Brown Adipose Tissue Regulates Small Artery Function Through NADPH Oxidase 4–Derived Hydrogen Peroxide and Redox-Sensitive Protein Kinase G-1α

Malou Friederich-Persson, Aurelie Nguyen Dinh Cat, Patrik Persson, Augusto C. Montezano, Rhian M. Touyz

Objective—Biomedical interest in brown adipose tissue (BAT) has increased since the discovery of functionally active BAT in adult humans. Although white adipose tissue (WAT) influences vascular function, vascular effects of BAT are elusive. Thus, we investigated the regulatory role and putative vasoprotective effects of BAT, focusing on hydrogen peroxide, nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4), and redox-sensitive signaling.

Approach and Results—Vascular reactivity was assessed in wild-type and Nox4-knockout mice (Nox4−/−) by wire myography in the absence and presence of perivascular adipose tissue of different phenotypes from various adipose depots: (1) mixed WAT/BAT (inguinal adipose tissue) and (2) WAT (epididymal visceral fat) and BAT (intrascapular fat). In wild-type mice, epididymal visceral fat and perivascular adipose tissue increased EC50 to noradrenaline without affecting maximum contraction. BAT increased EC50 and significantly decreased maximum contraction, which were prevented by a hydrogen peroxide scavenger (polyethylene glycated catalase) and a specific cyclic GMP–dependent protein kinase G type-1α inhibitor (DT-3), but not by inhibition of endothelial nitric oxide synthase or guanylate cyclase. BAT induced dimerization of cyclic GMP–dependent protein kinase G type-1α and reduced phosphorylation of myosin light chain phosphatase subunit 1 and myosin light chain 20. BAT from Nox4-knockout mice displayed reduced hydrogen peroxide levels and no anticontractile effects. Perivascular adipose tissue from β3 agonist–treated mice displayed brown perivascular adipose tissue and an increased anticontractile effect.

Conclusions—We identify a novel vasoprotective action of BAT through an anticontractile effect that is mechanistically different to WAT. Specifically, BAT, via Nox4-derived hydrogen peroxide, induces cyclic GMP–dependent protein kinase G type-1α activation, resulting in reduced vascular contractility. BAT may constitute an interesting therapeutic target to restore vascular function and prevent vascular complications in cardiovascular diseases.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:455-465. DOI: 10.1161/ATVBAHA.116.308659.)

Key Words: brown adipose tissue ■ cardiovascular disease ■ catalase ■ contractility ■ myosin light chain ■ perivascular adipose tissue ■ vascular function

Until recently, perivascular adipose tissue (PVAT) was regarded as a structural element responsible for support and mechanical protection of the associated vessel. However, in 1991, Soltis and Cassis demonstrated that PVAT influences vascular function by decreasing contractile responses in rat aorta. PVAT is now widely recognized as a functional regulator of vascular tone through its secretion of relaxing and mechanical protection of the associated vessel. However, in 1991, Soltis and Cassis demonstrated that PVAT influences vascular function by decreasing contractile responses in rat aorta. PVAT is now widely recognized as a functional regulator of vascular tone through its secretion of relaxing and constraining factors acting on the vasculature. Studies have suggested relaxing factors to be nitric oxide (NO), adiponecin, angiotensin 1–7, prostacyclins, hydrogen sulfide, and hydrogen peroxide (H2O2). These factors are counteracted by contracting factors, such as superoxide anion (O2−), tumor necrosis factor-α, and prostanoids. The ultimate regulatory effect on vascular tone is reflected by the balance of PVAT-derived vasconstrictor and vasodilator factors.

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The final determinant of vascular contraction is the phosphorylation status of myosin light chain 20 (MLC20), which is regulated by myosin light chain kinase and myosin light chain phosphatase. Myosin light chain phosphatase activity is inhibited by myosin light chain phosphatase regulatory subunit 1 (MYPT1), and myosin light chain kinase activity is regulated by intracellular calcium (Ca2+) concentration. Counterbalancing vascular contraction is the vasodilatory pathway mediated by cyclic GMP–dependent protein kinase G type 1 (PKG-1). Classically, activation of PKG-1 is mediated via endothelium-dependent increases in cyclic GMP, resulting in phosphorylation of myosin light chain phosphatase activity, which is essential for vasodilator function. Reduced availability of cyclic GMP leads to reduced phosphorylation and increased calcium sensitivity of the contractile apparatus.

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Rho kinase activity and consequent reduced phosphorylation of MYPT1. Together, these pathways converge on decreased phosphorylation of MLCK and, thereby, reduce vascular contraction.\(^4\) Recent studies have also implicated PKG-1 in the anticontractile effects of PVAT.\(^5,1^8\)

Adipose tissue comprises 2 subtypes: white (WAT) and brown (BAT) adipose tissues. WAT is known for its capacity to store lipids and its endocrine role, whereas BAT is characterized by energy expenditure in favor of heat generation (non-shivering thermogenesis). Previously thought only to be present in newborns, BAT has recently been identified in adults.\(^1^9,2^0\) In pathologies such as obesity and hypertension, WAT is characterized by low-grade inflammation and is associated with insulin resistance and vascular dysfunction.\(^2^1,2^2\) These changes are also evident in PVAT, where the anticontractile effect is lost,\(^2^3,2^9,3^0\) resulting in a procontractile phenotype.\(^2^3\) We previously demonstrated, in experimental models of obesity-related type 2 diabetes mellitus, that PVAT-derived factors induce endothelial dysfunction and regulate vascular contractility and remodeling.\(^3^1\) These processes were associated with increased vascular inflammation and vascular smooth muscle cell proliferation.\(^3^2,3^4\)

A major source of both \(O_2^-\) and \(H_2O_2\) are the nicotinamide adenine dinucleotide phosphate oxidases (Nox). Seven isoforms with tissue-specific expression patterns have been identified and characterized, including Nox1, Nox2, Nox3, Nox4, and Nox5 and the dual oxidases 1 and 2.\(^3^5\) Nox4 is the predominant isoform expressed in murine and human adipocytes. In adipose tissue, however, both Nox2 and Nox4 are expressed at the mRNA level.\(^3^6,3^8\) Nox4 produces mainly \(H_2O_2\) whereas the other Nox isoforms produces \(O_2^-\). This difference has pathophysiological relevance where \(H_2O_2\)-producing Nox4 activity has been shown to have protective effects on the vasculature, whereas \(O_2^-\)-producing Nox isoforms in many studies are shown to be detrimental.\(^4^1,4^2\) While PVAT of resistance vessels is phenotypically characterized as WAT, the PVAT of the aorta is of a BAT phenotype.\(^3^7\) Periaortie BAT has anticontractile\(^4^1,2^6,4^2\) and anti-inflammatory properties in obesity and may, thus, have vasoprotective effects.\(^4^3\)

In the present study, we examined the role of BAT on vascular function of small resistant arteries, and we elucidated some of the underlying molecular mechanisms whereby BAT influences endothelial function and vascular contractility. Our study focuses on small resistance arteries, important in blood pressure regulation and development of hypertension. Arteries were exposed to adipose tissue from different adipose depots: epididymal visceral adipose tissue (E VAT), which is phenotypically WAT, inguinal subcutaneous adipose tissue (iWAT) and mesenteric PVAT comprising both WAT and BAT, and interscapular adipose tissue, which is solely BAT.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Phenotyping of Adipose Tissue From Different Depots

iWAT, EVAT, and mesenteric PVAT are generally characterized as WAT. However, because WAT can convert into a brown-like phenotype, called beige adipose tissue (BeAT), a small amount of BeAT may be present in adipose tissue classically considered as WAT. To confirm the nature of the adipose tissue that we studied, we investigated the adipose phenotype by analyzing mRNA levels of expression of leptin and transcription factor 21 as markers for WAT, zinc finger of the cerebellum and purinergic receptor P2X5 as specific markers for BAT, and transmembrane protein-26, Cd-137, and T-box protein 1 as specific markers for BeAT. Uncoupling protein-1 and transcription factor Pat2 are joint markers for both BAT and BeAT. These markers have been previously investigated and evaluated in the phenotyping of adipose tissue.\(^3^5,3^6\)

Interscapular BAT displayed high expression of brown/beige markers uncoupling protein-1 and Pat2, as well as the specific markers zinc finger of the cerebellum and purinergic receptor P2X5 (Figure I in the online-only Data Supplement). iWAT expressed brown/beige marker uncoupling protein-1 and Pat2 (Figure I in the online-only Data Supplement), as well as the specific markers for BeAT transmembrane protein-26, T-box protein 1, and CD-137 (Figure II in the online-only Data Supplement). EVAT displayed only white markers (Figure III in the online-only Data Supplement). PVAT expressed WAT-specific marker transcription factor 21, as well as BeAT-specific marker T-box protein 1 and Bat/BeAT marker Pat2 (Figures II and III in the online-only Data Supplement). Hence, in our study, we used the paradigm that iWAT and PVAT display a mixed adipose phenotype, whereas EVAT is primarily WAT.

#### BAT Exerts an Anticontractile Effect

Contractile responses of mouse mesenteric arteries were evaluated using wire myography and showed that the presence of iWAT did not affect vascular contraction in response to increasing doses of noradrenaline (Figure 1A). The presence of mesenteric PVAT or EVAT increased the sensitivity (\(EC_{50}\)) to noradrenaline but did not affect maximum contraction to noradrenaline (Figure 1B). However, the presence of

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BeAT</td>
<td>beige adipose tissue</td>
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<tr>
<td>BK_{Ca}</td>
<td>big conductance calcium-sensitive potassium channel</td>
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<tr>
<td>EVAT</td>
<td>epididymal visceral adipose tissue</td>
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<tr>
<td>H_2O_2</td>
<td>hydrogen peroxide</td>
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<tr>
<td>iWAT</td>
<td>inguinal subcutaneous adipose tissue</td>
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<tr>
<td>MLC</td>
<td>myosin light chain</td>
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<tr>
<td>MYPT1</td>
<td>myosin light chain phosphatase subunit 1</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nox</td>
<td>NADPH oxidase</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycolated</td>
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<tr>
<td>PKG-1</td>
<td>cyclic GMP dependent protein kinase G type-1</td>
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<tr>
<td>PVAT</td>
<td>perivascular adipose tissue</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<td>WAT</td>
<td>white adipose tissue</td>
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BAT markedly reduced contraction to noradrenaline, phenylephrine, or serotonin, demonstrating a potent anticontractile effect (Figure 1C; Figure IV A and IVB in the online-only Data Supplement). The anticontractile effect of BAT was independent of sex and background strain (Figure IVC and IVD in the online-only Data Supplement).

**H2O2 Is Among the Anticontractile Factors Produced by BAT**

A media transfer approach was used to investigate whether the anticontractile effect of BAT required its immediate localization to the vessel or if the effect was mediated by a secreted factor. Transfer of media preconditioned with BAT displayed a similar anticontractile effect as BAT directly surrounding the vessel (Figure 1D), indicating the presence of a diffusible factor, secreted by BAT and acting in a paracrine manner. The presence of polyethylene glycated catalase (PEG catalase), which scavenges H2O2 prevented the anticontractile effect of BAT (Figure 2A). Blockade of voltage-gated potassium channels by 4-aminopyridine or XE-991, angiotensin 1–7 (Mas) receptors by A779, and production of hydrogen sulfide by β-cyanoalanine did not influence the anticontractile effect of BAT (Figure V in the online-only Data Supplement). Also, endothelial denudation and blockade of production of prostacyclin by indomethacin, did not affect the anticontractile effect of BAT (Figure VIA and VIB in the online-only Data Supplement).

**BAT Does Not Influence Vascular Relaxation**

As demonstrated in Figure VII in the online-only Data Supplement, endothelium-dependent vascular relaxation in response to acetylcholine and endothelium-independent relaxation in response to sodium nitroprusside were not affected by BAT.

**Nox4 Plays a Role in BAT-Secreted H2O2**

As shown in Figure 2, Nox4 mRNA levels were increased in BAT and iWAT versus iWAT, but not Nox1 or Nox2. Protein expression of Nox4 was increased only in BAT (Figure VIIID in the online-only Data Supplement). Because Nox4 is a constitutively active producer of H2O2, we further explored the possibility that Nox4 may be important in vascular tone regulation by BAT-secreted H2O2, by studying mice deficient for Nox4 gene (Nox4−/− mice). As shown in Figure 2, H2O2 levels in all investigated adipose depots were reduced in Nox4 −/− mice (Figure 2B). BAT from Nox4−/− did not exert an anticontractile effect on arteries from wild-type mice (Figure 2C), while BAT from wild-type mice exerted an anticontractile effect on arteries from Nox4−/− mice (Figure 2D), demonstrating that the presence of Nox4 in BAT is crucial to induce an anticontractile effect, whereas Nox4 localization in the vessel does not participate in the anticontractile effect of BAT.

Because H2O2 may also be sourced from scavenging of O2·− by superoxide dismutases (SOD), we investigated the expression levels of other Nox enzymes that preferentially produce O2·− and levels of Sod and catalase (scavenger of H2O2) in BAT. Nox1 mRNA levels were similar in all adipose depots (Figure VIII A in the online-only Data Supplement), and BAT from mice deleted for Nox1 gene exhibited an anticontractile effect that was not different from wild-type BAT (Figure X in the online-only Data Supplement). Nox2 mRNA was lowest in BAT (Figure VIIIIB in the online-only Data Supplement).
Sod-1 and Sod-2 mRNA levels were increased in BAT, and catalase mRNA levels were similar in all depots (Figure X in the online-only Data Supplement). Therefore, although a contribution to total BAT H2O2 content from dismutation of O2·− by SOD1 to 2 cannot be excluded, the O2·−-producing enzymes Nox1 and 2 do not seem to be major sources of BAT-derived H2O2.

**BAT Influences Vascular Function Through H2O2-Induced Activation of PKG-1**

To investigate molecular mechanisms involved in BAT-induced anticontractility, we focused on PKG-1, which is activated by reactive oxygen species, such as H2O2, in vascular smooth muscle cells and which has been shown to attenuate vasoconstriction and promote vasodilation.47 The anticontractile effect of BAT was abolished in the presence of a potent and selective inhibitor of PKG-1α (DT-3; Figure 3A). Similar effects were obtained after selective blockade of BK Ca channels by iberiotoxin (Figures 3B). The anticontractile effect was also abolished after PKG inhibitor Rp-8-Br-PET-cyclic GMP and general blockade of Ca2+-sensitive potassium channels by tetraethylammonium (Figure 3C and 3D in the online-only Data Supplement). However, the effect of BAT was similar after blockade of NO synthesis by Nω-nitro-l-arginine methyl ester hydrochloride (Figure 3C) or of cyclic GMP production by ODQ (Figure 3D), suggesting that the classical NO-dependent pathway was not involved in mechanisms underlying H2O2-induced activation of PKG-1. Previous studies demonstrated that PKG-1 is activated by oxidants, which create a disulphide bond in the enzymatic homodimer.48 We found that BAT from wild-type mice increased dimerization of PKG-1α but BAT from Nox4−/− mice did not (Figure 4A). Furthermore, dithiothreitol, a reducing agent, abolished the anticontractile effect of BAT (Figure XI in the online-only Data Supplement).

**BAT Reduces Activation of MYPT1 and MLC20**

To further confirm the anticontractile effect of BAT, we investigated effects of BAT on the activations of MYPT1 and MLC20, downstream proteins ultimately responsible for smooth muscle contraction. As shown in Figure 4, incubation of vascular tissue with BAT resulted in significantly reduced phosphorylation of vascular MYPT1 and MLC20 (Figure 4B and 4C).

**Differential Mechanisms Underlie the Anticontractile Effects of VAT and BAT**

We further investigated whether the molecular pathway identified for BAT was also present in mesenteric PVAT. The anticontractile effect of mesenteric PVAT was inhibited by 4-aminopyridine but not by PEG-catalase (Figure XIV in the online-only Data Supplement). However, 4-aminopyridine did not alter the anticontractile effect of BAT (Figure XIII in the online-only Data Supplement).
not prevent the anticontractile effect of BAT (Figure VIA in the online-only Data Supplement), indicating that the anticontractile effects of BAT and mesenteric PVAT are mechanistically different.

**Browning of PVAT Increases the Anticontractile Effect Through H$_2$O$_2$-Dependent Mechanisms**

To elucidate whether the changing of PVAT phenotype into a BAT/BeAT phenotype affects the anticontractile effect, we treated mice with a β$_3$ agonist, CL-316,243, a well-established method to induce browning in mice. Indeed, β$_3$ treatment increased BAT/BeAT markers in PVAT (Figure XV in the online-only Data Supplement), and PVAT from these animals displayed an increased anticontractile effect (Figure 5A). The increased anticontractile effect was reduced but not gone after blockade of H$_2$O$_2$ with PEG-catalase and PKG-1α by DT-3 (Figure 5B and 5C), indicating that the increased anticontractility after browning of resident PVAT is similar in mechanism to that of interscapular BAT.

**Discussion**

Our study demonstrates that interscapular BAT has anticontractile properties that may be vasoprotective. We elucidated putative novel molecular mechanisms underlying this process and showed that H$_2$O$_2$ released from BAT activates vascular PKG-1α through oxidant-induced dimerization. PKG-1α subsequently phosphorylates BK$_{Ca}$ channels and reduces phosphorylation of MYPT1 and MLC$_{20}$, with pathways ultimately converging to attenuated vascular contraction. These phenomena are BAT-specific because they did not occur in WAT. Importantly, increased browning of PVAT enhances the anticontractile effect, and this increase was shown to be mediated through H$_2$O$_2$ and PKG-1α-dependent mechanisms, demonstrating that increasing the amount of resident BAT can affect vascular function on small resistance vessels. Our study concludes that BAT induces a potent anticontractile effect in small resistant arteries through Nox4-dependent mechanism, in particular H$_2$O$_2$-induced activation of PKG-1α-dependent signaling.

PVAT of thoracic aorta is primarily a BAT phenotype and has been shown to exert an anticontractile effect, in part through H$_2$O$_2$. Gao et al reported that effects of aortic PVAT were prevented by catalase and mimicked by H$_2$O$_2$. This study identified 2 separate pathways of aortic PVAT, an unknown factor inducing endothelium-dependent relaxation and that of H$_2$O$_2$ as the endothelium-independent mechanism. It may be suggested that the presence of 2 mechanisms might reflect the combined effect of both BAT and WAT in aortic PVAT.

Mammalian target of rapamycin complex 2 may be partly responsible for aortic PVAT function. In animals lacking rapamycin-insensitive companion of mTOR and, thus, mammalian target of rapamycin complex 2, aortic PVAT anticontractility was impaired, possibly mediated through increased levels of inflammatory cytokines tumor necrosis factor-α and interleukin-6. The observed loss of PVAT function was still evident in denuded arteries, supporting an endothelial-independent mechanism for aortic brown PVAT, which is in accordance with the present study where endothelial denudation did not eliminate the anticontractile effect of BAT.
The central player in our described mechanism, H$_2$O$_2$, has been the focus of intense research, and its role in the vasculature has been extensively reviewed. $^{52,53}$ In the vasculature, H$_2$O$_2$ has been suggested to have both detrimental $^{54}$ and protective effects. $^{41}$ These conflicting results may be because of differences in subcellular localization of H$_2$O$_2$ and other reactive oxygen species and may relate in part to different cell types, preferentially producing H$_2$O$_2$. $^{55,56}$ In the present study, H$_2$O$_2$ mediates a protective role, activating PKG-1$\alpha$, ultimately resulting in reduced vascular contraction. H$_2$O$_2$ can originate from the dismutation of O$_2$$^•$− by SOD but also from a dedicated H$_2$O$_2$ producer: Nox4. Because of its vascular localization, $^{57}$ constitutively active function, and production of H$_2$O$_2$, Nox4 is generally regarded as a vasoprotective Nox. Indeed, it is highly expressed in BAT and pivotal to the anticontractive effect we observed because BAT from Nox4$^{-/-}$ mice failed to exhibit a vascular effect. However, BAT from wild-type mice mediated an anticontractile effect on vessels from Nox4$^{-/-}$ mice, suggesting that the perivascular production of H$_2$O$_2$ is critical for the anticontractile effect of BAT, rather than H$_2$O$_2$ produced locally in smooth muscle cells in the vascular media. Also, the need for a threshold level of H$_2$O$_2$ is evident because the H$_2$O$_2$ levels were reduced but not abolished in adipose tissue from Nox4$^{-/-}$ mice, despite the anticontractile effect being abrogated. Importantly, we have previously shown that Nox4 is expressed in human adipocytes. $^{58}$

In the present study, vessels devoid of PVAT from Nox4$^{-/-}$ mice displayed increased contraction, and BAT from these mice lacked an anticontractile effect. However, Nox4$^{-/-}$ mice display similar blood pressure as wild-type mice under basal

**Figure 4.** A, Dimerization of PKG-1$\alpha$ is induced by BAT from wild-type mice but not by BAT from Nox4$^{-/-}$ mice. Phosphorylation of MYPT1 (B) and MLC$_{20}$ (C) is decreased by BAT from wild-type mice (WT) but not with BAT from Nox4$^{-/-}$ mice. Values are expressed as means±SD. n=4 to 6 animals per group. One-way ANOVA, followed by Bonferroni multiple comparison test. * denotes P<0.05 vs clean. ANOVA indicates analysis of variance; BAT, brown adipose tissue; DTT, dithiothreitol; H$_2$O$_2$, hydrogen peroxide; MLC$_{20}$, myosin light chain 20; MYPT1, myosin light chain phosphatase regulatory subunit 1; Nox4, NADPH oxidase 4; and PKG, cyclic GMP–dependent protein kinase G.
conditions and after angiotensin II infusion, and basal blood pressure is reduced only after endothelial overexpression of Nox4. Taken together, this suggests that Nox4 may not be a critical regulator of blood pressure under basal conditions but may be important for blood pressure regulation in conditions where it is increased.

Whether BAT-derived H₂O₂ mediates an anticontractile effect directly or is determined by downstream signaling through PKG-1α remains unclear. Importantly, H₂O₂ is known to activate PKG-1α by oxidizing cysteine residues, creating a disulfide bond between the PKG-1α homodimers. Subsequently, there is a conformational change that exposes the phosphorylation site and downstream signaling occurs. Eaton et al demonstrated that mice that do not activate PKG-1α in this manner become hypertensive under normal conditions, demonstrating the importance of the H₂O₂-PKG-1α signaling axis in regulating normal blood pressure. In our study, BAT induced dimerization of PKG-1α, but when dimerization was prevented by dithiothreitol, the anticontractile effect was abolished. Interestingly, blockade of H₂O₂ to PKG-1α signaling by dithiothreitol revealed a procontractile effect of BAT, suggesting that when the beneficial part of H₂O₂-dependent signaling is removed, remaining signaling or other procontractile factors such as O₂⁻ can exert a vascular contractile action.

In our study, blockade of PKG-1α itself or its downstream target, BKCa channels, attenuated the anticontractile effect of BAT. This is in accordance with previous studies implicating the BKCa channels as mediators of the anticontractile effect of adipose tissue. Importantly, Withers et al found the anticontractile effect of PVAT to be absent in mice lacking PKG-1.

Our study highlights the fact that various adipose depots have anticontractile properties but that underlying molecular mechanisms may differ. Previous studies of mesenteric...
PVAT reported the involvement of voltage-gated potassium channels, in some cases further specifying it to Kv7 K⁺ channels. In the present study, the effect of mesenteric PVAT was inhibited by a voltage-gated potassium channel blocker, but the anticontractile effect of BAT was not affected. Similarly, scavenging of H₂O₂ removed the anticontractile effect of BAT but had no effect on mesenteric PVAT. Additionally, inhibition of mechanisms previously shown to be involved in vasoprotective effects of PVAT, such as hydrogen sulfide, NO, prostacyclins, and angiotensin 1–7 had no effect on the anticontractile effect of BAT. Taken together, these findings suggest that the anticontractile processes of mesenteric PVAT and BAT involve distinct signaling pathways. However, it should also be highlighted that mesenteric PVAT could be mediated by the small degree of BAT or BeAT within the depots commonly characterized as white. Originating from white adipocytes, inactive beige adipocytes localized within WAT can be mobilized into BeAT by exposure to cold or stimulation of β₃ receptors, resulting in a process called browning. Importantly, BeAT is morphologically and functionally similar to classical brown adipocytes.

Our phenotyping revealed that although EVAT was characterized as WAT, both PVAT and iWAT displayed BeAT markers and were, thus, characterized as a mix of WAT and BeAT. Interestingly, iWAT did not display an anticontractile effect, and the anticontractile effect of PVAT was not affected by PEG catalase. Either BeAT does not behave in a similar manner to BAT in terms of vascular effect or a critical level
of BAT or BeAT needs to be present before the anticontractile effect is functional. To investigate if an increased amount of BeAT may also exert an anticontractile effect, we induced browning through a β3 agonist. In these mice, PVAT displayed an increased anticontractile effect compared with PVAT from controls, an effect that was sensitive to both scavenging of H2O2 and blockade of PKG-1α, suggesting that the mechanisms of BAT and BeAT may indeed be similar if a critical threshold of BeAT is reached. BAT did not affect vascular relaxation and may relate to the fact that the NO synthase–NO pathway was not influenced by BAT.

From a pathophysiological viewpoint, our findings may be important because several clinical and experimental studies have reported the loss of the PVAT anticontractile effect in obesity, type 2 diabetes mellitus,27–29 and hypertension.30–33 Patients after bariatric surgery displayed improved insulin sensitivity, reduced blood pressure, and a restored anticontractile effect of PVAT.34 Interestingly, BAT surrounding the aorta has been reported to be resistant to obesity-induced inflammation, suggesting that BAT may help to protect the vasculature during pathological conditions. Furthermore, transplantation of BAT into the visceral cavity corrects the metabolic phenotype of rodents with diabetes mellitus and metabolic syndrome, and transplantation of BAT has been suggested to be of future importance in metabolic diseases.35–37 Based on the novel results of our study, BAT clearly demonstrates a redox-sensitive vasoprotective effect, and as such exposing small arteries to BAT, or increased browning of PVAT, may be an interesting strategy to improve vascular function in pathological conditions.

In conclusion, Nox4 in BAT produces H2O2, leading to oxidant-induced activation of PKG-1α and its downstream pathway. This pathway ultimately converges to influence signaling that decreases phosphorylation of MYPT1 and MLC20, a pathway. This pathway ultimately converges to influence signaling that decreases phosphorylation of MYPT1 and MLC20, a protective effect, and as such exposing small arteries to BAT, or increased browning of PVAT, may be an interesting strategy to improve vascular function in pathological conditions.

Acknowledgments

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Disclosures

None.

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References


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**Highlights**

- **Vascular dysfunction is a common feature in hypertension, diabetes mellitus, and obesity, and perivascular adipose tissue has emerged as an important regulator of vascular function.**

- The present study demonstrates that brown adipose tissue can modulate the function of small resistance vessels. This anticontractile effect is higher than the one induced by perivascular adipose tissue in physiological conditions.

- The mechanisms identified include nicotinamide adenine dinucleotide phosphate oxidase 4–derived H2O2 from brown adipose tissue, which activates cyclic GMP–dependent protein kinase G type-1α and its downstream pathways, resulting in reduced vascular contraction.

- By clearly elucidating the mechanisms of how brown adipose tissue influences vascular tone, our findings provide a rationale to target brown adipose tissue for new therapeutic strategies aiming to preserve vascular function and to prevent vascular complications in cardiovascular diseases such as diabetes mellitus, obesity, and hypertension.
Brown Adipose Tissue Regulates Small Artery Function Through NADPH Oxidase 4–Derived Hydrogen Peroxide and Redox-Sensitive Protein Kinase G-1α

Malou Friederich-Perssson, Aurelie Nguyen Dinh Cat, Patrik Persson, Augusto C. Montezano and Rhian M. Touyz

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BROWN ADIPOSE TISSUE

Nox4

H₂O₂

PKG-1α
oxidant activation

MLC₂₀(P)

Reduced vascular contraction
SUPPLEMENTAL MATERIAL

Brown adipose tissue regulates small artery function through NADPH oxidase 4-derived hydrogen peroxide and redox-sensitive protein kinase G-1α.

Friederich-Persson M, Nguyen Dinh Cat A, Persson P, Montezano AC, Touyz RM.
Institute of Cardiovascular Medicine and Sciences, University of Glasgow, Glasgow, United Kingdom.

Wire myography
All animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local animal ethics committee. Mesenteric vessels (2nd order, <190 μm diameter) from male and female mice of FVb, C57bl6 and mixed background were cut into 2 mm segments and mounted on 25 μm wires in a wire myograph (Danish Myotechnology, AD Instruments Ltd, Oxford, United Kingdom) and bubbled at 37°C in physiological saline (PSS, in mmol/L: 119.0 NaCl, 4.7 KCl, 1.2 MgSO4, 24.9 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, 11.1 glucose, pH 7.4). Vessels were normalized as described previously. The internal circumference, L100, corresponding to a transmural pressure of 100 mm Hg for a relaxed vessel in situ was calculated and vessels set to the internal circumference L1, given by L1=L1/π and was between 150 and 190 μm. Normalization was followed by 1 h resting period and wake-up protocol consisting of two additions with high potassium PSS (KPSS, containing 120 mM K), one addition of 1x10^{-5} M noradrenaline (NA) and 1x10^{-5} M acetylcholine (ACh) after preconstriction with 3x10^{-6} M NA to check that endothelium was intact.

Contraction responses to cumulative doses of NA (1x10^{-9} to 1x10^{-4.5} M), phenylephrine (Phe 1x10^{-9} to 1x10^{-4} M), serotonine (5-hydroxytryptamine (5-HT), 1x10^{-9} to 1x10^{-8} M) were evaluated. Endothelium-independent and dependent vasorelaxation were evaluated by sodium nitroprusside (SNP; 1x10^{-9} to 3x10^{-5} M) and acetylcholine (ACh; 1x10^{-9} to 1x10^{-4.5} M) respectively after preconstriction to 3x10^{-6} M NA in order to achieve approximately 60 to 80% of maximal response. In some vessels, endothelium was mechanically denuded using a 40 μm wire and loss of endothelium was determined as <30% relaxation to ACh. Vessels were evaluated as clean vessels devoid of any fat and after addition of 2-3 mg of mesenteric perivascular adipose tissue (PVAT), epididymal visceral adipose tissue (EVAT), inguinal subcutaneous adipose tissue (iWAT) or interscapular brown adipose tissue (BAT) to surround the vessel. For curves with adipose tissue-conditioned media, adipose tissue was incubated in PSS in a separate bath bubbled at 37°C, 30 minutes prior the concentration response curves and the solution minus adipose tissue added to the vessel. The amount of adipose tissue added to the vessels was kept constant and corresponding to a healthy amount of PVAT. Genetically modified mice lacking nicotinamide adenine dinucleotide phosphatase (NAPDH) oxidase (Nox) 1 or 4 was investigated as indicated throughout the figures. Effects of adipose tissue was
evaluated in combination with drugs: NG-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor 100 µmol/L), polyethylene glyced catalase (PEG-catalase, scavenger of H₂O₂, 300 U/mL), 4- aminopyridine (4-AP, blocker of voltage-dependent K-channels, 1 mmol/L), Xe-991 (10 µmol/L, selective blocker of KNCQ5-channels) indomethacin (non-selective inhibitor of cyclooxigenases, 10 µmol/L), A779 (inhibitor of Mas-receptor, 10 µmol/L), β-cyano-l-alanine (inhibitor of H₂S-producing enzyme cystathione γ-lyase, 1 mmol/L), DT-3 (selective PKG-1α inhibitor, 1 µmol/L), Rp-8-Br-PET-cGMP (3 µmol/L) iberiotoxin (blocker of large-conductance calcium-activated potassium (BK)-channels, 100 nmol/L), tetraethylammonium (TEA, blocker of calcium-activated potassium channels, 1 mmol/L), ODQ (selective inhibitor of soluble guanylyl cyclase, 10 µmol/L), PKI 5-24 (protein kinase A inhibitor, 5 µmol/L), KT-5720 (protein kinase A-inhibitor, 200 nmol/L) and DTT (general reducing agent, 1 mmol/L).

Treatment with β3-agonist

A separate cohort of male C57/Bl6J mice was treated with the β3-agonist CL-316,243, 1 mg/kg bw/day by osmotic minipumps (Alzet, Cupertino, USA) for 7 days, and vascular function evaluated by wire myography as described above. Browning of PVAT was confirmed by mRNA expression of BAT and BeAT markers.

Western blotting

PKG-1α dimerization was evaluated as described previously. Briefly, isolated mesenteric arteries were homogenized in 100 mM Tris-Hcl pH 6.8 with protease inhibitors leupeptin, pepstatin and aprotinin (1 mg/mL) and 100 mM maleimide. Protein lysates were diluted in non-reducing sample buffer (containing in mM: 100 maleimide, 2% sodium dodecyl sulphate, 10% glycerol, 0.005% bromophenol blue, pH 6.8) and analysed using western blotting technique (Bio-Rad, Hertfordshire, UK). Protein lysates were separated on 12% Tris-HCl gels under non-reducing conditions, transferred to nitrocellulose membrane. Blocking for 1 h at room temperature with 5% non-fat dried milk in Tris-buffered saline (TBS) + 0.1% Tween-20 (TBST) was followed by incubation with primary antibody for PKG-1α (goat-anti PKG1α, Santa Cruz #sc13030, 1:1000 in 5 % bovine serum albumin (BSA) overnight, 4°C, monomer appears at 75 kDa, dimer at 150 kDa). Densitometric analysis of bands were corrected for β-actin (mouse-anti β-actin, 1:10 000, Sigma-Aldrich) and % dimerization calculated as intensity at 150 kDa/(75 +150 kDa)*100. DTT (1 mM) and H₂O₂ (100 µM) was used as controls for PKG-1α dimer and monomer respectively. Horseradish peroxidase conjugated secondary antibodies was used at 1:2000 diluted in 5% non-fat dry milk for 1 h at room temperature (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA).

Phosphorylation of myosin light chain 20 (p-MLC20, Cell Signalling #3674, 1:1000) and myosin phosphatase target subunit 1 (p-MYPT1, Santa Cruz #sc-17556, 1:1000) was analysed in a separate set of mesenteric vessels homogenized in lysis buffer (in mmol/L: sodium pyrophosphate 50, NaF 50, NaCl 50, EDTA 5, EGTA 5, HEPES 10, Na₃VO₄ 2, phenylmethylsulfonyl fluoride (PMSF) 50, Triton 100 0.5% and leupeptin/aprotinin/pepstatin 1 mg/mL), diluted in reducing sample buffer and analysed under reducing conditions. Primary antibodies were incubated overnight at 4°C and HRP-conjugated secondary antibodies were incubated with membranes for
1 hour at room temperature and signal developed using chemiluminescence and visualized by autoradiography followed by densitometric analysis using Image Studio Lite 5.2.

Nox4 protein was determined in a separate set of EVAT, iWAT, mesenteric PVAT and BAT homogenized in lysis buffer (in mmol/L: 10 NaF, 80 Tris, pH 7.5, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing enzyme inhibitors (Phosphatase inhibitor cocktail-2; 10 µl/ml, and Complete Mini; 1 tablet/1.5 ml; Roche Diagnostics, Mannheim, Germany). Protein lysates were separated on 12.5% Tris-HCl gels (Criterion Precast system, Bio-Rad) under reducing conditions, transferred to nitrocellulose membrane, blocked 1 h at room temperature with 5% non-fat dry milk in TBST, followed by primary antibody against Nox 4 (Anti-NADPH oxidase 4 antibody, Abcam #133303, 1:1000 in 5% BSA, 4°C, overnight). Total protein staining by Ponceau was used as a loading control as β-actin showed significant variations between different depots. This is a well-known problem with loading controls for western blotting and total staining for protein has been highlighted as a good option for this issue.3

qPCR
Quantitative real-time PCR (Applied Biosystems, Carlsbad, CA, USA) was used to analyse mRNA expression. In brief, total RNA was isolated with the TRizol lysis approach. After DNAse-treatment (0.1 U/ml, 1 hour, 37°C) 1 µg RNA underwent reverse transcription using High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). For real-time PCR, 3 µl cDNA were mixed with 5 µl SYBR Green PCR master mix and 300 nmol/L of primers in a total volume of 10 µl. The gene of interest was corrected for the average of Importin 8 (IPO8), F-box and leucine-rich repeat protein-10 (FBX10) and ubiquitin C (UBC). The relative copies number of the target genes was calculated with the -ΔΔCt method, after assessment that PCR efficiency was 100%. Results were reported in relative quantification (RQ) as $2^{(-\Delta\Delta Ct)}$. Primer sequences were designed using Primer BLAST (NCBI) and are shown in Table 1. Analysis of Cd-137, leptin, purinergic receptor P2X (P2RX), transcription factor Pat2, T-box protein 1 (Tbx1), transcription factor 21 (tcf21), transmembrane protein 26 (Tmem-26), uncoupling protein-1 (Ucp-1), and zinc finger of the cerebellum (Zic1) was used to investigate adipose tissue phenotype in terms of brown, beige and white phenotype. These markers have been previously investigated and validated by others.4,5

Amplex red
The fluorescence assay Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes) was used to evaluate levels of H$_2$O$_2$ in AT according to the manufacturer's instructions. Levels were corrected for protein content, measured by Protein DC-assay (BioRad, Hertfordshire, UK), and expressed as µmol H$_2$O$_2$/g protein.

Statistical analysis
Dose-response curves from wire myography studies were fitted using non-linear regression and analysed for half maximal effective concentration (EC$_{50}$) and maximal contraction (MC). Parameters were analysed using 2-way analysis of variance
(ANOVA) with Dunnett’s posthoc test or 2-tailed Student’s t-test as appropriate. p<0.05 was considered statistic and all values are presented as mean±SD.

Supplemental references

1. MJ M. Procedures for investigating small vessels using wire myograph. DMT Danish Myo Technology. 2004
SUPPLEMENTAL MATERIAL

Brown adipose tissue regulates small artery function through NADPH oxidase 4-derived hydrogen peroxide and redox-sensitive protein kinase G-1α.

Friederich-Persson M, Nguyen Dinh Cat A, Persson P, Montezano AC, Touyz RM. Institute of Cardiovascular Medicine and Sciences, University of Glasgow, Glasgow, United Kingdom.

Supplementary table

Table 1. Mouse primer sequences for real time PCR.

<table>
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<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 3’-5’</th>
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<td>mSod1</td>
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CD137: tumor necrosis factor receptor superfamily member 9; FBX10: F-box and leucine-rich repeat protein-10; Ipo8: importin 8; m: mouse; Nox: NADPH oxidase;
Ubc: ubiquitin C; Ucp: uncoupling protein; P2RX5: purinergic Receptor P2X 5; Sod: superoxide dismutase; Tbx1: T-box protein 1; tcf21: transcription factor 21; Tmem-26: transmembrane protein 26; Zic1: Zinc finger of the cerebellum.

Supplemental figures and legends

Supplemental Figure I. A) Joint BAT and BeAT marker Ucp-1 is expressed in BAT and iWAT but at low levels in PVAT and EVAT. Specific BAT marker Zic1 (B) and P2RX5 (C) is expressed in solely BAT. D) Joint BAT and BeAT marker Pat2 is expressed in iWAT, PVAT and BAT. Values are expressed as means±SD. N=6-8 animals per group. One-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. EVAT, BAT: brown adipose tissue; BeAT: beige adipose tissue; EVAT: epididymal visceral adipose tissue; iWAT: inguinal subcutaneous white adipose tissue; P2RX5: purinergic receptor 2X; PVAT: perivascular adipose tissue; Ucp-1: uncoupling protein 1; WAT: white adipose tissue; Zic: zinc finger of the cerebellum.
Supplemental Figure II. Specific BeAT markers \textit{Tmem-26} (A) and \textit{CD137} (B) are expressed in BAT and iWAT but at low levels in PVAT and EVAT. C) Specific BeAT marker \textit{Tbx1} expressed in iWAT and PVAT. Values are expressed as means±SD. N=6-8 animals per group. One-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. EVAT, BAT: brown adipose tissue; BeAT: beige adipose tissue; EVAT: epididymal visceral adipose tissue; iWAT: inguinal subcutaneous white adipose tissue; PVAT: perivascular adipose tissue; Tbx: T-box protein; Tmem: transmembrane; WAT: white adipose tissue.
Supplemental Figure III. (A) WAT marker *leptin* is expressed in EVAT. (B) Specific WAT marker *Tcf21* is expressed in EVAT and PVAT. Values are expressed as means±SD. N=6-8 animals per group. One-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. EVAT, BAT: brown adipose tissue; EVAT: epididymal visceral adipose tissue; iWAT: inguinal subcutaneous white adipose tissue; PVAT: perivascular adipose tissue; Tcf: transcription factor; WAT: white adipose tissue.
Supplemental Figure IV. The anticontractile effect of BAT is evident in response to both Phe (A, 2by2 interaction: ns) and 5-HT (B, 2by2 interaction: ns). The anticontractile effect of BAT is similar regardless of gender (C, 2by2 interaction: ns) and strain (D (2by2 interaction: ns). Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs clean vessels. 5-HT: 5-hydroxytryptamine; BAT: brown adipose tissue; NA: noradrenaline; KPSS: potassium-rich physiological saline solution; Phe: phenylephrine.
Supplemental Figure V. The anticontractile effect of BAT is not affected by voltage-gated K-channels by 4-AP blockade (A, 2by2 interaction: p<0.05) or XE-991 (B, 2by2 interaction: p<0.05), angiotensin 1-7 receptor blockade by A779 (C, 2by2 interaction: ns) or blockage of γ-cystathione lyase (producer of H₂S) inhibition by β-cyanoalanine (β-cyano) (D, 2by2 interaction: ns). Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. clean vessels. 4-AP: 4-aminopyridine; β-cyano: β-cyanoalanine; BAT: brown adipose tissue; H₂S: hydrogen sulphide; KPSS: potassium-rich physiological saline solution; NA: noradrenaline.
Supplemental Figure VI. The anticontractile effect of BAT is not affected by removal of the endothelium (A, 2by2 interaction: ns), indomethacin (B, 2by2 interaction: ns). The anticontractile effect was blocked by PKG-inhibitor Rp-8-Br-PET-cGMP (C, 2by2 interaction: p<0.05) and calcium-sensitive potassium channel blocker TEA (D, 2by2 interaction: p<0.05). Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. clean vessels. BAT: brown adipose tissue; KPSS: potassium-rich physiological saline solution; NA: noradrenaline; PKG: protein kinase G; TEA: tetraethylammonium.
Supplemental Figure VII. A) BAT does not affect endothelium-dependent relaxation in response to ACh. B) BAT does not affect endothelium-independent relaxation in response to SNP. Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Bar graphs show level of preconstriction in response to NA. ACh: acetylcholine; BAT: brown adipose tissue; KPSS: potassium-rich physiological saline solution; NA: noradrenaline; SNP: sodium nitroprusside.
Supplemental Figure VIII. Figure 2. A) Nox1 expression does not vary between adipose depots. B) Nox2 expression is decreased in BAT. C) Nox4 is increased in iWAT and BAT. D) Nox 4 protein level is increased in BAT. Values are expressed as means ± SD. A-C: n=8 animals per group, D: n=3-7 per group. One-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. EVAT. BAT: brown adipose tissue; EVAT: epididymal visceral adipose tissue; iWAT: inguinal white adipose tissue; PVAT: perivascular adipose tissue.
Supplemental Figure IX. BAT from Nox1⁻/⁻ mice exerts a similar anticontractile effect as BAT from wildtype mice. Values are expressed as means ± SD. In brackets are indicated the number of animals per group. One-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. clean vessels. BAT: brown adipose tissue; KPSS: potassium-rich physiological saline solution; NA: noradrenaline; Nox: NADPH oxidase.
Supplemental Figure X. BAT displayed increased mRNA levels of Sod1 (A) and Sod2 (B). Catalase mRNA levels were not changed between adipose depots (C). Values are expressed as means ± SD. n=8 animals per group. One-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs EVAT. BAT: brown adipose tissue; EVAT: epididymal visceral adipose tissue; iWAT: inguinal subcutaneous white adipose tissue; PVAT: perivascular adipose tissue; Sod: superoxide dismutase.
Supplemental Figure XI. DTT prevents the anticontractile effect of BAT. Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. clean vessels, 2by2 interaction: p<0.05. BAT: brown adipose tissue; DTT: dithiotreitol; KPSS: potassium-rich physiological saline solution; NA: noradrenaline.
Supplemental Figure XII. Using phenylephrine as the contractile agent, the anticontractile effect of BAT is removed by PEG-catalase (A, 2by2 interaction: p<0.05) and IBTX (B, 2by2 interaction: p<0.05) but not by ODQ (C, 2by2 interaction: ns). Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs. clean vessels. BAT: brown adipose tissue; IBTX: iberiotoxin; KPSS: potassium-rich physiological saline solution; PEG: polyethylene glycol; Phe: phenylephrine.
Supplemental Figure XIII. Protein kinase A inhibition by PKI 5-24 (A, 2by2 interaction: ns) or KT-5720 (B, 2by2 interaction: p<0.05) does not affect the anticontractile effect of BAT. Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, * P<0.05 vs clean vessels. BAT: brown adipose tissue; KPSS: potassium-rich physiological saline solution; NA: noradrenaline.
Supplemental Figure XIV. The anticontractile effect of mesenteric PVAT is prevented after blockade of Kv-channels (A, 2by2 interaction: *p<0.05) and not affected by H₂O₂ scavenging by PEG-catalase (B, 2by2 interaction: ns). Values are expressed as means ± SD. In brackets are indicated the number of animals per group. Repeated measured two-way ANOVA, followed by Bonferroni multiple comparison test, *P<0.05 vs. clean. 4-AP: 4-aminopyridine; BAT: brown adipose tissue; KPSS: potassium-rich physiological saline solution; Kv-channel: voltage gated potassium-channel; PEG: polyethylene glycated; PVAT: perivascular adipose tissue.
Supplemental Figure XV. Mesenteric PVAT obtained from mice treated with β₃-agonist Cl-316,243 display increased of joint BAT and BeAT marker Ucp1 (A) as well as specific BeAT markers tmem-26 (B), Tbx1 (B) and CD137 (D). Values are displayed as mean ± SD, N=5-7 per group. * P<0.05 vs PVAT, Student’s t-test. BAT: brown adipose tissue; BeAT: beige adipose tissue; PVAT: perivascular adipose tissue; tbx: T-box protein; tmem: transmembrane protein; ucp: uncoupling protein.