The α7 Nicotinic Acetylcholine Receptor: A Promising Target for the Treatment of Fibrotic Skin Disorders

Agatha Stegemann1, Damian Flis2, Wiesław Ziółkowski3, Jörg H.W. Distler4, Kerstin Steinbrink1 and Markus Böhm1

Targeting neuroendocrine receptors can be considered as another interesting approach to treating fibrotic disorders. Previously, we could demonstrate that tropisetron, a classical serotonin receptor blocker, can modulate collagen synthesis and acts in vitro through the α7 nicotinic acetylcholine receptor (α7nAChR). Here, we used a pharmacologic approach with specific α7nAChR agonists to validate this hypothesis. PHA-543613, an α7nAChR-specific agonist, not only prevented but also reversed established skin fibrosis of mice injected with bleomycin. Interestingly, agonistic stimulation of α7nAChR also attenuated experimental skin fibrosis in the non-inflammation driven adenovirus coding for TGFβ receptor 1αct mouse model, indicating fibroblast-mediated and not only anti-inflammatory effects of such agents. The fibroblast-mediated effects were confirmed in vitro using human dermal fibroblasts, in which the α7nAChR-specific agonist strongly reduced the impact of TGFβ1-mediated expression on collagen and myofibroblast marker expression. These actions were linked to modulation of the redox-sensitive transcription factor JunB and impairment of the mitochondrial respiratory system. Our results indicate that pharmacologic stimulation of the α7nAChR could be a promising target for treatment of patients with skin fibrotic diseases. Moreover, our results suggest a mechanistic axis of collagen synthesis regulation through the mitochondrial respiratory system.

INTRODUCTION

Systemic sclerosis (SSc) is a chronic inflammatory connective tissue disease. It can lead to life-threatening conditions affecting not only the skin but also the internal organs (Asano, 2018; Hunzelmann and Krieg, 2010). Excessive tissue fibrosis occurring because of profibrotic cytokines like TGFβ1 and vascular damage are key pathogenetic events of this autoimmune disease (Laesk and Abraham, 2004; Varga and Whitfield, 2009). The pathogenesis of SSc is still incompletely understood, and currently, no fully effective antifibrotic therapies are available (Distler et al., 2017).

An approach for the treatment of SSc could consist of the modulation of fibroblast activation and collagen metabolism through targeting specific neuroendocrine receptors that are expressed by resident skin-cell types (Böhm et al., 2004; Kokot et al., 2009). Accordingly, we could show that stimulation of the melanocortin-1 receptor through α-melanocyte-stimulating hormone can prevent experimentally induced skin fibrosis. Moreover, tropisetron, a classical serotonin receptor antagonist approved for the treatment of chemotherapy-induced nausea, turned out to have beneficial effects on experimentally induced skin fibrosis. In vitro, these effects of tropisetron appeared to work through the stimulation of a serotonin receptor-unrelated target, the α7 nicotinic acetylcholine receptor (α7nAChR) (Stegemann et al., 2013).

Of note, α7nAChR is one of the most abundant nAchRs and not only expressed in the brain but also in non-neuronal cells including the skin (Kurzen et al., 2007). It consists of five identical α7 subunits and is essential for the cholinergic anti-inflammatory action as shown in α7nAChR-deficient animals (Wang et al., 2003). Classical signal transduction of the α7nAChR leads to an increase in intracellular Ca2+. However, stimulation of the α7nAChR can also activate the Jak and/or signal transducer and activator of transcription and the NF-kB pathway (Arredondo et al., 2006; de Jonge et al., 2005; Saeed et al., 2005). In addition, cholinergic agonists can modulate several other signaling pathways such as extracellular signal-regulated kinases 1 and 2 (MAPK) (Heeschen et al., 2002) and PGE2 and/or protein kinase A (Takahashi et al., 2006).

In this study, we employed a pharmacologic approach with specific α7nAChR agonists to test whether these agents can affect experimentally induced skin fibrosis. First, we utilized the bleomycin (BLM)-induced scleroderma model, which is considered an inflammation-driven model of skin fibrosis. Second, we used the adenovirus coding for TGFβ receptor 1 (AdTBRIαct) mouse model that acts independently of inflammatory cells to induce collagen expression directly in dermal...
fibroblasts. We also investigated in vitro in human dermal fibroblasts (HDFs) distinct intracellular pathways by which agonistic stimulation of the α7nAchR could suppress fibroblast activation and thus, skin fibrosis.

RESULTS

The α7nAchR pharmacologic agonist PHA-543613 antagonizes BLM-induced skin fibrosis

First, we used the well-established BLM mouse model of SSC (Yamamoto et al., 1999a) to test the impact of PHA-543613 (Wishka et al., 2006) on skin fibrosis. Repetitive subcutaneous injections of BLM increased mRNA levels of Col1α1 and α-Sma compared with those in the control mice (Figure 1a). PHA-543613 significantly reduced the mRNA expression levels of both Col1α1 and α-Sma in skin samples of BLM-treated mice (Figure 1a). At protein level, BLM treatment likewise resulted in increased collagen amounts as shown by SDS-PAGE following pepsin digestion of skin samples. This profibrotic effect of BLM was again suppressed by PHA-543613 (Figure 1b). In accordance with this, the treatment of mice with BLM led to increased dermal thickness, whereas PHA-543613 significantly reduced this effect (Figure 1c). The used PHA-543613 dose was based on the literature and our previous experiments (Supplementary Figure S2a).

To further test whether PHA-543613 is also operative on established skin fibrosis, we used a therapeutic approach described initially by Yamamoto et al. (1999b). In fact, PHA-543613 strongly reduced BLM-induced collagen induction after the onset of skin fibrosis as shown by mRNA (Figure 2a) and protein read-outs (Figure 2b). Interestingly, mRNA levels of another fibroblast activation marker Ctgf were also significantly reduced by injections with PHA-543613. Indeed, the increased dermal thickness was normalized to control levels after PHA-543613 treatment in comparison with sodium chloride-treated skin (Figure 2c). The α7nAchR antagonist a-bungarotoxin was used in vitro to further clarify the role of this receptor in fibrosis and collagen synthesis. In HDFs, a-bungarotoxin neutralized the suppressive effect of AR-R17779 and PHA-543613 on TGFβ1-induced collagen mRNA expression, suggesting that the observed in-vivo effect of these agents is also mediated through this receptor (Supplementary Figure S2b). Taken together, these findings strongly suggest that PHA-543613, presumably through pharmacologic activation of the α7nAchR, has both preventive and therapeutic antifibrotic effects.

Pharmacologic activation of the α7nAchR acts independently of inflammation to reduce experimentally skin fibrosis

Because the BLM mouse model is considered an inflammation-driven model, we next investigated whether the beneficial effect of α7nAchR activation is directly related to effects on fibroblasts. Thus, we used the AdTBRIact mouse model, in which skin fibrosis was induced by injection with AdTBRIact (Akhmetshina et al., 2012; Zhang et al., 2017). In this model, skin collagen synthesis was turned on by transfection with a constitutive active TGFβ1 receptor, resulting in fibroblast activation and collagen synthesis independent of inflammation. Mice carrying TBRIact revealed elevated mRNA expression of collagen type I and the myofibroblast marker α-Sma at RNA level. Cotreatment with either PHA-543613 or another α7nAchR agonist, AR-R17779, resulted in reduced expression of Col1α1 and α-Sma in murine skin (Figure 3a). At protein level, reduction in collagen type I expression was apparent for both AR-R17779 and PHA-543613 in comparison with the expression in TBR-treated skin alone (Figure 3b). These
results were confirmed by measurements of hydroxyproline content in murine skin. Whereas hydroxyproline expression was upregulated in TBR-treated skin compared with LacZ skin, both α7nAchR agonists significantly reduced hydroxyproline expression compared with TBR alone (Figure 3c). Visualization of skin thickness was finally performed by histologic H&E staining. TBR murine skin showed increased thickening of the dermis, whereas dermal thickness in skin treated with both α7nAchR agonists was comparable to that in LacZ skin (Figure 3d). These results clearly confirm the modulatory effects of α7nAchR agonists, which act independently of inflammation and directly at the fibroblast level, that is, through modulation of collagen synthesis.

**The redox-sensitive transcription factor JunB is an intracellular target for α7nAchR agonists**

To shed light on the mode of action of the antifibrotic effect of α7nAchR agonists, we analyzed a distinct set of signaling transduction cascades that orchestrate fibroblast activation and collagen synthesis. We focused on TGFβ1-mediated pathways that are not only directly implicated in the
effector pathway of fibrosis in the AdTBRI act mouse model but also indirectly in the BLM-induced SSc model. Using HDFs as an in vitro model, TGF-β1 readily increased mRNA expression of COL1α1, α-SMA, and CTGF genes as shown by real-time PCR. However, in accordance with our in-vivo data, coincubation with both AR-R17779 and PHA-543613 strongly suppressed this effect of TGF-β1 (Figure 4 a). Then, we concentrated on the activator protein-1 (AP-1) signaling pathway, which can be activated by TGF-β1 (Avouac et al., 2012). AP-1 transcription factors belong to a family of structurally related factors composed of protein complexes derived from the Fos and Jun families. These redox-sensitive proteins can bind to cyclic adenosine monophosphate response elements or tetracycline response elements (Wisdom, 1999). Treatment of HDFs with TGF-β1 led to an upregulation of JunB, c-Fos, and Fra-1 within 60 minutes as shown by quantitative real-time RT-PCR (Figure 4 b, Supplementary Figure S1 a and b). Simultaneous stimulation of cells with AR-R17779 and/or PHA-543613 and TGF-β1 strongly suppressed TGF-β1-mediated JunB mRNA expression but did not affect c-Fos and Fra-1 mRNA expression (Figure 4 b, Supplementary Figure S1 a and b). In accordance with these findings, TGF-β1-mediated JunB protein expression was reduced after stimulation with AR-R17779 and, to a lesser extent, with PHA-543613 (Figure 4 c). JunB activity assay was performed to determine JunB expression levels in a more quantitative manner. JunB activity was strongly induced after TGF-β1 treatment, whereas both co-incubated α7nAchR agonists almost completely abrogated TGF-β1-induced elevation of JunB activity in HDFs (Figure 4 d). In accordance with these results, immunofluorescence analysis of JunB disclosed an intensive nuclear signal after TGF-β1 in HDFs, whereas the intensity of this nuclear staining was strongly diminished with co-incubated α7nAchR agonists (Supplementary Figure S1 c). In summary, JunB was the only factor of the AP-1 signaling pathway that was modulated by α7nAchR agonists, suggesting that it is the mediator of the suppressive effect of α7nAchR activation on TGF-β1-mediated induction of extracellular matrix (ECM) genes in HDFs. Of note, the SMAD pathway, a prototypical signal transduction pathway activated by TGF-β1, was not affected by
both α7nAchR agonists in HDFs (Supplementary Figure S2c).

The α7nAchR agonists AR-R17779 and PHA-543613 modulate TGFβ1-activated respiration states of mitochondria in HDFs

Oxidative stress and ROS generation, which dysfunctional mitochondria are one of the major sources, are known to play a role in fibrosis and scleroderma (Doridot et al., 2019; Sekiguchi et al., 2019). Considering that JunB is a redox-sensitive factor (Rao et al., 1999), we addressed the mechanistic aspect and investigated whether respiratory chain dysfunction in mitochondria of HDFs could trigger collagen synthesis and would be a target for α7nAchR agonists.

After stimulation of HDFs with TGFβ1, TGFβ1 in combination with AR-R17779 or PHA-543613 for 24 hours of proton leak (state L) was measured. TGFβ1 led to a significant induction of proton release compared with untreated cells (Figure 5a). Coincubation with either AR-R17779 or PHA-543613 almost completely abrogated this leak of protons in the mitochondrial respiration, whereas the 2 agonists alone did not exhibit any effect on state L in HDFs (Figure 5a). In our system, TGFβ1 stimulation resulted in a significant lesser oxidative phosphorylation (OXPHOS) coupling efficiency than in untreated cells (Figure 5b). Cotreatment with both α7nAchR agonists restored the coupling efficiency of HDFs to normal control levels (Figure 5b). Other respiration parameters (state CI, state CI + CII, state E, and state R) were not significantly changed (Supplementary Table S1). ROS measurements by FACS analysis using a redox-sensitive probe confirmed that TGFβ1 treatment of HDFs leads to accumulation of ROS, whereas coincubation with α7nAchR agonists reduced TGFβ1-induced ROS production in the HDFs (Supplementary Figure S2d). In summary, TGFβ1 as a profibrotic factor leads to a dysfunction of the respiratory system in HDFs, pointing to fibrotic changes possibly owing to the

Figure 4. The anticollagenic effect of α7nAchR agonists is mimicked and mediated by JunB in HDFs. (a) AR and PHA inhibit TGFβ1-induced expression of ECM genes in HDFs. Cells were treated with TGFβ1 (10 ng/ml) or co-incubated with AR (50 μM) or PHA (10 μM) for 24 hours followed by real-time RT-PCR. *P < 0.05 versus TGFβ1. (b) α7nAchR agonists alter JunB mRNA expression in HDFs. The donors (n = 5) were stimulated as described followed by real-time RT-PCR. *P < 0.05 versus TGFβ1. (c) Western immunoblotting of JunB. Lamin B antibody served as the loading control. Depicted is one out of three experiments. (d) α7nAchR agonists decrease TGFβ1-induced JunB activity in HDFs. Cells were treated as indicated. Nuclear fractions were subjected to JunB ELISA. OD was determined at 450 nm (n = 4). *P < 0.01. α7nAchR, α7 nicotinic acetylcholine receptor; ECM, extracellular matrix; HDF, human dermal fibroblast; OD, optical density.
accumulation of ROS. Therefore, activation of α7nAChR by AR-R17779 and PHA-543613 abrogates the TGFβ1-mediated negative effects, suggesting that modulation of mitochondrial ROS production is at least a part of the mechanism by which α7nAChR agonists exert antifibrotic effects on activated dermal fibroblasts.

**DISCUSSION**

In this report, we provide evidence for pharmacologic targeting of α7nAChR, which could be useful in the future treatment of fibrotic skin diseases such as SSc. Previous findings from our group have already pointed to this particular neuroendocrine receptor as a potential target (Stegemann et al., 2013). According to anecdotal reports (Müller et al., personal communication), this classical serotonin receptor agonist was reported to have some beneficial effects through the α7nAChR. Using two well-established agonists, PHA-543613 and AR-R17779, we now show that the pharmacologic α7nAChR is druggable in the context of preclinical models of SSc and also in HDFs in vitro. Notably, PHA-543613 did not only prevent BLM-induced skin fibrosis but also had an antifibrotic effect on an established fibrosis, the latter suggesting a therapeutic potential of this agent in patients with SSc with already advanced skin and organ fibrosis. Both PHA-543613 and AR-R17779 also had attenuating effects in the TBR-induced skin fibrosis model. It would be interesting to learn about the impact of α7nAChR antagonists such as α-bungarotoxin or methyllycaconitine in our skin fibrosis studies. Because such antagonists have rarely been used in in-vivo settings, experiments with α7nAChR-deficient animals are planned and should further increase our knowledge of the role of the α7nAChR in skin fibrosis. Importantly, our results on the beneficial effects of α7nAChR agonists in models of skin fibrosis are strongly supported by studies on mice with experimentally induced liver fibrosis. Accordingly, hepatic fibrosis induced by high-fat diet and experimentally induced kidney fibrosis are strongly increased in α7nAChR-deficient animals compared with wild-type animals (Kimura et al., 2019; Truong et al., 2015). At present, we do not know whether the expression of the α7nAChR is altered in its activity in patients with SSc or related fibrotic diseases. Thus, future studies have to define whether the α7nAChR is also druggable in the human system in vivo.

Because inflammation is a key pathogenetic element in SSc, we do not believe that any anti-inflammatory action induced by agonistic activation of the α7nAChR would be a limitation. In fact, in animal models of inflammation, selective α7nAChR agonists suppress macrophage cytokine production and inflammatory responses (Pavlov et al., 2007; van Maanen et al., 2009; van Westerloo et al., 2006).

In our subsequent in vitro studies on the mode of action of α7nAChR agonists, we detected the modulation of the AP-1 transcription factors (Supplementary Figures S1a and b), and cJun (Stegemann et al., 2013) after TGFβ1 treatment. However, other studies showed that after testing several AP-1 members for modulation in response to TGFβ1, only cJunB results in collagen type I accumulation and development of dermal fibrosis in patients with SSc (Ponticos et al., 2015). Fra-2 is a known key regulator of ECM in SSc (Reich et al., 2010). Busnadiego et al. (2013) showed that Fra-2 requires dimerization with JunB to form the AP-1 complex capable of binding to DNA. It seems that the interaction between the AP-1 transcription factors is accounted for in the action of TGFβ. Our studies revealed an induction of c-Fos, Fra1 (Supplementary Figure S1a and b), and cJun (Stegemann et al., 2013) after TGFβ1 treatment. However, other studies showed that after testing several AP-1 members for modulation in response to TGFβ, only JunB and slightly Fra-2 protein expression was elevated in fibroblasts (Ponticos et al., 2015). Most interestingly, modulation of this redox-sensitive transcription factor was related to the regulation of the respiratory state in the mitochondria of HDFs. We observed a higher...
proton leakage after TGFβ1 treatment in HDFs, which may be associated with electron leak linked to ROS production and showed a clear protective effect of both agonists on the TGFβ1-induced dysfunction of the respiratory state in mitochondria of HDFs. It was reported that ROS is increased and the expression of mitochondrial membrane potential, ATP content, and mitochondrial complex protein is reduced by TGFβ (Zhang et al., 2018). On the molecular level, TGFβ affects mitochondrial function in fibroblasts by decreasing PTEN-induced putative kinase Parkin and SIRT3, leading to mitochondrial respiratory chain dysfunction and mitochondrial DNA mutagenesis. All of these factors contribute to fibroblast-to-myofibroblast differentiation (Zank et al., 2018). Studies with isolated mitochondria show that ROS production is high during state IV respiration, which reflects conditions where there is an excess of ATP and deficiency of ADP (Liu et al., 2002). The ATP synthase inhibitor oligomycin is used to fix mitochondria in state IV respiration inhibiting the conversion of ADP into ATP (Liu and Schubert, 2009), showing that oligomycin induces the highest cellular ROS levels of all respiration inhibitors. Owing to this fact, we assume that higher proton leakage observed after oligomycin addition is influenced by electron leak—induced ROS generation. The observed antioxidative effects of both PHA-543613 and AR-R17779 on OXPHOS are in accordance with the observation that another α7nAChR agonist, PNU, protects obese mice from experimentally induced hepatic steatosis through suppression of oxidative stress (Hasan et al., 2018). Moreover, treatment of astrocytes with the α7nAChR agonist GTS-21 upregulates canonical nuclear factor erythroid 2-related factor 2 (NRF2) antioxidant gene products (Kalkman and Feuerbach, 2016). Therefore, we suggest that reduction of intracellularly generated ROS is a key mechanism in the antifibrotic effect of α7nAChR agonists (Figure 6).

In summary, we have demonstrated here that pharmacologic targeting with specific full α7nAChR agonists has beneficial effects in mouse models of skin fibrosis. α7nAChR stimulation directly affects dermal fibroblast function as not only demonstrated by an inflammation-independent mouse model but also by addressing the effect of such agonists on collagen synthesis of HDFs in vitro. Mechanistically, modulation of JunB activity, presumably through modulation of mitochondrial ROS release, appears to be a key mechanism in the mode of action of α7nAChR agonists in the context of collagen synthesis.

MATERIALS AND METHODS

Cell culture and reagents

Normal neonatal HDFs (Tebu-bio, Offenbach, Germany) from five different donors were cultured as outlined earlier (Kokot et al., 2009). TGFβ1 was obtained from PeproTech (Hamburg, Germany) and PHA-543613 and AR-R17779 from Tocris Bioscience (Eching, Germany).

RNA extraction and real-time RT-PCR

Total RNA was prepared from cells by InnuPrep RNA Kit (AnalytikJena, Jena, Germany) or from skin by Trizol (Invitrogen, Darmstadt, Germany). cDNA synthesis was performed by employing the Revert Aid cDNA Kit (Fermentas Life Sciences, St. Leon-Rot, Germany). Primers used for real-time RT-PCRs are listed in Supplementary Table S2. Quantification of gene expression of each sample was done by the 2−ΔΔCT method using β-actin as an internal standard and depicted as n-fold induction or ct-values.

Western immunoblot

Cell pellet was suspended in low salt buffer and/or protease inhibitors. 10% NP40 was added and incubated for 30 minutes. For the nuclear fraction, the pellet was resuspended in high salt buffer and/or protease inhibitors and incubated for 20 minutes. Lysates were separated by SDS-PAGE followed by immunoblotting as described earlier (Kokot et al., 2009) and incubated with a JunB antibody (Cell Signaling). Equal protein loading was assured by an antibody against Lamin B (Cell signaling). Western immunoblots were repeated at least three times.

JunB transcription activity assay

JunB activity assay was performed according to the manufacturer’s protocol using a commercially available kit that measures DNA binding (RayBiotech, Norcross, GA). An amount of 5 μg protein per well was subjected to the assay. Absorbance was measured with an ELISA reader (BioTek, Bad Friedrichshall, Germany) at 450 nm.

BLM mouse model of scleroderma

BLM-induced skin fibrosis model described by Yamamoto et al. (1999a) was used. Animal experiments were performed with permission from the local veterinary authorities of the University of Münster, Münster, Germany. Male mice were treated in the preventive approach with BLM (10 μg), PHA-543613 (10 μM), BLM plus PHA-543613, or sodium chloride (negative control) for 21 days. For the therapeutic approach, mice were injected with BLM (10 μg) for 21 days followed by injections with sodium chloride (negative control) or PHA-543613 (10 μM) for further 14 days. Skin biopsies were collected for RNA extraction, collagen protein determination, and immunohistochemistry.

AdTBRIαct mouse model of fibrosis

Male C57BL/6j mice were injected subcutaneously with adenovirus coding for TBRIRαct on day 1 and day 28 of the experiment. Controls received injections with adenovirus coding for LacZ. Further animal groups received daily subcutaneous injections with AR-R17779 (50 μM) and PHA-543613 (10 μM) over 8 weeks from day 1. All animal experiments were performed with permission from the local veterinary authorities of the University of Erlangen, Erlangen, Germany.
Skin biopsies were collected for RNA extraction, collagen protein determination, hydroxyproline assay, and immunohistochemistry.

**Hydroxyproline assay**

Hydroxyproline content in skin samples was determined according to Woessner (1961). Briefly, a 3-mm-punch biopsy per sample was digested in 6 M hydrochloric acid (Roth, Karlsruhe, Germany) at 120 °C for 3 hours. Chloramine T (Roth) was added for 20 minutes at room temperature. Subsequently, p-dimethylaminobenzaldehyde digested in 6 M hydrochloric acid (Roth, Karlsruhe, Germany) at 120 °C for 3 hours. Chloramine T (Roth) was added for 20 minutes at 60 °C. Optical density was determined in triplicate aliquots photometrically at 557 nm.

**Histochemistry**

Skin sections from mice treated as indicated were stained with H&E. Sections were viewed under a Zeiss Axiopt microscope (Zeiss, Oberkochen, Germany).

**Determination of collagen protein content**

Skin-punch biopsies were incubated in 0.5 M acetic acid with protease-inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). We added 1 mg pepsin (Sigma, Kanagawa, Japan) to each sample followed by incubation for 24 hours at 4 °C. After centrifugation, supernatants were precipitated with methanol and/or chloroform. Samples were subjected to SDS-PAGE and stained with 0.5% Coomassie Brilliant Blue (Bio-Rad Laboratories, Munich, Germany).

**High-resolution respirometry**

Mitochondrial respiration in HDF was measured in MiR05 medium in a high-resolution respirometer using an Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria). According to Pesta and Gnaiger (2012), after routine respiration, cells were permeabilized with digitonin (10 μg/ml) followed by addition of pyruvate, glutamate, and malate (5 mM, 5 mM, and 0.5 mM, respectively). OXPHOS capacity of complex I (state CI) was evaluated by adding ADP (2.5 mM). Succinate (10 mM) was added to obtain maximal OXPHOS capacity with convergent input through both complex I and complex II (state CI + CII). Oligomycin (1 μg/ml) was used to inhibit the ATP synthase and induce leak respiration (state L). The uncoupler carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (0.5 μM steps) was used to determine electron transfer system capacity (state E). Rotenone (0.5 μM) and antimycin-A (2.5 μM) were added for the determination of residual oxygen consumption. Data were evaluated using DatLab software (Oroboros Instruments). OXPHOS coupling efficiency was calculated with the formula [1 – (state L)/(state CI + CII)]. A lower value of OXPHOS coupling efficiency means lesser coupling of the oxidation and phosphorylation after addition of ADP (van Schaardenburgh et al., 2017).

**Statistical analysis**

All experiments were performed at least three times. Expression levels were calculated as means ± SD or ± SEM, and deviations from normality were assessed by Kolmogorov-Smirnov and the Shapiro–Wilks test. Statistical significance between two mean values was determined by ANOVA with Tukey post hoc test, Mann–Whitney U test, and Wilcoxon signed-rank test. Differences were considered significant at P < 0.05.

**Data availability statement**

Datasets related to this article are available on request from Agatha Stegemann, Department of Dermatology, University of Münster, Münster, Germany.

**ORCIDs**

Agatha Stegemann: http://orcid.org/0000-0001-5583-1950

Damian Flis: http://orcid.org/0000-0002-6176-4536
Wiesław Ziolkowski: http://orcid.org/0000-0001-8371-4153
Jörg H.W. Distler: http://orcid.org/0000-0001-7408-9333
Kerstin Steinbrink: http://orcid.org/0000-0002-0500-2158
Markus Böhm: http://orcid.org/0000-0001-7338-7734

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

The authors are grateful to Lena Summa and Katja Dreißigacker for expert technical assistance. This work was supported by the German Research Foundation (DFG; STE 2312/1-3 to Agatha Stegemann).

**AUTHOR CONTRIBUTIONS**


**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.04.006.

**REFERENCES**


van Schaardenburgh M, Wohlwend M, Rognno O, Mattsson EJR. Exercise in claudicants increase or decrease walking ability and the response relates to mitochondrial function. J Transl Med 2017;15:130.


