sex	Age (years old)	Group	Experiments
HPK11197	Breast	Woman	26	Young	Calcium imaging ,ATP, ,Rt-qPCR
HPK22045	Abdomen	Woman	29	Young	Calcium imaging ,ATP, Rt-qPCR, Luminex
HPK20060	Abdomen	Woman	30	Young	Calcium imaging ,ATP, Rt-qPCR, Luminex
HPK090317	Abdomen	Man	36	Young	Calcium imaging ,ATP, ,Rt-qPCR, Luminex
HPK20057	Abdomen	Woman	42	Young	Calcium imaging ,ATP, ,Rt-qPCR, Luminex
HPK18TL053546	Back	Woman	75	Old	Calcium imaging ,ATP, ,Rt-qPCR, Luminex
HPK12015	Face	Woman	80	Old	Calcium imaging ,ATP, ,Rt-qPCR, Luminex
HPK18TL150624	Abdomen	Man	80	Old	Calcium imaging ,ATP, ,Rt-qPCR, Luminex
HPK19TL150604	Abdomen	Man	82	Old	Calcium imaging ,ATP, Rt-qPCR, Luminex
HPK19TL149593	Buttock	Woman	92	Old	Calcium imaging ,ATP, ,Rt-qPCR, Luminex

Supplementary Table 3 : List of primers pairs used for qPCR analysis and RT-qPCR program

Gene	Origin	Primer	Sequence (5'-3')
<i>TBP</i>	Human	Forward	TCA AAC CCA GAA TTG TTC TCC TTA T
		Reverse	CCT GAA TCC CTT TAG AAT AGG GTA GA
<i>RPL13A</i>	Human	Forward	CTC AAG GTC GTG CGT CTG AA
		Reverse	TGG CTG TCA CTG CCT GGT ACT
<i>TRPV3</i>	Human	Forward	GAC ATG CGG TGA TCT CAG GG
		Reverse	AGT GTG CAC TCT TCT TTG TGG G
<i>IL-1β</i>	Human	Forward	ACA-GAT-GAA-GTG-CTC-CTT-CCA
		Reverse	GTC-GGA-GAT-TCG-TAG-CTG-GAT
<i>IL-8</i>	Human	Forward	TCT GCA GCT CTG TGT GAA GG
		Reverse	ACT TCT CCA CAA CCC TCT GC
<i>IL-6</i>	Human	Forward	GAA CTC CTT CTC CAC AAG CGC CTT
		Reverse	CAA AAG ACC AGT GAT GAT TTT CAC CAG G
<i>NGF-β</i>	Human	Forward	ATA CAG GCG GAA CCA CAC TC
		Reverse	TGC TCC TGT GAG TCC TGTT G
<i>Rpl13a</i>	Mouse	Forward	AGA AGC TCT TGA GGT TAC GG
		Reverse	GGA GTC CGT TGG TCT TGA GG
<i>Eif4h</i>	Mouse	Forward	ATG ACT TCA ACT CTG GCT ACA GG
		Reverse	GAG GTC TCT GTG CTC GTT CC
<i>Trpv3</i>	Mouse	Forward	TCA CCA AGA CCT CTC CAC C
		Reverse	CGC GGT ACC ACC GAC GTT TCT GGG AAT TC

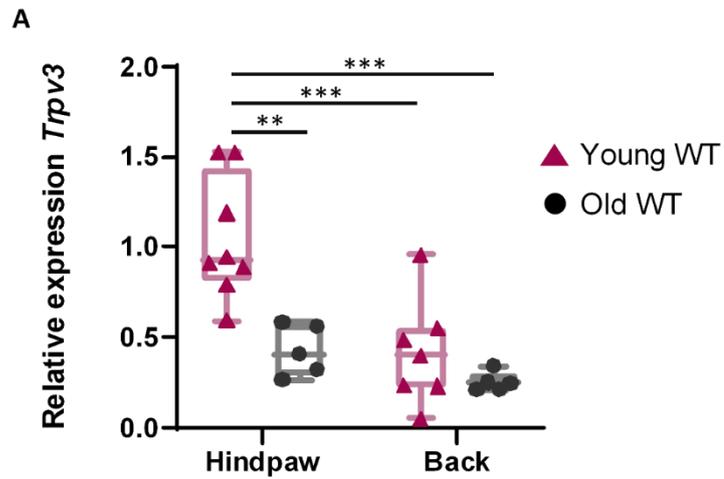
Reverse transcription	Time	Temperature (°C)	
	15 min	37	
	5 sec	95	
q-PCR			
Denaturation	30 sec	95	
Amplification	5 sec	95	40 cycles
	30 sec	60	
	30 sec	72	
Melting curve	1 min	95	
	30 sec	60	
	30 sec	95	

Supplementary Figures



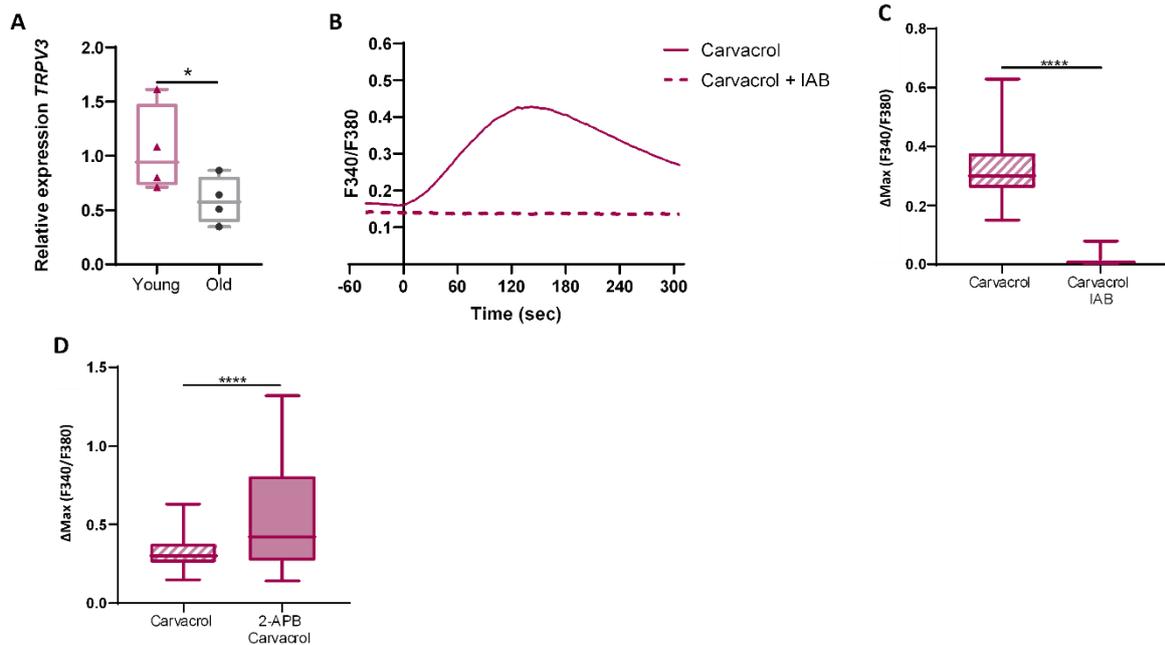
Supplementary Figure 1: Heat-induced vasodilation is reduced in a similar fashion in Trpv3-KO and old mice

Skin blood vessels integrity was analyzed using (A) acetylcholine (ACh) and (B) sodium nitroprusside (SNP) iontophoresis on mouse back skin of WT 2 months (ACh: n=8 ♂ and n=8 ♀; SNP: n=13 ♂ and n=10 ♀), WT 22 months (ACh: n=5 ♂ and n=7 ♀; SNP: n=7 ♂ and n=7 ♀), and *Trpv3*-KO 2 months (ACh: n=11 ♂ and n=6 ♀; SNP: n=14 ♂ and n=3 ♀). Data were expressed as percentage from baseline. Basal systolic arterial blood pressure (C) and basal cutaneous skin temperature (D) were measured on WT 2 months (n=12 ♂ and n=15 ♀), WT 22 months (n=11 ♂ and n=13 ♀); and *Trpv3*-KO 2 months (n=12 ♂ and n=11 ♀) mice. Sensory nerve integrity was assed using tail flick (E) and hot plate (F) test on WT 2 months (n=5 ♂ and n=12 ♀), WT 22 months (n=16 ♂ and n=12 ♀); and *Trpv3*-KO 2 months (TF: n=8 ♂ and n=10 ♀; HP: n=8 ♂ and n=8 ♀) mice. Data were expressed as time of latency (sec). A One-way ANOVA or Kruskal-Wallis was done to determine significance between mouse-dependent groups. Statistic tests were chosen according to the normality (Shapiro-Wilk's test). Data are expressed as mean \pm SD.



Supplementary Figure 2: *TRPV3* expression is decreased with aging in mouse hindpaw

(A) Relative expression of *Trpv3* normalized with *Rpl13a* in young (5 months) and old (22-24 months) mouse hindpaw and back skin. A Two-way ANOVA with Tuckey's multiple comparison was used to compare *Trpv3* expression in the skin of young (n=8; ♂=4, ♀=4) and old (n=5; ♂=4, ♀=1) mice. Data are expressed as median (IQR).



Supplementary Figure 3: Aging is associated with lower TRPV3 activity in human primary keratinocytes

(A) Relative expression of *TRPV3* normalized with *TBP* and *RPL13A* in primary human keratinocytes from young (mean age 33.5 years) and old (mean age 81.8 years) individuals (n=4/group) (median (IQR)). (B) Changes in the intracellular Ca^{2+} concentration (mean curve) and (C) maximal Ca^{2+} response from baseline, expressed as fluorescence intensity ratio at 340 and 380 nm (F340/F380) in human primary keratinocytes (mean age 33,5 years) stimulated with 300 μM carvacrol (5 min) in the absence (B: solid line; C: cross-hatched box) or presence (B: dotted line; C: empty box) of 100 μM IAB (n=5) (median (IQR)). (D) Maximal Ca^{2+} response from baseline (F340/F380) of young keratinocytes after 300 μM carvacrol (n=5, mean age 33.5 years) (cross-hatched box) or, 50 μM 2-APB (2 min) and 300 μM carvacrol (5 minutes) stimulation (n=4, mean of age 37 years) (full box) (median (IQR)). A t-test or Mann-Whitney was performed according to the Shapiro-Wilk's test. Activity of TRPV3 was assessed with at least 40 cells recorded in each biological replicate.