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$TRPV_1$ Channels in Human Skeletal Muscle Feed Arteries: Implications for Vascular Function

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Abstract

To examine the role of transient receptor potential vanilloid type 1 ($TRPV_1$) ion channel in the vascular function of human skeletal muscle feed arteries (SMFAs) and if activation of this heat-sensitive receptor could be involved in modulating vascular function, SMFAs from 16 humans (63±5, 41–89 yrs) were studied using wire myography with capsaicin ($TRPV_1$ agonist) and without (control). Specifically, phenylephrine (PE, α_1-adrenergic receptor agonist), dexemedetomidine (DEX, α_2-adrenergic receptor agonist), acetylcholine (ACh), and sodium nitroprusside (SNP) concentration response curves (CRCs) were performed to assess the role of TRPV1 channels in α-receptor-mediated vasocontraction as well as endothelium-dependent and independent vasorelaxation, respectively. Compared to control, capsaicin significantly attenuated maximum vasocontraction in response to PE (control: 52±8; capsaicin: 21±5 %length-tension$_{max}$ (LT_{max})) and DEX (control: 29±12; capsaicin: 2±3 %LT_{max}), while robustly enhancing maximum vasorelaxation with ACh (control: 78±8; capsaicin: 108±13 %vasorelaxation), and less clearly enhancing the SNP response. Denudation of the endothelium greatly attenuated the maximum ACh-induced vasorelaxation equally in the control and capsaicin conditions (~17 %vasorelaxation), and abolished the attenuating effect of capsaicin on the maximal PE response (denuded+capsaicin: 61±20 %LT_{max}). Immunohistochemistry identified a relatively high density of $TRPV_1$ channels in the endothelium compared to the smooth muscle of the SMFAs, but, due to the far greater volume of smooth muscle, total $TRPV_1$ protein content was not significantly attenuated by denudation. Thus, SMFAs ubiquitously express functional $TRPV_1$ channels, which

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alter vascular function, in terms of α_1-receptors, in a predominantly endothelium dependent manner, conceivably contributing to the functional sympatholysis and unveiling a therapeutic target.

Keywords

heat; blood flow; sympatholysis

INTRODUCTION

The skeletal muscle feed artery (SMFA) has been recognized as a key site of skeletal muscle blood flow regulation in both animals (Segal & Duling, 1986; Williams & Segal, 1993; Lash, 1994; Segal, 2000; VanTeeffelen & Segal, 2003, 2006), and humans (Ives *et al.*, 2012b). Thus, understanding the factors contributing to the basic function of the feed artery is important in terms of understanding the mechanisms that regulate muscle blood flow. However, currently there is a paucity of studies that address the basic physiology of human SMFAs. Recent work from our group has provided evidence of a regulatory role for human SMFAs (Ives *et al.*, 2012b) and that these vessels may be susceptible to "metabolic inhibition" of sympathetically-mediated vasocontraction via metabolic by-products such as heat (Ives *et al.*, 2011a; Ives *et al.*, 2012a) and acidosis (Ives *et al.*, 2013). Furthermore, we (Ives *et al.*, 2012a) and others (Harris *et al.*, 2003), have found that the endothelium and the endothelial nitric oxide synthase (eNOS) pathway appear to be integral components of this heat-induced sympatholysis, however, the mechanisms and receptors contributing to NOS activation in human SMFAs are still not well understood.

There is a growing interest in the transient receptor potential (TRP) family of ion channels and their role in the vasculature (Ramsey *et al.*, 2006; Venkatachalam & Montell, 2007; Baylie & Brayden, 2011). Specifically, although the vanilloid type TRP channels (TRPV), have classically been suggested to play a role in the afferent neuronal sensation of temperature (Wang *et al.*, 2006b; Light *et al.*, 2008; Yang *et al.*, 2010b) or noxious stimuli (Ramsey *et al.*, 2006; Baylie & Brayden, 2011), recent work by our group (Gifford *et al.*, 2014), investigated the role of TRPV channels, particularly the $TRPV_4$ channel, in the heat induced sympatholysis of SMFAs. Whether using non-specific TRPV inhibition (Ruthenium Red, RR) or $TRPV_4$ specific inhibition (RN-1734), the heat-induced suppression of the vasocontractile response to PE was abolished, suggesting TRPV channels, particularly the $TRPV_4$ channels, are activated by heat and antagonize adrenergic-mediated vascular responses. Interestingly, TRPV inhibition also blunted the ACh response, indicative of an endothelial-dependent TRPV-mediated response, which was confirmed by denuding the endothelium. While it appears likely that the $TRPV_4$ channel accounts for the majority of the functional sympatholytic effect of heat from muscle contraction, it is important to recognize that several factors associated with exercise (*e.g.* elevated reactive oxygen species (ROS), temperature, levels of anandamide, and decreased pH), probably acting synergistically (Ho *et al.*, 2008; Roy *et al.*, 2012), would also activate the vascular $TRPV_1$ channels (Chuang & Lin, 2009; Mergler *et al.*, 2010). Therefore, given the effect that non-specific TRPV inhibition, which includes $TRPV_1$ inhibition, had on vascular function (Gifford *et al.*, 2014)

and the established relationship between the $TRPV_1$ channels and endothelial function in mice (Yang *et al.*, 2010a), it is conceivable that the $TRPV_1$ channels play a similar role to the $TRPV_4$ channel in modulating vascular function in the SMFAs of humans. Indeed, coupled with epidemiological data that suggests regular ingestion of capsaicin-rich foods (e.g. peppers from capsicum frutescent plants) reduces cardiovascular-related mortality (Lv *et al.*, 2015), there is the implication that $TRPV_1$ channels may play a significant role in vascular function. However, there is actually very little known about the potential role of the $TRPV_1$ channels in modulating endothelial or vascular smooth muscle function in humans.

Consequently, the purpose of this study was to determine if human SMFAs express functional $TRPV_1$ channels, what, if any, potential role they may play in vascular function, and, using an agonist approach, look for evidence that activation of $TRPV_1$ channels could, conceivably, play a role in the functional sympatholysis associated with exercise. Additionally, by denuding the endothelium, we sought to determine if the endothelium plays a role in mediating any of the observed $TRPV_1$ responses. We hypothesized that human SMFAs will express $TRPV_1$ channels, which, upon activation, will significantly alter vascular function, specifically, reducing adrenergic mediated vasocontraction in a similar fashion to heat-induced sympatholysis, and this phenomenon would be predominantly endothelium-dependent.

METHODS

Subjects and General Procedures

A heterogeneous group of subjects agreed to have their vessels harvested during melanoma-related surgeries and used in this study (Table 1). Although medical conditions and medications were noted, by means of medical records, there were no exclusions based on this information. All subjects included in this study had not received chemotherapy, as this was a contraindication for surgery. All protocols were approved by the Institutional Review Boards of the University of Utah (IRB#32786) and the Salt Lake City VA Medical Center, and written informed consent was obtained from all subjects. This study was performed in accordance with the latest revision of the *Declaration of Helsinki,* except for registration in a database.

Vessel Harvest

Human SMFAs from the axillary and inguinal regions were obtained during melanoma-related node dissection surgeries at the Huntsman Cancer Hospital, University of Utah. Patients were anaesthetized using a standard protocol including: propofol, fentanyl, benzodiazepines, and succinylcholine. After removal of sentinel lymph nodes or lymph node dissection, SMFAs in the axillary (e.g. serratus anterior, or latissimus dorsi) or inguinal (e.g. hip adductors, or quadriceps femoris) regions were identified and classified as SMFAs based on entry into a muscle bed, structure, coloration, and pulsatile bleed pattern. The vessels were ligated, excised, and immediately placed in iced normal physiological saline solution (PSS) and brought to the laboratory within 15 minutes of harvesting.

Wire Myography

Vessels were dissected under a stereo microscope in cold (~4°C) normal physiological saline solution (NPSS) (125 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 18 $NaHCO_3$, 0.026 Na_2EDTA, and 11.2 Glucose mM). All NPSS solutions and drugs were prepared fresh daily. Vessel internal diameter was measured using a calibrated micrometer eyepiece and reported in micrometers (μm). Perivascular adipose tissue was dissected from the SMFAs. The NPSS was continuously aerated with carbogen gas (95% oxygen, 5% carbon dioxide), and pH was monitored at regular intervals and maintained at pH 7.35 – 7.45 by altering the amount of aeration (Orion 3 Star, Thermo Scientific, Waltham MA).

Vessels were cut into four rings measuring approximately 2 mm in length, and mounted in wire myography baths (700 MO, DMT Systems, Aarhus, DK) to be studied using the isometric tension technique, as previously utilized by our group (Ives *et al.*, 2011b). Once the vessels were mounted, the vessel baths were also aerated with the same carbogen gas mixture, and the media in the bath was exchanged at 10 minute intervals, except during the assessment of cumulative drug dose responses. Vessel baths were warmed to 37°C over a 30 minute equilibration period prior to the start of a protocol.

All vessel segments underwent length tension procedures at 37°C under control conditions to determine the length at which the vessels produced the greatest tension in response to a single dose of 100mM KCl (LT_{max}) (Symons *et al.*, 2002). LT_{max} was operationally defined as less than a 10% improvement in developed tension in response to 100mM KCl.

Capsaicin and Vascular Reactivity

An overview of the experimental procedures is presented in Figure 1. To activate $TRPV_1$ channels, capsaicin (1 μM) was added to two of the four baths 15 minutes prior to the start of the concentration response curves (CRCs), and was maintained in the designated baths throughout the experiment. This concentration of capsaicin was chosen as it is a commonly used dose sufficient to elicit significant $TRPV_1$ channel activation in the vasculature (Poblete *et al.*, 2005; Czikora *et al.*, 2012). The capsaicin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1mM, from this concentrated stock, 8 uL was added to the 8mL bath, resulting in a low concentration (0.1%/vol.) of DMSO, a concentration that has previously been determined to have no dilatory effect on isolated arteries (Pitts *et al.*, 1986). In a balanced order, the following CRCs were performed: PE (10^{-9} – 10^{-3} log M), DEX (10^{-10} – 10^{-3} log M), ACh (10^{-7} – 10^{-3} log M), and SNP (10^{-9} – 10^{-4} log M) to determine α-receptor-mediated vasocontraction as well as endothelium-dependent and independent vasorelaxation. The CRCs were performed in a balanced manner, minimizing an order effect, and overall reducing the impact of neuronal $TRPV_1$ receptors, due to repeated exposure of capsaicin and subsequent depletion of sensory neurotransmitters such as calcitonin gene related peptide (Zygmunt *et al.*, 1999). It also should be noted that each bath contained, originally contiguous, vessel rings which were simultaneously exposed to the capsaicin or control conditions for each CRC. This approach was adopted to minimize the effect of time on a given CRC. To normalize vasocontraction data to the individual maximal response, as described elsewhere (Jarajapu *et al.*, 2001; Wareing *et al.*, 2002; Kluess *et al.*, 2005; Ives *et al.*, 2013), all vasocontractile responses are expressed as a percent of the

individual maximal response to 100mM KCl during the length tension procedure (%LT_{max}) obtained during the length tension protocol, which typically yields the greatest tension development (unpublished observations). All vasorelaxation responses are expressed as percent relaxation (%) from approximately 60–70% PE pre-contraction (Ives *et al.*, 2011a). All chemicals were obtained from Sigma Aldrich (St Louis, MO). All data were acquired at 4Hz using an analog to digital data acquisition system (Biopac Systems, Goleta, CA) to monitor vessel tension and allow later offline analyses.

The Role of the Endothelium in Capsaicin-Mediated Responses

To determine the potential role of the endothelial $TRPV_1$ channels, additional control and capsaicin experiments were performed in vessel rings, from the same subject, that either had an intact endothelium or had been denuded of endothelial cells. Denudation was achieved by passing 2 ml of air through the lumen of the artery before it was dissected and mounted onto the myograph chambers, as described previously (Gifford *et al.*, 2014). Specifically, CRCs for PE, ACh, and SNP were performed on these intact and denuded vessels with and without capsaicin.

$TRPV_1$ mRNA and Protein Expression

Using quantitative PCR (q-PCR) analysis, samples of the human SMFAs were probed for the gene expression of $TRPV_1$ channels, α_{1A}-, and α_{2B}-adrenergic receptors (Rudner *et al.*, 1999; Kable *et al.*, 2000) using GAPDH as the normalizing gene. After homogenization in ice cold buffer containing RNAse inhibitors, RNA was extracted using RNAeasy mini kits (Qiagen, Valencia, CA) and was immediately converted to a cDNA library and stored at -20°C until further analysis. After which, cDNA libraries were analyzed using the ABI quantitative real time PCR system on the ABI Sequence detection system (SDS) platform (v 2.4.1, ABI, Foster City, CA). Using ABI Taqman master mix and Taqman gene expression assay primer probes for $TRPV_1$, α_{1A}-, α_{2B}-adrenergic receptors, and GAPDH for a control gene, as well as no template controls, q-PCR was performed. Gene expression was quantified using the comparative Ct ($2^{-\Delta ct}$) method.

Frozen endothelium intact and endothelium denuded vessels were thawed on ice and then homogenized in 200 μL of ice-cold homogenization buffer containing a protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO)] and centrifuged for 15 min at 13,800 g at 4°C. The supernatant was then collected and total protein concentrations determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) as a standard (Pierce Chemical Company, Rockford, IL). Supernatants were stored at -80°C. Using standard western blotting methods membranes were probed with primary antibodies for $TRPV_1$ receptor (sc12498, Santa Cruz, San Juan Ranch, CA), and GAPDH (ab9485, Abcam, Cambridge, MA) and visualized with enhanced chemiluminescence (ECL) (Pierce detection kit, Rockford, IL) using a digital imaging system (BioRad Chemidoc XRS Imager, Bio-Rad Laboratories, Hercules, CA). Membranes were stained with Coomassie blue (BioRad Laboratories, Hercules, CA) for a loading/transfer control. The $TRPV_1$ signal was then normalized to the loading control signal (GAPDH).

To determine the locus of the $TRPV_1$ channels, immunohistochemistry of the vessel sections was performed. Again, the primary antibodies for $TRPV_1$ receptor (ab3487, Abcam, Cambridge, MA) were used to visualize the distribution of $TRPV_1$ channels.

Statistical Analyses

Two way repeated measures ANOVA were used to determine significant responses for each CRC, between conditions (control vs. capsaicin) (SPSS v.16, Chicago, IL). Where significant main effects were identified, Tukey's HSD *post hoc* tests were utilized ($\alpha = 0.05$). Vasocontraction was calculated as follows: vasocontraction ($\%LT_{max}$) = (Dose induced change in tension (change from pre-drug baseline)/maximal change in tension observed during LT)*100. Vasorelaxation (%) was calculated as follows: vasorelaxation (%) = (Dose induced change in tension (change from pre-contraction)/pre-contraction induced change in tension (pre-contraction − baseline)) *100. For the CRCs, to account for potential differences in the concentration which elicited the greatest response, maximal responses were determined on an individual basis and compared between conditions. The $logEC_{50}$ was individually calculated using a sigmoidal parameter to estimate vascular sensitivity to each agonist (biodatafit v.1.02, Castro Valley, CA) and was compared between conditions using t-tests. To compare the effects of $TRPV_1$ activation on adrenergic receptor subtype, percent sympatholysis was calculated (%Sympatholysis = (control $_{response}$ − capsaicin $_{response}$)/ control $_{response}$*100) and compared using t-tests. All data are expressed as mean ± SEM[1] for better visual clarity in the figures, and thus, for consistency, throughout the paper.

RESULTS

Subject Characteristics

SMFAs were successfully harvested from 16 volunteers (63 ± 5 yrs, 9 male, 7 female) (Table 1). None of these subjects had overt coronary artery, peripheral vascular, or cerebrovascular disease, or a history of MI/stenting/angioplasty. Due to the heterogeneity of the subjects and the origin of the vessels, this study was inadequately powered to detect differences in vascular function between sex, age, vessel location, medication use, complete blood count, or blood chemistry in any of the outcome variables. The average basal internal diameter for these SMFAs was 652 ± 96 μm. Twelve of the sixteen vessels obtained were harvested from the inguinal region, and 4 were obtained from the axillary region.

$TRPV_1$ Activation and Adrenergic Mediated Vasocontraction

Baseline vascular tension was modestly, and transiently, increased by capsaicin, after which capsaicin tended to reduce baseline tension (588 ± 211 vs. 334 ± 117 mg, control vs. capsaicin, $p > 0.05$). Vessel function protocols revealed a significant main effect of concentration on the contraction response to the α_1-receptor agonist PE and the α_2-receptor agonist DEX (Figure 2A and B). For the PE CRC, there was a significant condition (control vs. capsaicin) by concentration interaction effect, indicating a reduced responsiveness with increasing concentrations of PE in the presence of capsaicin (Figure 2A). Here, there was also a significant main effect for condition (control vs. capsaicin, $p < 0.05$), indicating an

[1]All data are expressed as mean ± SEM for better visual clarity in the figures

overall blunted response to PE in the presence of capsaicin such that the individual maximal response to PE was attenuated (52 ± 8; 21 ± 5 %LTmax, control vs. capsaicin, respectively). The α_2-receptor CRC assessments also displayed a significant condition by concentration interaction, revealing a blunted vascular response to increasing concentrations of DEX in the presence of capsaicin (Figure 2B). The main effect for condition, indicating an overall reduction in the vascular response to DEX, tended to be attenuated, but did not reach statistical significance ($p = 0.06$). The individual maximal response to α_2-receptor stimulation was significantly blunted in the capsaicin condition (30 ± 13 vs. 4 ± 2 $\%LT_{max}$, control vs. capsaicin, $p < 0.05$). The $logEC_{50}$ was unaffected by capsaicin in response to either PE (-4.7 ± 0.3 vs. -4.7 ± 0.2 log M) or DEX (-5.8 ± 0.3 vs. -5.9 ± 0.7 log M) (control vs. capsaicin, respectively). Calculation of percent sympatholysis, revealed a significantly greater attenuating effect of $TRPV_1$ activation on α_2-receptor versus α_1-receptor-mediated vasocontraction (PE 50 ± 10 vs. DEX 79 ± 16 % Sympatholysis).

$TRPV_1$ Activation and Endothelium-Dependent and -Independent Vasorelaxation

The SMFAs exhibited significant vasodilation ($p < 0.05$) in response to both the endothelium dependent agonist ACh and the endothelium-independent agonist SNP (Figure 3A and B). For the ACh CRC, there was a significant concentration by condition interaction effect, indicating an enhanced endothelial responsiveness with increasing concentrations of ACh in the presence of capsaicin. Here, there was also a significant main effect for condition (control vs. capsaicin, $p < 0.05$), indicating an overall greater endothelial response to ACh in the capsaicin condition such that the maximal response to ACh was increased (78 ± 8 vs. 108 ± 13 % vasorelaxation, capsaicin vs. control, respectively, Figure 3A). The $logEC_{50}$ for ACh was not significantly altered with capsaicin. On a *post hoc* basis, as capsaicin was determined to alter PE-induced vasocontraction, the level of pre-contraction and maximal ACh-induced relaxation were entered into a simple linear regression, and there was no evidence of a relationship ($r^2 = 0.07$, $p > 0.05$), suggesting that the level of pre-contraction was not influential in determining the relaxation response.

For the SNP CRC, there was not a significant interaction effect between condition and concentration ($p = 0.2$) (Figure 3B). However, there was a significant main effect for condition (control vs. capsaicin, $p < 0.05$), indicating an overall greater vasorelaxation response to SNP in the capsaicin condition, but the difference in the maximal response between conditions did not achieve statistical significance (87 ± 3 vs. 105 ± 6 %vasorelaxation, control vs. capsaicin, respectively, Figure 3B, $p = 0.07$). The $logEC_{50}$ for SNP was significantly increased in the presence of capsaicin (-6.9 ± 0.4 vs. -7.6 ± 0.3 log M, control vs. capsaicin, respectively), indicating an enhanced vascular sensitivity to exogenous NO.

The Role of the Endothelium in the Capsaicin-Mediated Responses

Removal of the endothelium abolished the attenuating effect of capsaicin on PE (capsaicin: 32 ± 8 vs. denuded+capsaicin: 61 ± 20 $\%LT_{max}$) and restored vasocontraction in the control condition (62 ± 16$\%LT_{max}$) (Figure 4A). Denudation of the endothelium significantly reduced the maximal vasorelaxation response to ACh in both the control (control: 82 ± 12 vs. control+denuded: 19 ± 6 % vasorelaxation) and capsaicin (capsaicin: 98 ± 27 vs.

capsaicin+denuded: 16 ± 4 % vasorelaxation) conditions (Figure 4B). An original tracing of the impact of Capsaicin and/or endothelial denuding on the vasocontraction response to Phenylephrine in human skeletal muscle feed arteries is presented in Figure 5.

$TRPV_1$ mRNA, Protein Expression, and Immunohistochemistry

Using quantitative PCR analysis, the SMFAs were determined to express significant levels of $TRPV_1$ mRNA (0.05 ± 0.01 mRNA relative to GAPDH), which was on par with the level of adrenergic receptor mRNA (α_{1A} 0.03 ± 0.00 and α_{2B} 0.02 ± 0.01 mRNA relative to GAPDH). Immunohistochemistry of the SMFAs revealed ubiquitous expression of $TRPV_1$ in both the endothelial and smooth muscle layers, however, notably, there appeared to be a relatively high $TRPV_1$ prevalence in the relatively small volume of the endothelium (Fig 6A and B). Correspondingly, Western blots indicated expression of $TRPV_1$ channels which was not greatly reduced following denudation (Figure 6).

DISCUSSION

The main finding of this study is that human skeletal muscle feed arteries express functional $TRPV_1$ channels, in both the endothelial and smooth muscle layers, which are capable of significantly altering vascular function. Specifically, pharmacological activation of vascular $TRPV_1$ channels with capsaicin, blunted sympathetic vasocontraction mediated through either the α_1- or α_2-receptors. There was apparent receptor specificity, such that the α_2-receptors were far more suppressed by $TRPV_1$ activation than the α_1-adrenergic receptors. The mechanism responsible for these findings is, at least in part, mediated by the endothelium, as denudation of this layer abolished the effects of capsaicin on α_1-receptor mediated vasocontraction. In support of this, capsaicin enhanced endothelium-dependent vasorelaxation, as measured by ACh-induced vasorelaxation, which was also ablated by denudation of the endothelium. These findings suggest an important role of vascular endothelial $TRPV_1$ channels in modulating vascular function, likely through an endothelium dependent increase in NO bioavailability, which can oppose α_1-receptor mediated vasocontraction. Given the well documented sensitivity of $TRPV_1$ channels to heat and other physiological activators such as those encountered during exercise (e.g. elevated reactive oxygen species, anandamide, and decreased pH), and the likely synergy of these stimuli, the results of this study suggest that these ion channels have the potential to contribute to sympatholysis during exercise. Furthermore, pharmacological activation of these channels via capsaicin might also be of therapeutic value.

The role of $TRPV_1$ channels on vasocontraction

Previous investigations have revealed that $TRPV_1$ activation can significantly alter myogenic tone (Scotland *et al.*, 2004), induce vasodilation (Poblete *et al.*, 2005; Wang *et al.*, 2006a; Hoi *et al.*, 2007; Bratz *et al.*, 2008) or vasoconstriction (Lizanecz *et al.*, 2006; Cavanaugh *et al.*, 2011), depending upon capsaicin dose (10^{-8} vs. 10^{-6}) (Kark *et al.*, 2008), vessel location, and location of $TRPV_1$ channels (neural compared to vascular) (Cavanaugh *et al.*, 2011; Czikora *et al.*, 2012). Using a dose of capsaicin (1 μM) that has been previously reported to induce either vasoconstriction (Kark *et al.*, 2008; Czikora *et al.*, 2012) or vascular NO release (Poblete *et al.*, 2005), this study revealed no sustained effect of

capsaicin alone on baseline tension and a blunted vasocontractile response to both α_1- and α_2-adrenergic receptors. Finally, removal of the endothelium prevented the capsaicin-induced reduction in α_1-receptor mediated vasocontraction, suggesting that the $TRPV_1$ activity is likely endothelium-dependent.

Interestingly, much of the work investigating the effects of $TRPV_1$ activation on vascular responses, utilizing capsaicin, implies a neural mechanism, specifically though perivascular sensory neuronal release of vasoactive substances (Wang *et al.*, 2006a; Kark *et al.*, 2008). Thus, the possibility of neuronal $TRPV_1$ channel involvement, and subsequent capsaicin-induced release of substance P or calcitonin gene related peptide (Kark *et al.*, 2008) cannot be definitively excluded. However, the response, at least for α_1-receptors, appears to be endothelium dependent (Figure 4A), which strongly implicates the vascular, not neuronal, $TRPV_1$ receptors in modulating vascular function.

The current study revealed a significant effect of activating vascular $TRPV_1$ channels, via capsaicin, on vasocontractile function in human feed arteries in the form of blunting adrenergic receptor mediated vasocontraction. These results suggest antagonism of G-protein coupled receptor function and subsequent signaling which is likely the result of antagonistic vasorelaxation initiated by the endothelium, as denuding restored PE-induced vasocontraction, and the endothelium specific agonist ACh, was enhanced in the presence of capsaicin. This observation is in agreement with the findings of others suggesting that the activation of $TRPV_1$ channels in vascular endothelial cells activates the eNOS pathway, through both the activation (Yang *et al.*, 2010a) and the removal of inhibition (Ching *et al.*, 2013) of eNOS. Therefore, this study suggests that $TRPV_1$ channels reduce adrenergic mediated vasocontraction in human feed arteries, in which multiple $TRPV_1$ channel stimuli (i.e. heat, ROS, anandamide, and reductions in pH) may contribute to the functional sympatholysis associated with exercise, but this warrants further investigation.

The role of $TRPV_1$ channels in vasorelaxation

Previous investigations focusing on the role of $TRPV_1$ channels on vasorelaxation and the endothelium, provide convincing evidence of a role for endothelial $TRPV_1$ channels in producing NO. Specifically, $TRPV_1$ channels are Ca^{++} conducting channels, allowing Ca^{++} influx, and potentially activating Ca^{++} sensitive pathways. Given the well described role for Ca^{++} in activating eNOS (Dimmeler *et al.*, 1999), it stands to reason that $TRPV_1$ activation, either through exogenous capsaicin or endogenously through anandamide, heat, or both, would result in endothelial NO production (Poblete *et al.*, 2005). In agreement with this concept, this study revealed that capsaicin enhanced the vasorelaxation response to ACh, suggestive of a synergistic effect of the endothelial receptor-mediated second messenger and $TRPV_1$ channel-induced calcium signaling pathways, leading to greater endothelial NOS activity and NO bioavailability. In addition to documenting functional responses, this study followed up with gene and protein expression, and immunohistochemistry assays. Immunohistochemistry identified a relatively high density of $TRPV_1$ channels in the endothelium compared to the smooth muscle of the SMFAs, but, due to the far greater volume of smooth muscle, total $TRPV_1$ protein content was not significantly attenuated by denudation (Figure 6). Interestingly, SNP-induced vasorelaxation tended to also be

augmented with capsaicin (Figure 3B), implying that the vascular response to exogenous NO had been sensitized, likely due to mechanisms aside from eNOS derived NO. One such mechanism could be the $TRPV_1$-mediated release of endothelial derived hyperpolarizing factors (Baylie & Brayden, 2011), or direct hyperpolarization of the vascular smooth muscle (Bratz *et al.*, 2008) through $TRPV_1$ channels in the smooth muscle. Clinically, such knowledge may be of use in terms of targeting $TRPV_1$ to improve endothelial function, which might help to explain the recently documented link between ingesting capsaicin-rich foods and reduced cardiovascular mortality (Lv *et al.*, 2015), and to that end capsaicin has been used to prevent hypertension in mice (Yang *et al.*, 2010a), though this remains to be explored in humans.

$TRPV_1$ versus $TRPV_4$ Ion channels in SMFAs

In a previous study we examined the role of the $TRPV_4$ ion channels in modulating vascular function in human feed arteries (Gifford *et al.*, 2014). Although the methods by which the ion channels were activated differed in these two studies, the current study utilizing a ligand and the other using heat, the results still offer an opportunity to compare the roles of these two ion channels in feed arteries. First, it should be noted that the activation of either $TRPV_1$ or $TRPV_4$ ion channels resulted in attenuated α_1 adrenergic vasocontraction, but despite a tendency for decreased α_2-induced contraction under $TRPV_4$ activated conditions, only $TRPV_1$ channel activation with capsaicin significantly inhibited both α_1 and α_2-receptor mediated vasocontraction. Second, it should be noted that while $TRPV_4$ channel activation, with heat, inhibited adrenergic contraction in an endothelium-dependent manner, there was only a weak tendency to potentiate ACh-induced vasorelaxation while $TRPV_1$ activation with capsaicin significantly augmented ACh-induced vasorelaxation. Thus, while activation of both types of ion channels results in a similar sympatholytic response, $TRPV_1$ activation may present a more potent stimulus. This, teleologically, makes sense because temperatures known to activate $TRPV_1$ channels (40+°C) are much higher and more noxious than those that activate the $TRPV_4$ channels (25–39°C) (Baylie & Brayden, 2011), perhaps demanding a greater response. Alternatively, unlike $TRPV_4$ channels, $TRPV_1$ are known to be sensitized by other metabolic factors aside from temperature, such as reductions in pH (Faisy *et al.*, 2007; Gao *et al.*, 2007), ROS, as well as the endogenous ligand anandamide (Poblete *et al.*, 2005; Chuang & Lin, 2009; Mergler *et al.*, 2010). During exercise pH can be reduced and the circulating levels of ROS (Bailey *et al.*, 2003) and anandamide increased (Heyman *et al.*, 2012), both of which likely lower the activation threshold of $TRPV_1$ (Ho *et al.*, 2008; Roy *et al.*, 2012), and/or increase $TRPV_1$ activity under more physiological conditions than previously thought. Therefore, the activation of $TRPV_1$ ion channels with heat, ROS, acidosis, and/or anandamide may, in combination with the $TRPV_4$ channels (Gifford *et al.*, 2014), also contribute to functional sympatholysis, but this speculation awaits further investigation.

Experimental Considerations

The subjects who took part in this study were certainly heterogeneous in terms of age, gender, and health, but, although exhibiting a tendency to be overweight and some evidence of systolic hypertension (although it should be noted that these measurements were obtained during pre-operative examination), they were taking minimal medications and had normal

blood chemistry and complete blood count data (Table 1). Thus, interpretation of the current data must be taken in this context and may not apply to young healthy humans. Additionally, given the disparate vessel harvest location it is possible that differences in muscle fiber type might complicate the current results. However, unlike murine models, where muscle fiber type is far more dichotomous, human muscle is quite mosaic, likely minimizing this as a confounding issue. Thus, despite a group of heterogeneous subjects, varied vessel harvest location (i.e. axillary and inguinal), and potential pathology, the notion that $TRPV_1$ activation elicited profound effects on vascular function speaks to the robust nature of these findings as they relate to vascular function and blood flow regulation in humans. However, at present, the potential role of age in these findings cannot be ruled out, as previously, albeit in subjects a decade older than the current average subject age, our group has documented age-related differences in vascular function using pressure myography (Park *et al.*, 2016). Therefore, it would be useful for future studies to explore $TRPV_1$ involvement in vascular function across the lifespan. Additionally, due to the relative scarcity of human SMFAs for research and the apparent predominant role of the α1-adrenergic receptors at this point in the vascular tree (Figure 2), it should be noted that the role of the α_2-adrenergic receptors was not assessed with and without an intact endothelium. Thus, although we expect a similar important role for the endothelium in the $TRPV_1$ modulation of α_2-adrenergic receptor vasoconstriction, this remains to be confirmed.

Conclusion

Utilizing an isolated *in vitro* approach to study human SMFA function, this study reveals that these arteries express functional vascular $TRPV_1$ channels in both the endothelial and smooth muscle layers. Activation of these channels significantly increased endothelium-dependent vasorelaxation and opposed α-adrenergic-mediated vasocontraction, effects that could be reversed with denudation of the endothelium, as assessed by α_1-receptor mediated responses. Thus, skeletal muscle feed arteries ubiquitously express functional $TRPV_1$ channels, which alter vascular function in a predominantly endothelium-dependent manner, perhaps presenting as a novel therapeutic target, and could, conceivably, contribute to the functional sympatholysis associated with exercise.

Acknowledgments

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NEW FINDINGS

This study sought to determine if human skeletal muscle feed arteries (SFMAs) express TRPV1 channels and what role do they play in modulating vascular function. Human SMFAs do, in fact, express functional $TRPV_1$ channels that modulate vascular function, specifically opposing α-adrenergic receptor-mediated vasocontraction and potentiating vasorelaxation, in an endothelium-dependent manner, as evidenced α_1-receptor mediated responses. Thus, the vasodilatory role of $TRPV_1$ channels, and their ligand, capsaicin, could be a potential therapeutic target for improving vascular function. Additionally, given the "sympatholytic" effect of $TRPV_1$ activation and known endogenous activators (anandamide, ROS, H^+, etc.) $TRPV_1$ channels may contribute to functional sympatholysis during exercise.

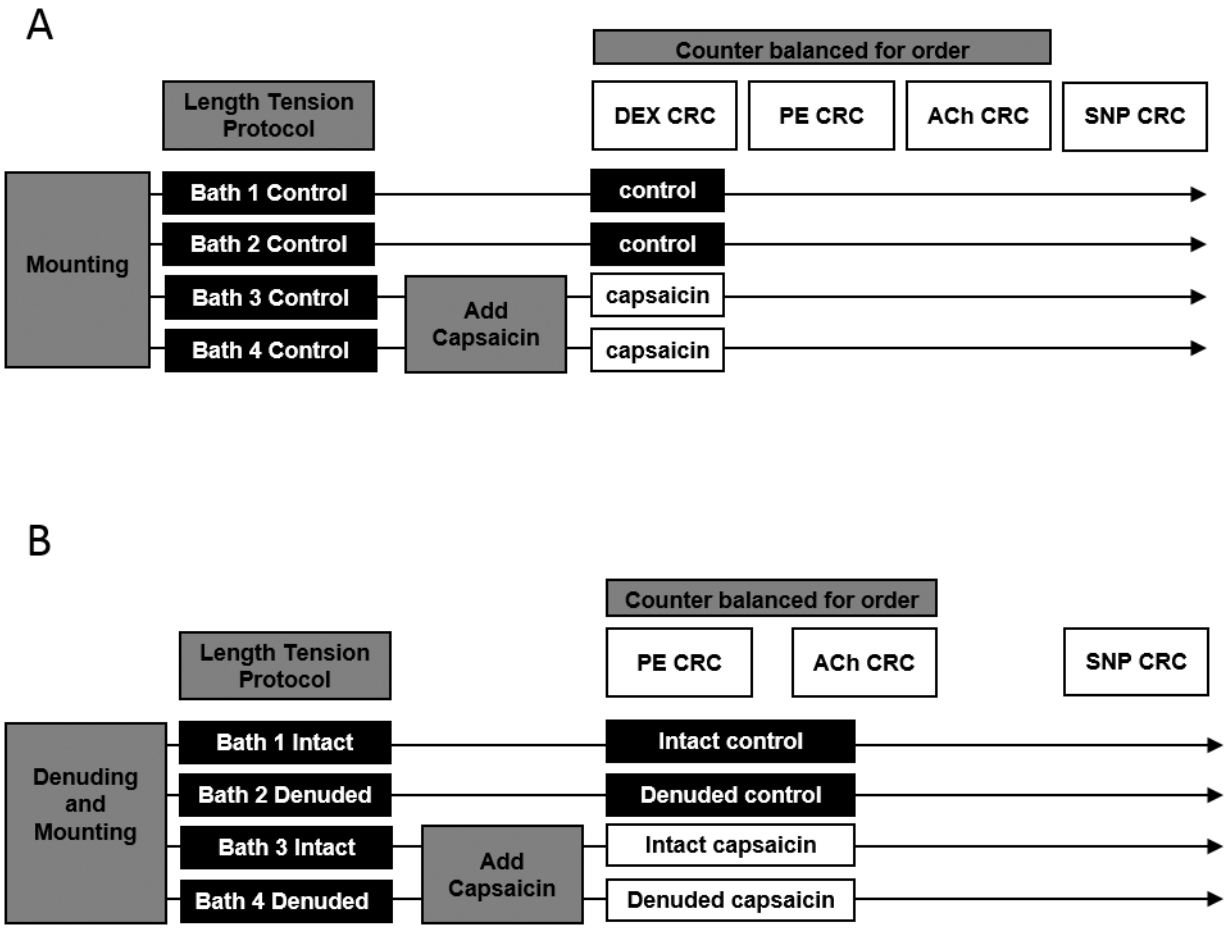

Figure 1. Overview of the Experimental Approaches

PE; Phenylephrine, DEX; Dexemedetomidine, ACh; Acetylcholine, SNP; Sodium Nitroprusside, CRC; Concentration Response Curve.

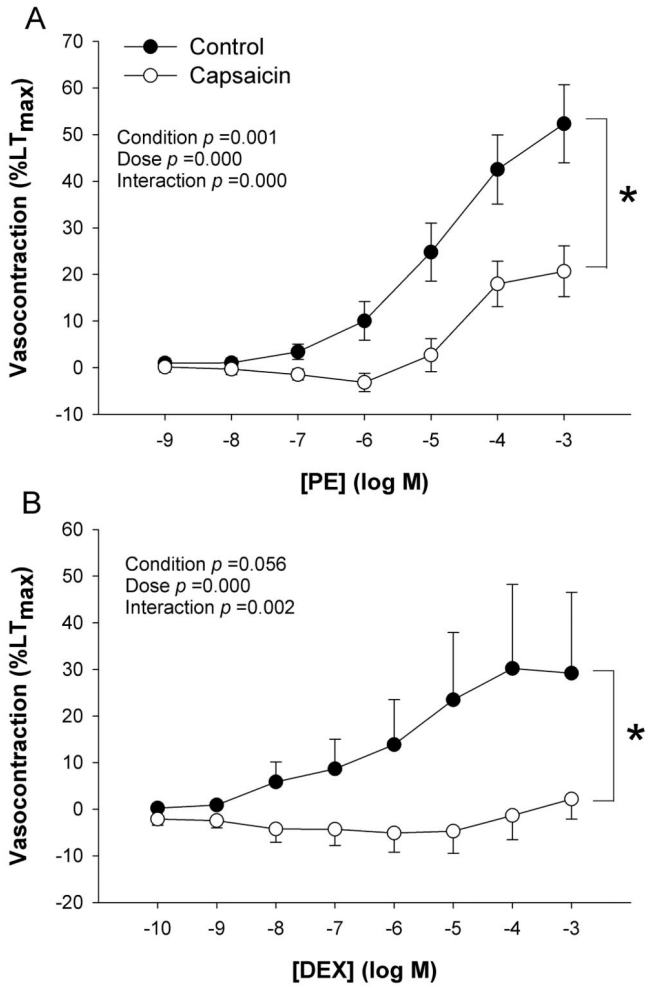

Figure 2. The effect of $TRPV_1$ activation on vasocontraction in human skeletal muscle feed arteries

A) Phenylephrine (PE) concentration response curve (CRC) for α_1-mediated vasocontraction, B) Dexemedetomidine (DEX) CRC for α_2-mediated vasocontraction (n=16). * $p < 0.05$ control vs. capsaicin for maximal responses. Data are presented as mean ± SE.

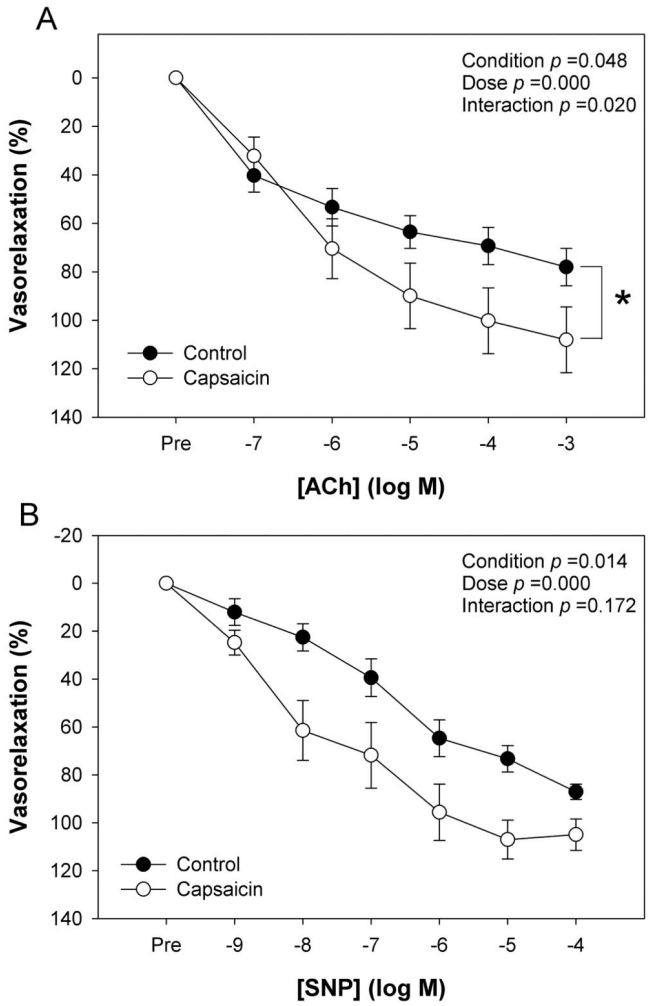

Figure 3. The effect of $TRPV_1$ activation on vasorelaxation in human skeletal muscle feed arteries

A) Acetylcholine (ACh) concentration response curve (CRC) for endothelium dependent vasodilation, B) Sodium Nitroprusside (SNP) CRC for endothelium independent vasodilation, (n=16). * $p < 0.05$ control vs. capsaicin for maximal responses. Data are presented as mean ± SE.

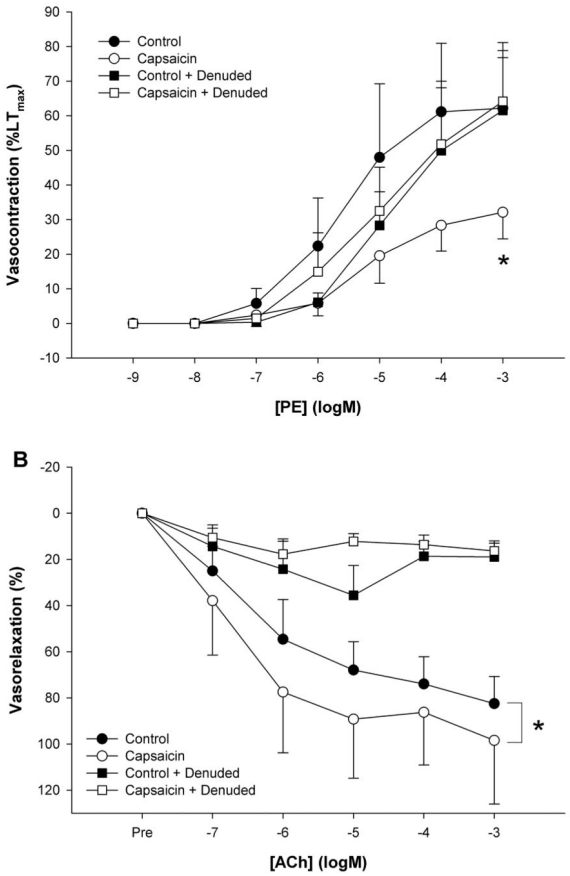

Figure 4. Endothelial contribution to the effect of TRPV$_1$ channel activation on α_1-receptor mediated vasocontraction and vasorelaxation in human skeletal muscle feed arteries

A) Phenylephrine (PE) concentration response curve (CRC) for α_1-receptor mediated vasocontraction. * $p < 0.05$ control vs. capsaicin. B) Acetylcholine (ACh) concentration response curve (CRC) for endothelium dependent vasodilation. * $p < 0.05$ endothelium intact vs. endothelium denuded (n=6). Data are presented as mean ± SE.

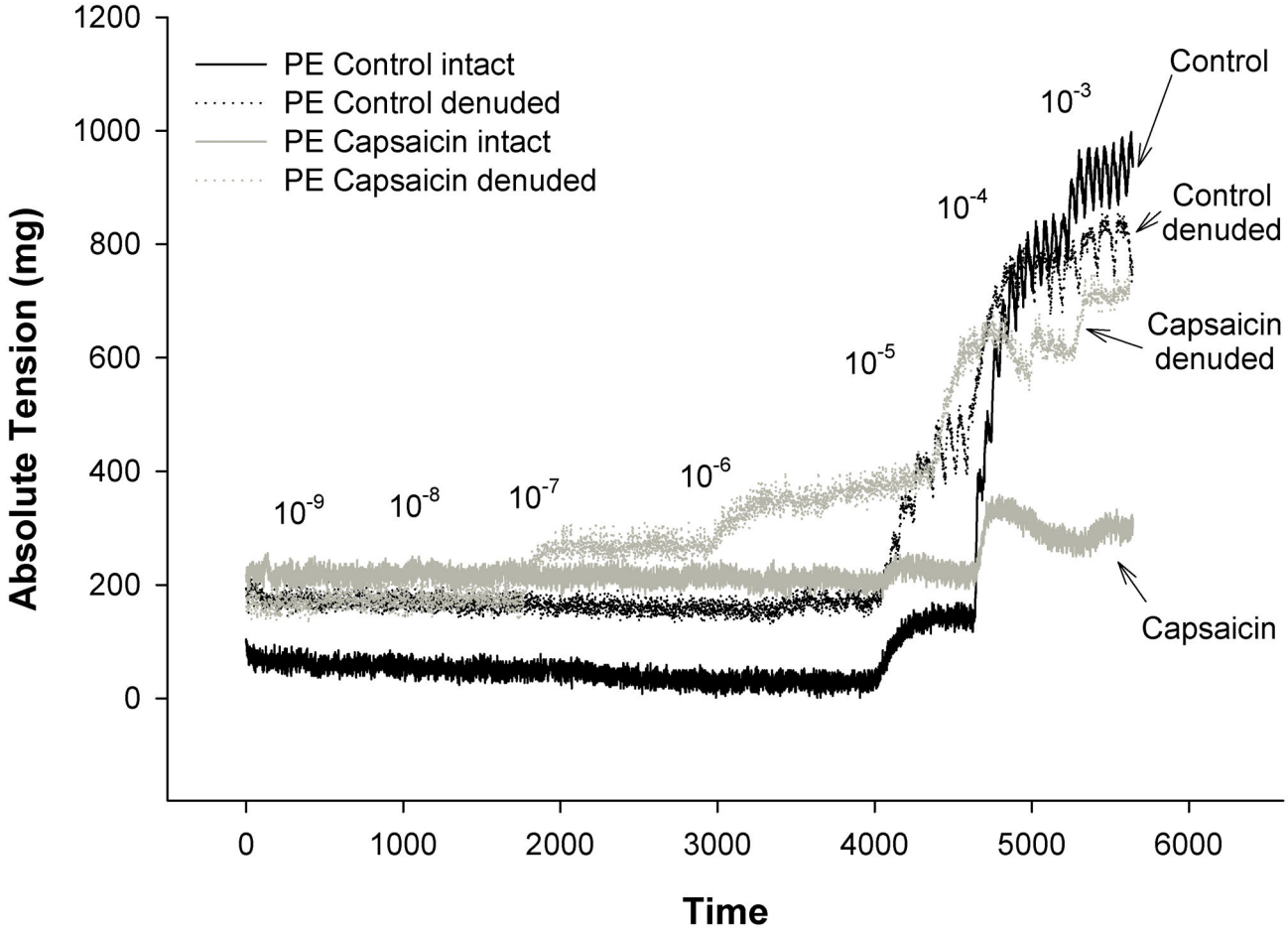

Figure 5.
Original tracings of the impact of Capsaicin and/or endothelial denuding on the vasocontraction response to Phenylephrine in human skeletal muscle feed arteries.

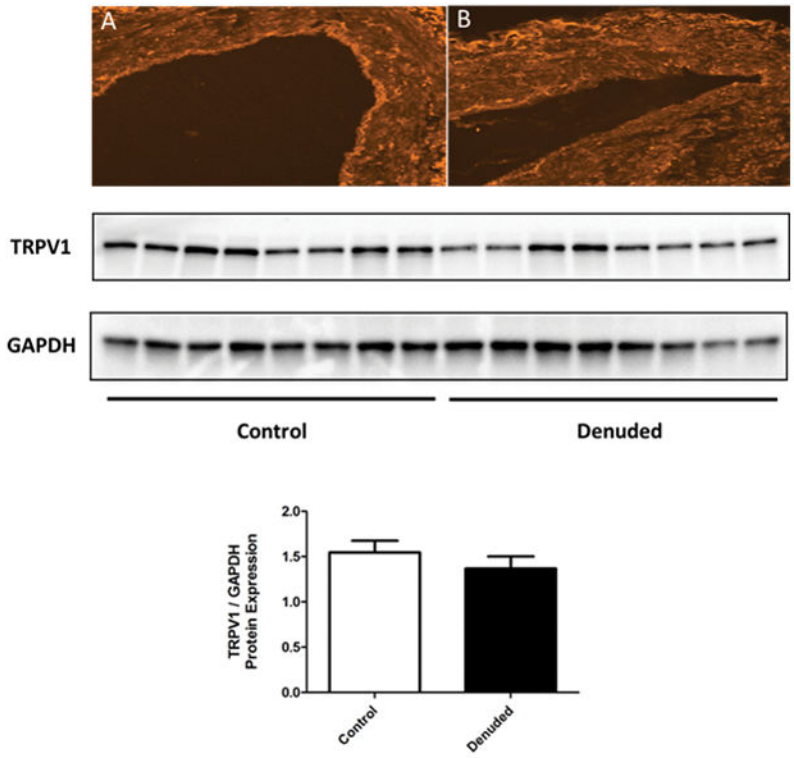

Figure 6. Immunohistochemistry and protein expression of $TRPV_1$ receptors in human skeletal muscle feed arteries (SMFA)

A) $TRPV_1$ receptors (bright signal) evident both in the endothelium and smooth muscle in a control (endothelium intact) SMFA. B) $TRPV_1$ receptors (bright signal) evident only in the smooth muscle in a denuded SMFA. Western blot analysis for $TRPV_1$ protein content in control and denuded SMFAs with GAPDH as a loading control (n=8). Mean ± SE.

Table 1

Subject Characteristics.

	Mean ± SE	Normal Range
Age (yr)	63 ± 5 (41–89)	--
Males/Females (n)	9/7	
Height (cm)	169 ± 9	--
Body Mass (kg)	93 ± 10	--
BMI (kg/m^2)	33 ± 3	< 30
Systolic Blood Pressure (mmHg)	133 ± 5 †	≤ 120
Diastolic Blood Pressure (mmHg)	82 ± 3 †	≤ 80
MAP (mmHg)	99 ± 4	--
Glucose (mg/dl)	100 ± 8	65 – 110
Blood Urea Nitrogen (mg/dl)	16 ± 1	6 – 21
Creatinine (mg/dl)	0.8 ± 0.1	0.52 – 0.99
Albumin (g/dl)	3.9 ± 0.1	3.3 – 4.8
Bilirubin (mg/dl)	0.4 ± 0.1	0.2 – 1.3
Lactate Dehydrogenase (U/L)	458 ± 39	300 – 600
WBC (K/uL)	6.3 ± 1.1	3.2 – 10.6
Platelets (K/uL)	230 ± 18	150 – 400
RBC (M/uL)	4.9 ± 0.1	4 – 5.2
Hemoglobin (g/dl)	14 ± 0.4	12 – 16
Hematocrit (%)	42 ± 0.8	36 – 46
Medications (users/n)		
Cardiovascular		
Statin	6/16	
Ca^{++} Channel Blocker	1/16	
Beta Blocker	1/16	
ACE inhibitor	2/16	
Diuretic	2/16	
Other		
Hypothyroid	2/16	

† Data obtained during pre-operative examination, (n = 16).