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Adipocyte-derived factors impair insulin signaling in differentiated human vascular smooth muscle cells via the upregulation of miR-143

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ABSTRACT

Cardiovascular complications are common in patients with type 2 diabetes. Adipokines have been implicated in the induction of proliferative and pro-atherogenic alterations in human vascular smooth muscle cells (hVSMC). Other reports demonstrated the importance of the miRNA cluster miR-143/145 in the regulation of VSMC homeostasis and insulin sensitivity. Here we investigated whether the detrimental effects of adipokines on hVSMC function could be ascribed to alterations in miR-143/145 expression. The exposure of hVSMC to conditioned media (CM) from primary human subcutaneous adipocytes increased the expression of smooth muscle α-actin (SMA), and the miR-143/145 cluster, but markedly impaired the insulin-mediated phosphorylation of Akt and its substrate endothelial nitric oxide synthase (eNOS). Furthermore, CM promoted the phosphorylation of SMAD2 and p38, which have both been linked to miR-143/145 induction. Accordingly, the induction of miR-143/145 as well as the inhibition of insulin-mediated Akt- and eNOS-phosphorylation was prevented when hVSMC were treated with pharmacological inhibitors for Alk-4/5/7 and p38 before the addition of CM. The transfection of hVSMC with precursor miR-143, but not with precursor miR-145, resulted in impaired insulin-mediated phosphorylation of Akt and eNOS. This inhibition of insulin signaling by CM and miR-143 is associated with a reduction in the expression of the oxysterol-binding protein-related protein 8 (ORP8). Finally, the knock-down of ORP8 resulted in impaired insulin-mediated phosphorylation of Akt in hVSMC. Thus, the detrimental effects of adipocyte-derived conditioned media on insulin action in primary hVSMC can be ascribed to the Alk- and p38-dependent induction of miR-143 and subsequent downregulation of ORP8.

1. Introduction

Cardiovascular complications are common in patients with type 2 diabetes and a major cause of mortality [12]. Accumulating evidence shows that adipose tissue secreted factors, termed adipokines, may participate in the development of cardiovascular complications in patients with type 2 diabetes by affecting the function of cardiomyocytes and smooth muscle cells (SMC) [11,20,29].

Vascular endothelial cells and SMC represent the major cell types of the artery wall preserving vessel wall homeostasis. SMC are highly plastic and modulate their phenotype in response to physiological and pathological cues. Differentiated SMC are quiescent and contractile. In response to vascular injury or growth factor signaling, SMC de-differentiate and adopt a proliferative, migratory phenotype that contributes to vascular occlusion in a variety of disorders, including atherosclerosis [27]. Alterations in vascular insulin signaling may also participate in the development of cardiovascular dysfunction in type 2 diabetes. The insulin-mediated activation of phosphatidylinositol 3′-kinase (PI3K) results in the activation of Akt, which on its turn promotes the phosphorylation of endothelial nitric oxide synthase (eNOS) [24]. This results in activation of eNOS and an increase in bioavailable nitric oxide (NO) thereby promoting vasodilation [24]. In the vasculature of animal models of insulin resistance, such as obese Zucker (fa/fa) rats, the insulin-mediated activation of the PI3K-pathway is impaired [16]. Intriguingly, this is paralleled by an overactivation of the mitogen activated protein kinase (MAPK) pathway, which on its turn promotes proliferation of vascular cells [16,24]. Studies on mouse models which lack the insulin receptor in either the endothelium or the entire vasculature have further highlighted the importance of vascular insulin action, and specifically the Akt/eNOS-axis, for the regulation of vasorelaxation in vivo [9,30].

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Previously we showed that conditioned media (CM) prepared from human adipocytes induce pro-atherogenic changes in primary human vascular smooth muscle cells (hVSMC) as illustrated by an increase in proliferation and migration, thus indicating an involvement of adipokines in the development of atherosclerosis [20,29]. However, until now the underlying mechanisms, which are responsible for hVSMC dysfunction induced by adipokines remain incompletely understood. In cardiomyocytes, we observed that conditioned media generated from epicardial adipose tissue from patients with type 2 diabetes induce cardiomyocyte dysfunction as illustrated by contractile dysfunction and insulin resistance [11]. Furthermore, we found that the induction of insulin resistance in cardiomyocytes could be ascribed to the increases in miR-143 expression [1]. Also in other tissues, such as the liver and adipose tissue, the induction of miR-143 is associated with obesity and insulin resistance [18,31]. Interestingly, miR-143 and the co-transcribed miR-145 are highly expressed in the smooth muscle cell lineage [2]. Studies on miR143/145-deficient mice showed that this cluster is required for the acquisition of the contractile phenotype [2]. This is established through the activation of a transcriptional network, which promotes the differentiation of smooth muscle cells [8]. Among the genes targeted by the miR-143/145-cluster are the smooth muscle differentiation markers transgelin (TAGLN, also known as SM22), and smooth muscle α-actin (SMA) [8]. These observations suggest a dual role for the miR-143/145 in vascular smooth muscle cells. Therefore the aim of this study is to examine the role of the miR-143/145-cluster in primary human vascular smooth muscle cells (hVSMC) in more detail. This was achieved by studying the effects of CM on the expression of the miR-143/145-cluster and its potential target genes, and whether these effects associate with alterations in insulin action.

2. Material and methods

2.1. Cell culture and differentiation of smooth muscle cells

Primary human coronary artery smooth muscle cells (hVSMC) from two different donors (Caucasian, female, 55 and 56 years old) were purchased from tebu-bio (Offenbach, Germany) and Lonza (Basel, Switzerland). The hVSMC were supplied as proliferating cells and cultured according to the manufacturers' instructions. For experiments, differentiation was induced culturing the subconfluent cells of passage 3 for 14 days in smooth muscle cell differentiation medium (tebu-bio, Offenbach, Germany). The transition from the undifferentiated to the differentiated phenotype was judged on the basis of increases in the protein abundance of smooth muscle cell differentiation markers, such as SMA, the transforming growth factor β (TGFβ) receptor II, and the bone morphogenic protein receptor II [8,13], as well as the lack of proliferation. Following differentiation, hVSMC were incubated for 24 h in adipocyte-derived conditioned media or maintained in SMC serum-free basal medium (PromoCell, Heidelberg Germany). When indicated cells were treated for 1 h with 10 μM SB431542 (Sigma Aldrich, St. Louis, MO) or 2.5 μM SB203580 (Promega, Mannheim, Germany) prior to the addition of the media. For insulin signaling, the cultures were stimulated for 10 min with 100 nM insulin.

2.2. Adipocyte isolation, culture and generating of conditioned media

Conditioned media (CM) were generated from mature subcutaneous adipocytes that were differentiated from pre-adipocytes isolated from subcutaneous adipose tissue obtained from lean or moderately overweight women (n = 13, body mass index 28.1 ± 1.3, and aged 39.0 ± 3.9 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). All patients were healthy, free of medication and had no evidence of metabolic diseases according to routine laboratory tests. Pre-adipocytes were isolated by collagenase digestion of adipose tissue as described [14]. Isolated pre-adipocytes were resuspended in Dulbecco's modified Eagles/Hams F12 (DMEM/F12) medium supplemented with 10% FCS, seeded in 75 cm^2 culture flasks and maintained at 37 °C with 5% CO2. After overnight incubation, cultures were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 μmol/l biotin, 17 μmol/l D-pantothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 μg/ml apo-transferrin, 50 μg/μl gentamycin, 15 mmol/l HEPES, 14 nmol/l $NaHCO_3$, pH 7.4)

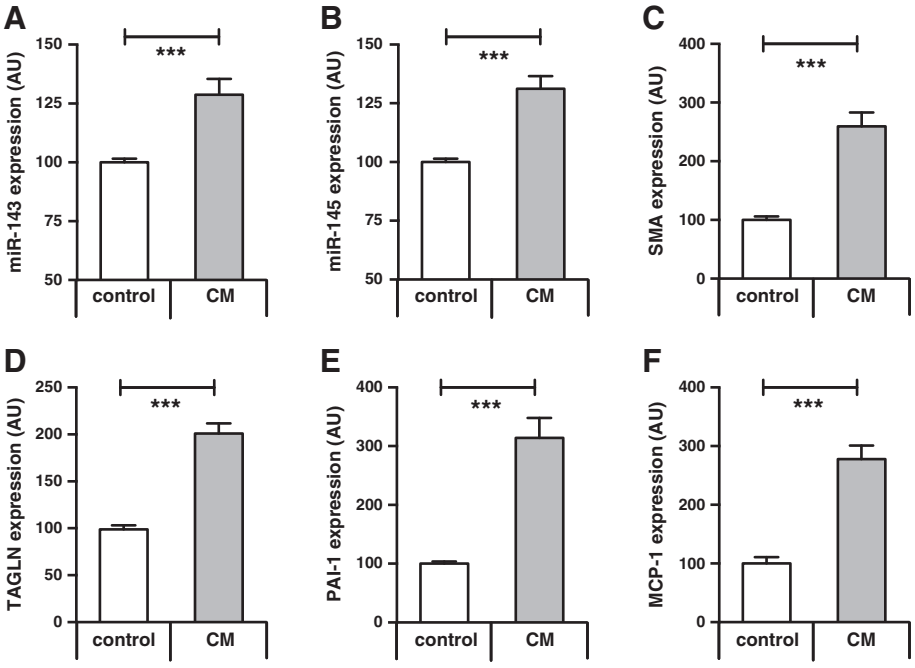

Fig. 1. Effect of conditioned media on miRNA and gene expression in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24 h and levels of miR-143 (A), miR-145 (B), smooth muscle actin (SMA) (C), transgelin (TAGLN) (D), PAI-1 (E), and MCP-1 (F) were quantified by real-time PCR. Data were collected in 16 independent experiments using 2 different donors and CM from 8 different preparations, and are expressed as mean ± standard error of the mean. Differences between the groups were evaluated using student's *t*-test. ***, indicates $P < 0.001$ versus control medium.

for 15 days with medium change every 2–3 days and addition of 5 μM troglitazone for the first 3 days. For the collection of CM, differentiated adipocytes were maintained for 48 h in SMC serum free basal medium (PromoCell) with the addition of 50 ng/ml fungizone and 50 μg/ml gentamycin. Then CM was collected and stored as aliquots at −80 °C until further use.

2.3. Analysis of protein expression

For analysis of protein expression and phosphorylation, hVSMC were lysed in 50 mM HEPES, pH 7.4, 1% TritonX100 supplemented with protease and phosphatase inhibitor cocktails (Complete, PhosStop; Roche Diagnostics, Mannheim, Germany). After incubation for 2 h at 4 °C on a rotation shaker, the suspension was centrifuged at 10,000 *g* for 15 min, and protein content was determined using Bradford reagent (Biorad Laboratories, Munich, Germany). Thereafter, five microgram of protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany) in a semi-dry blotting apparatus [36]. Membranes were blocked with Tris-buffered saline containing 0.1% Tween and 5% non-fat dry milk and subsequently incubated overnight at 4 °C with primary antibodies for Akt, phospho-Akt-Ser473, phospho-Akt-Thr308, phospho-proline-rich Akt substrate of 40-kDa (PRAS40) Thr246, phospho-p38-Thr180/Tyr182, phospho-SMAD2-Ser465/467 (all from Cell Signaling Technology, Danvers, MA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), oxysterol-binding protein-related protein 8 (ORP-8) (abcam Cambridge, UK), smooth muscle α-actin (Sigma Aldrich), α-tubulin (Calbiochem Merck Biosciences, Schwalbach, Germany) insulin receptor β-subunit (IRβ), and phospho-eNOS-Ser1177 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, membranes were incubated with corresponding secondary HRP-coupled antibody (Promega). Bound conjugate was detected using enhanced chemiluminescence using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and quantitated on a Versadoc work station (VersaDoc 4000 MP; BioRad, Munich, Germany) using Quantity One software (BioRad, version 4.6.7).

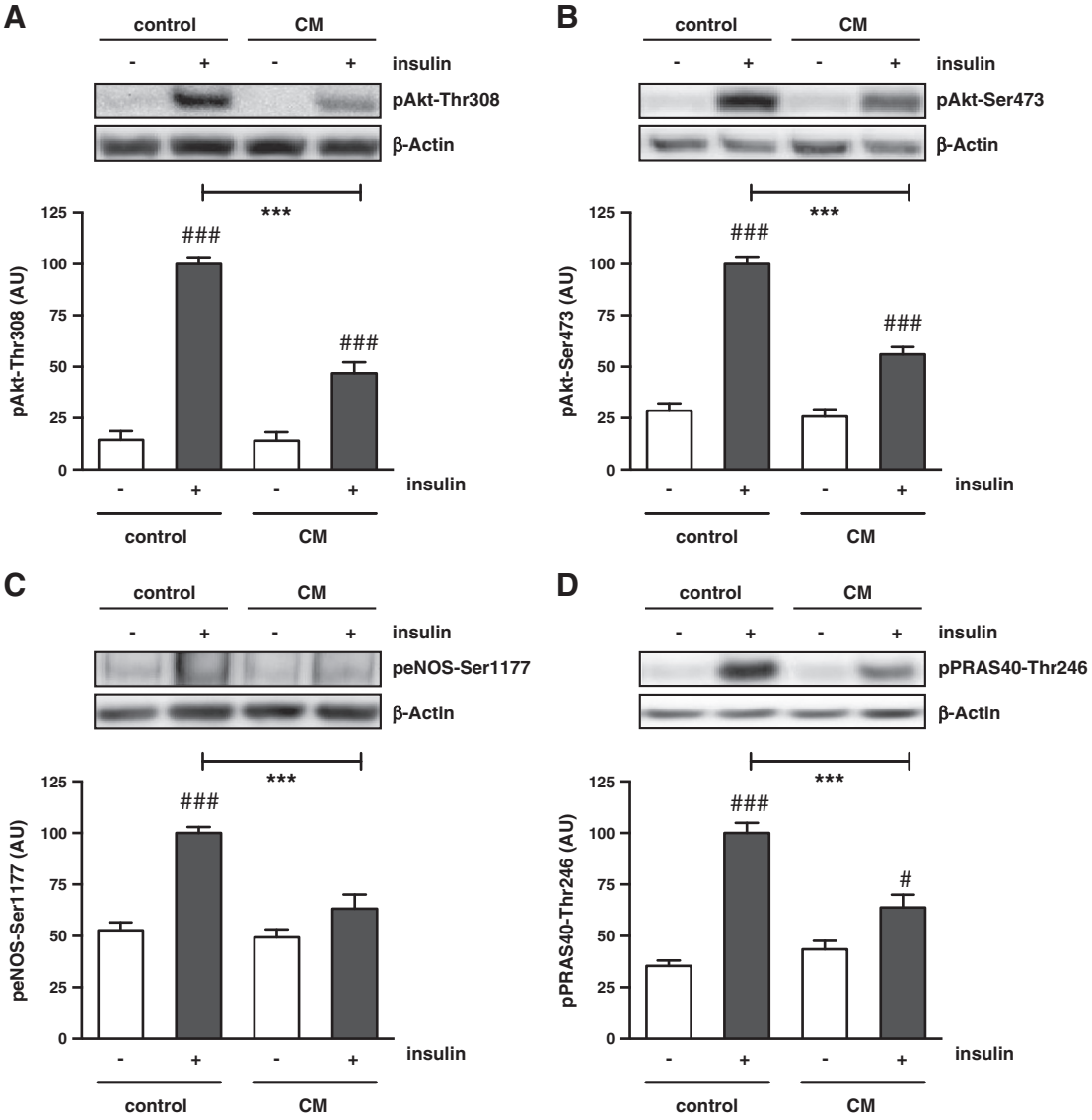

Fig. 2. Effect of conditioned media on insulin action in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24 h and kept untreated (−) or stimulated for 10 min with 100 nM insulin (+). Shown are representative Western blots and quantifications for the phosphorylation levels of Akt-Thr308 (A), Akt-Ser-473 (B), eNOS-Ser1177 (C), and PRAS40-Thr246 (D). Phosphorylation levels normalized for β-actin are expressed as mean ± standard error of the mean of at least 8 independent experiments using cells from 2 different donors and CM from 8 different preparations. Differences among the experimental conditions were evaluated using two-way ANOVA. ###, and # indicate $P < 0.001$ and $P < 0.05$, respectively, for the effect of insulin (+) versus cells kept untreated (−); *** indicates $P < 0.001$ for the effect of CM versus control medium.

2.4. RNA isolation, cDNA synthesis and qRT-PCR

For analysis of miRNA- and mRNA expression, total RNA was extracted using a miRNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using the miScript RT kit (Qiagen). Then miRNA expression levels were determined with miScript Primer Assays (Qiagen) using miScript SYBR Green (Qiagen) on a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Gene expression levels were determined after cDNA synthesis with the GoScript™ Reverse Transcription System (Promega) and GoTaq® qPCR Master Mix (Promega) using QuantiTect Primer Assays (Qiagen) for the amplification of SMA, TAGLN, PAI-1, MCP-1, activin A, and POLR2A. To amplify RPS28 the following primers were designed using the Primerblast-tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and ordered from Eurogentec (Seraing, Belgium): forward-5′-GGTCTGTCACAGTCTGCTCC-3′, and reverse-5′-CATCTCAGTTACGTGTGGCG-3′. Real-time PCR data were analyzed qualitatively with StepOne Plus software (version 2.1; Applied Biosystems) and quantitated using Qbase+ software (version 2.6; Biogazelle, Zwijnaarde, Belgium) in which the Ct-values obtained for RPS28 and POLR2A were used for normalization. The expression of RPS28 and POL2RA was not impacted by the experimental conditions tested as assessed with the GeNorm algorithm within the Qbase+ software [35].

2.5. Transfection of hVSMC with miRNA-precursor

To investigate the impact of differentially regulated miRNAs and their potential targets on insulin action, differentiated hVSMC were transfected in 6-well dishes with 30 nmol/l Cy3™-labeled pre-miR™ (negative control) or pre-miR™ miRNA-precursor (Ambion, Life Technologies, Darmstadt, Germany) using Hiperfect (Qiagen) as transfection reagent. 48 h after transfection in SMC serum free basal medium (PromoCell) cells are stimulated with insulin and lysed for protein isolation and western blot analysis.

2.6. Lentiviral vector-based silencing of ORP8

To silence ORP8, three validated MISSION® shRNA constructs (TRCN000014 -6765, -7289 -7487) targeting human ORP8 (NM_020841) (Sigma Aldrich) or empty vector were used to produce infectious virus particles (LV). Therefore, HEK293t were transfected with the shRNA constructs together with helper plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope as described previously [4]. For quantification of virus yield in the harvested medium, p24 antigen levels were determined using a HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corp., New York, NY, USA). hVSMC are transduced with a MOI of 2 for 24 h. Two days after transduction of hVSMC in serum free basal medium (PromoCell), cells were stimulated with 100 nM insulin for 10 min or kept untreated, and harvested.

2.7. Statistical analysis

Data are presented as means ± standard error of the mean. Significant differences between experimental conditions were evaluated as described in the legends to the tables and the figures using GraphPad

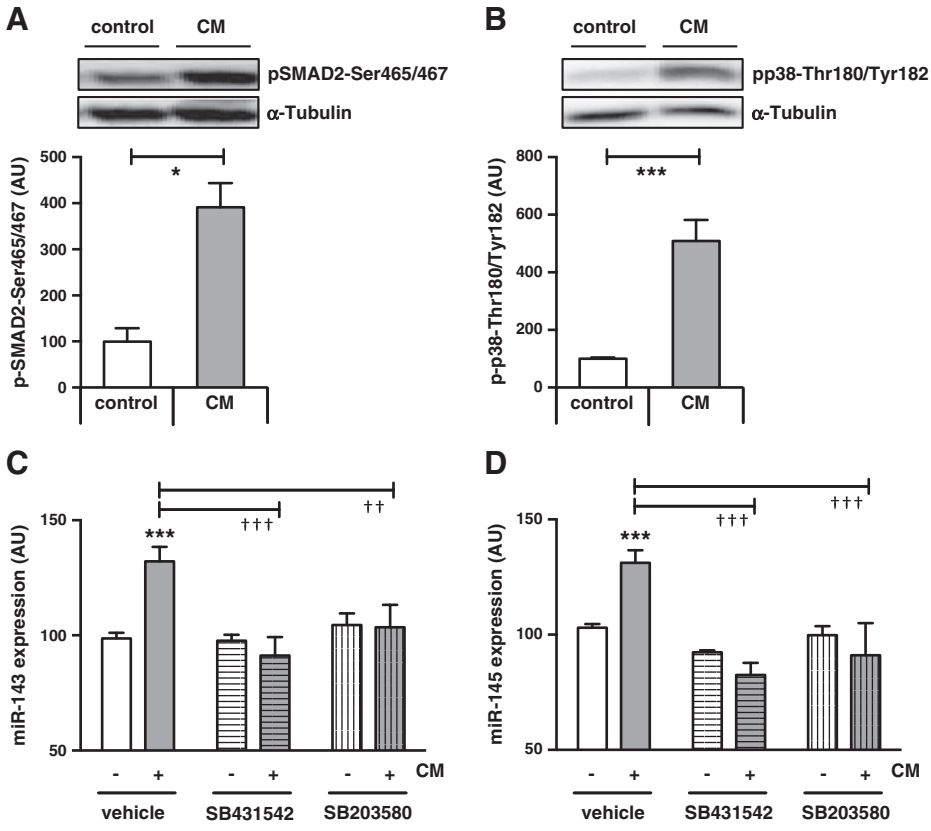

Fig. 3. Involvement of TGFβ receptor- and p38-signaling in the induction of the miR-143/145 cluster in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24 h. Shown are representative Western blots and quantifications for the phosphorylation levels of SMAD2-Ser465/467 (A), and p38-Thr180/Tyr182 (B). Phosphorylation levels normalized for α-tubulin are expressed as mean ± standard error of the mean of 14 independent experiments using cells from 2 distinct donors and CM from 7 different preparations. Differences between the groups were evaluated using student's *t*-test. ***, indicates $P < 0.001$; *, $P < 0.05$ versus control medium. C/D. Cells were exposed to DMSO (vehicle), or pharmacological inhibitors for Alk4/5/7 (SB431542), or p38 (SB203580) for 1 h prior to the addition of control medium (−) or CM (+), whereafter expression of miR-143 (C), and miR-145 (D) was determined via real-time PCR. Data are expressed as mean ± standard error of the mean of least 8 independent experiments using cells from 2 different donors and CM from 8 different preparations. Differences among the groups were analyzed using two-way ANOVA and Bonferroni multiple comparison analysis. ***, indicates $P < 0.001$ for CM versus control medium; †††, $P < 0.001$; ††, $P < 0.01$ for CM only versus inhibitors.

Prism 6 for Mac OS X (GraphPad, LA Jolla, CA) software. *P*-values of <0.05 were considered as statistically significant.

3. Results

3.1. Effects of adipocyte-derived factors on primary human vascular smooth muscle cells

The exposure of hVSMC to CM increased the expression of the miR-143/145 cluster by 1.3-fold ($P < 0.001$) as compared to cells kept in control medium (Fig. 1 A/B). Furthermore, CM increased the mRNA levels of the miR-143/145 regulated targets SMA and TAGLN by 2.6-fold and 2.0-fold respectively, and of the pro-inflammatory cytokines PAI-1 and MCP-1 by 3.1- and 2.8-fold, respectively (all $P < 0.001$) (Fig. 1 C/F). Exposing hVSMC to CM inhibited the insulin-mediated phosphorylation of Akt on Thr308 and Ser473, and its substrates eNOS on Ser1177, and PRAS40 on Thr246 by 53%, 44%, 37%, and 36% respectively versus cells kept in control medium (all $P < 0.001$) (Fig. 2). Incubation with CM did not affect the basal phosphorylation levels of these proteins (Fig. 2).

3.2. Effect of inhibition of TGFβ receptor- and p38-signaling on the CM-induced expression of miR-143/145 and inhibition of insulin action in hVSMC

A previous report ascribed the induction of the miR-143/145 cluster in hVSMC to the activation of the p38-signaling pathway by TGFβ [22]. Fig. 3A/B shows that the phosphorylation of SMAD2, a component of the TGFβ receptor signaling pathway, as well as the phosphorylation of p38 were increased by 3.9- and 5.1-fold, respectively in hVSMC exposed to CM versus cells kept in control medium. To examine whether the TGFβ receptor- and p38-signaling pathway(s) participate in the induction of the miR-143/145 cluster, pharmacological inhibitors were used. Pretreating the hVSMC with either SB431542, which inhibits the TGFβ type I receptors 'Activin Receptor-Like Kinase' (ALK) 4, 5 and -7, or with the p38 inhibitor SB203580, completely abolished the induction the miR-143/145-cluster by CM in hVSMC (Fig. 3C/D). Furthermore, the inhibition of insulin-induced phosphorylation of Akt-Thr308, Akt-Ser473, eNOS-Ser1177, and PRAS40-Thr246 by CM was reversed in hVSMC incubated with either SB431542 or SB203580 before exposure to CM (Fig. 4). Both inhibitors also blunted the induction of TAGLN in hVSMC by CM (supplementary Fig. 1). However, in contrast to the

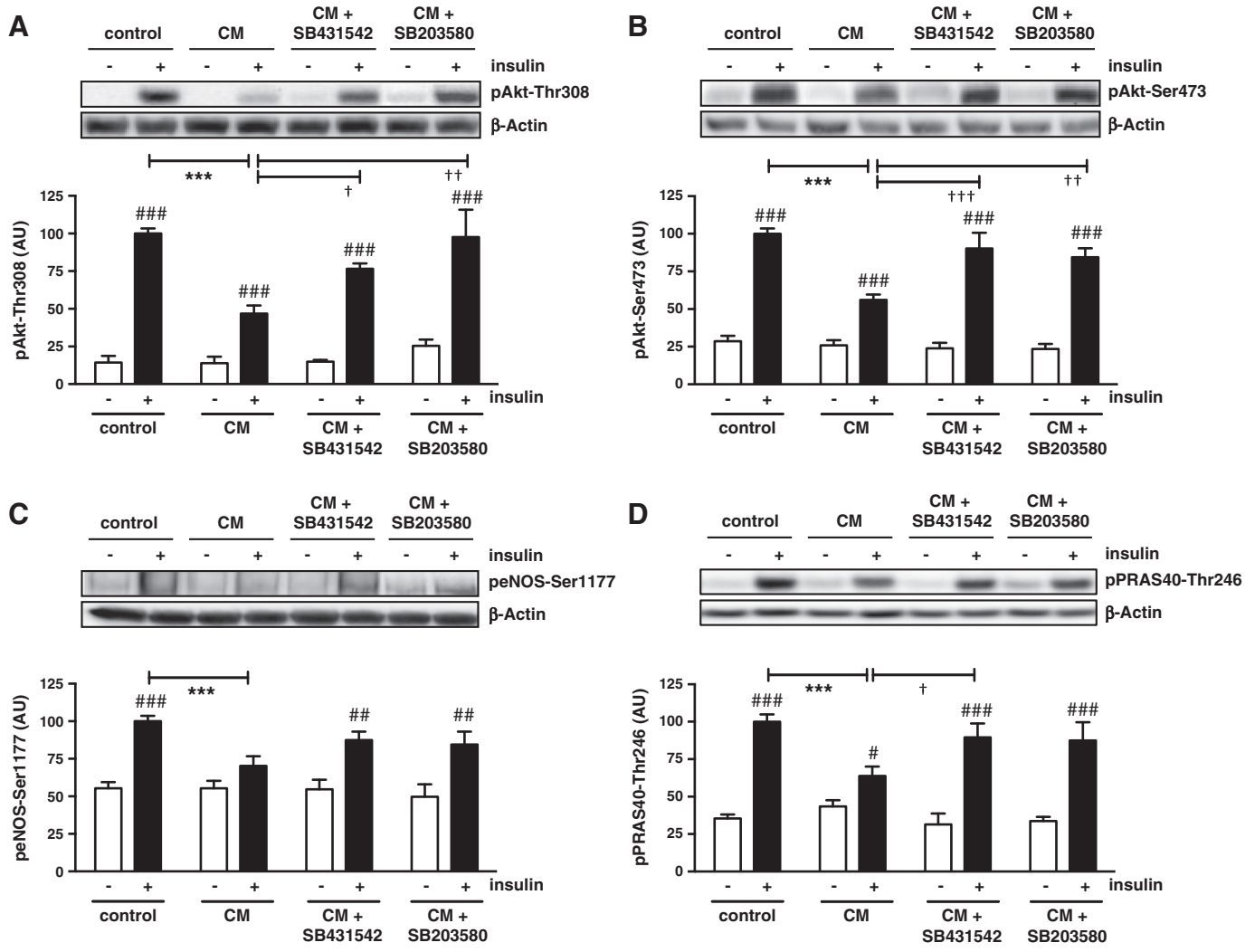

Fig. 4. Involvement of TGFβ receptor- and p38-signaling in insulin action in primary human vascular smooth muscle cells. Cells were exposed to DMSO (vehicle), or pharmacological inhibitors for Alk4/5/7 (SB431542), or p38 (SB203580) for 1 h prior to the addition of control medium (−) or conditioned media CM, (+). Shown are representative Western blots and quantifications for the phosphorylation levels of Akt-Thr308 (A), Akt-Ser-473 (B), eNOS-Ser1177 (C), and PRAS40-Thr246 (D). Phosphorylation levels normalized for β-actin are expressed as mean ± standard error of the mean of at least 4 independent experiments using cells from two distinct donors and CM from 4 different preparations. Differences among the experimental conditions were evaluated using two-way ANOVA. ###, $P < 0.001$; ##, $P < 0.01$; #, $P < 0.05$ for the effect of insulin versus untreated cells; *** indicates $P < 0.001$ for the effect of CM versus control medium; †††, $P < 0.001$; ††, $P < 0.01$; †, $P < 0.05$ for CM only versus CM + inhibitors.

effects on the miRNA-143/145 cluster and insulin action, the inhibitors did not or only partially prevent the induction of SMA, PAI-1 and MCP-1 in hVSMC by CM (supplementary Fig. 1).

3.3. Effect of miR-143 and miR-145 overexpression on insulin signaling

To investigate the impact of the miR-143/145 cluster on insulin action, hVSMC were transfected with precursors for miR-143 and miR-145, respectively. In line with a previous report [8], SMA protein abundance was increased in cells transfected with pre-miR-143 and pre-miR-145 by 9.0- and 2.0-fold respectively versus cells transfected with control pre-miR ($P < 0.05$) (Fig. 5A). Insulin-stimulated phosphorylation of Akt-Thr308, Akt-Ser473, and eNOS-Ser1177 was reduced by 42%, 44%, and 39%, respectively, in cells transfected with pre-miR-143 versus cells transfected with control pre-miR (Fig. 5B–D). In contrast, transfection with pre-miR-145 did not affect insulin action (Fig. 5B–D).

Several reports have linked miR-143 to an inhibition of insulin signaling through downregulation of ORP8. As shown in Fig. 6A, the transfection of hVSMC with pre-miR-143 reduced ORP8 protein levels by 25% ($P < 0.01$) versus cells transfected with either control pre-miR or pre-miR-145. Also exposure to CM lowered ORP8 levels by 30% ($P < 0.01$) versus cells kept in control medium, and this reduction in ORP8 abundance was not observed in hVSMC treated with either SB431542 or SB203580 prior to exposure to the CM (Fig. 6B). To examine whether the downregulation of ORP8 impairs insulin signaling in hVSMC, cells were transduced with lentiviruses encoding control shRNA or shRNA for ORP8. A 30% decrease in ORP8 abundance, which is comparable to that achieved by expression of miR-143 or exposure to CM, was found to inhibit insulin-mediated Akt-Ser473 phosphorylation by 25% (Fig. 6C/D).

4. Discussion

The present study shows that adipocyte-derived factors impair insulin signaling in hVSMC. Exposing the hVSMC to adipocyte-derived conditioned media was found to increase the expression of miR-143. The resulting decrease in the expression of the validated miR-143 target ORP8 resulted in inhibition of the insulin-mediated phosphorylation of

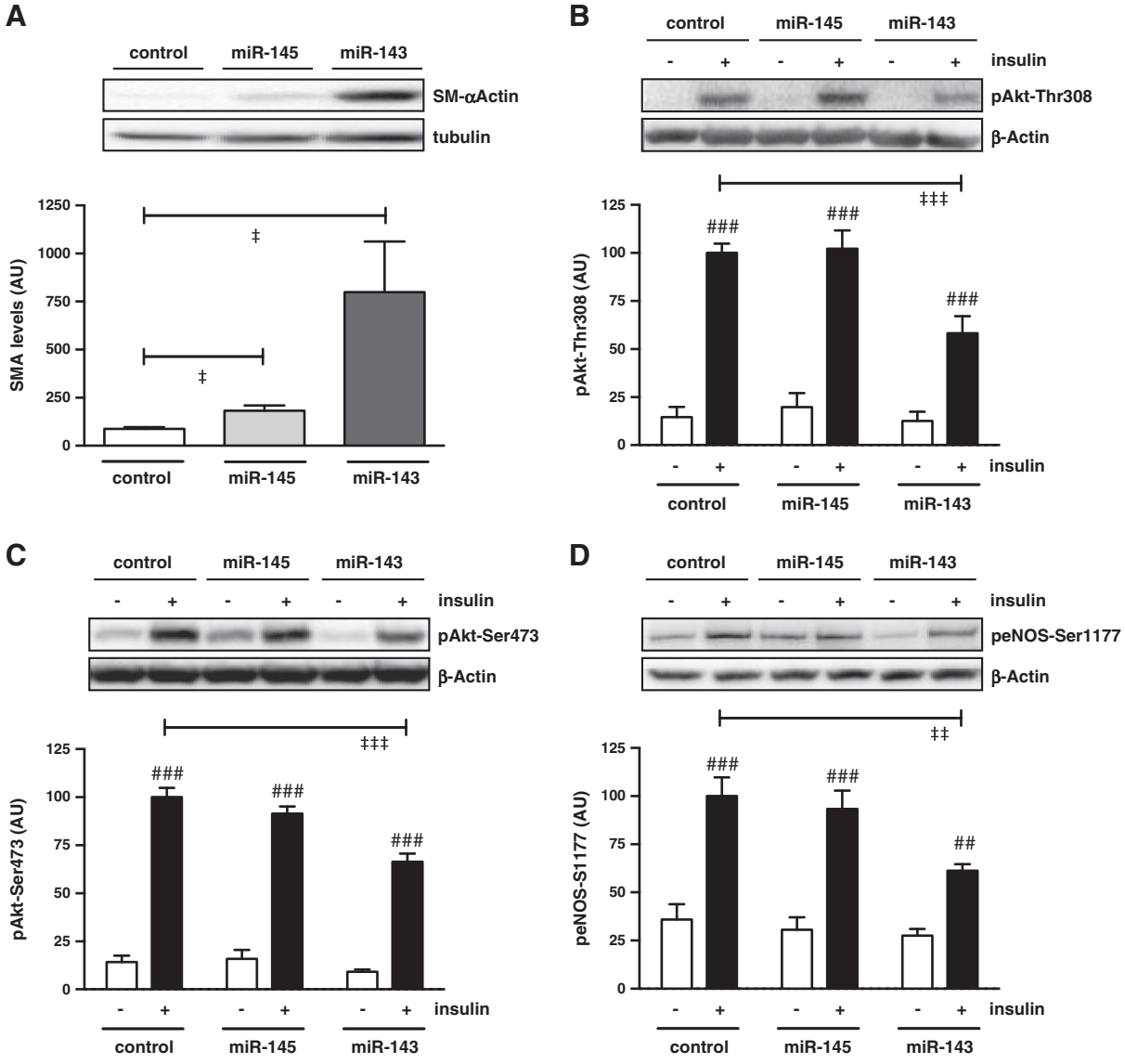

Fig. 5. Effect of miR-143 and miR-145 expression on insulin action in primary human vascular smooth muscle cells. Cells were transfected with control precursor miRNA or precursors for miR-143 or miR-145, respectively. Shown are representative blots and quantifications for the protein abundance of smooth muscle α-actin (A), and the phosphorylation levels of Akt-Thr308 (B), Akt-Ser-473 (C), and eNOS-Ser1177 (D). Signal normalized for tubulin (A) or β-actin (B–D) is expressed as mean ± standard error of the mean of 8 independent experiments performed on cells from two different donors. Differences among the experimental conditions were evaluated using ANOVA (A) or two-way ANOVA (B–D) followed by Bonferroni multiple comparison analysis. ###, indicates $P < 0.001$ for the effect of insulin versus untreated cells; ‡‡‡, $P < 0.001$; ‡‡, $P < 0.01$; ‡, $P < 0.05$ for miR-143 or miR-145 versus control pre-miR.

Akt/eNOS-signaling pathway. Furthermore, the activation of the miR-143/ORP8 pathway and the induction of TAGLN by CM were sensitive to inhibition of Alk4/5/7- and p38-signaling. In contrast, the induction of the inflammatory markers PAI-1 and MCP-1 by CM as well as the induction of SMA by CM was not fully reversed by Alk4/5/7- and p38-inhibition, indicating that these effects involve at least in part different pathways.

In contrast to classical target tissues for insulin action, like liver, fat and muscle, the function of proper insulin action in the vasculature is less well understood. Nevertheless, vascular smooth muscle cell function is impaired in patients with type 2 diabetes [23]. In vitro, physiological concentrations of insulin have been reported to stimulate the autophosphorylation of the insulin receptor in vascular smooth muscle cells [17,19,24,33,34]. Furthermore, insulin has been found to promote glucose uptake through translocation of the insulin-regulated glucose transported GLUT4 in vascular smooth muscle cells [15]. For these in vitro studies, it remains to be investigated whether these effects can be fully ascribed to activation of the insulin receptor or also to activation of hybrid insulin receptor/insulin like growth factor 1 receptor. Yet, studies using a vascular insulin receptor knock-out mouse have clearly illustrated the physiological relevance of activation of the Akt2/eNOS-pathway by insulin for vasorelaxation [9,30].

Whether an impaired insulin action in SMC also has detrimental effects on the progression of atherosclerosis is less well understood. One study reported that the presence of high palmitate induces a "selective" inhibition of insulin signaling in hVSMC with a profound abrogation of insulin induced PI3K-activation, whereas the activation of the MAPK-pathway is enhanced [5]. Additionally this study showed that the increased activation of MAPK-signaling by insulin is involved in hVSMC proliferation, migration, and inflammation [5]. Alternatively, in advanced plaque progression, inflammation and insulin resistance may promote apoptosis of SMC and therefore thinning of fibrous cap

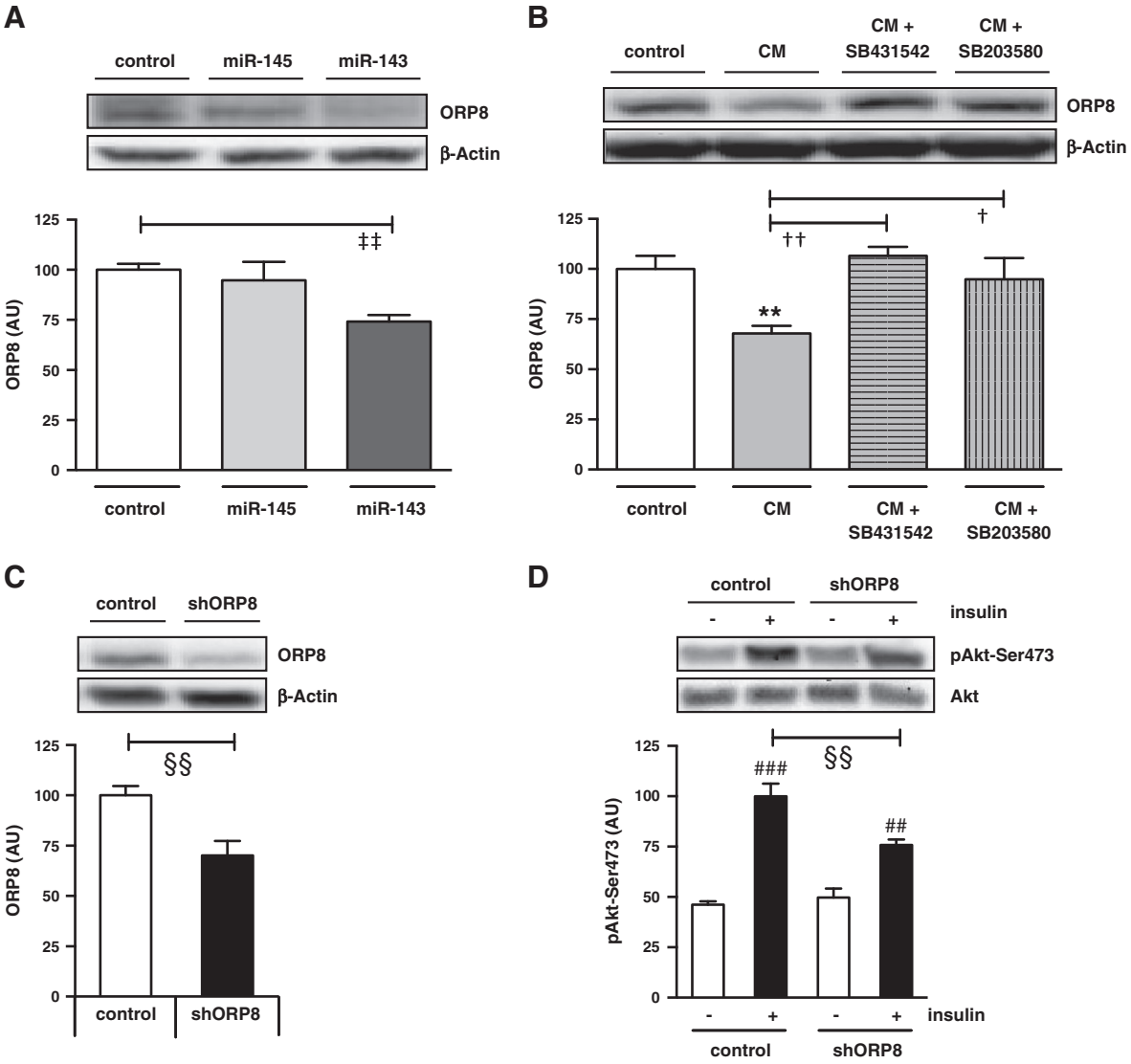

Fig. 6. Inhibition of insulin action by conditioned media involves downregulation of the miR-143 target ORP8. Primary human vascular smooth muscle cells were transfected with control precursor miRNA or precursors for miR-143 or miR-145 (A), or exposed to conditioned media (CM) in the presence or absence of SB431542 or SB203580 (B) whereafter protein levels of ORP8 were examined via Western blot analysis. Shown are representative blots and quantifications. ORP8 abundance normalized for β-actin is expressed as mean ± standard error of the mean of 16 independent experiments using cells from 2 distinct donors and CM from 8 different preparations. Differences among the experimental conditions were evaluated using ANOVA followed by Bonferroni multiple comparison analysis. **, indicates $P < 0.01$ for the effect of CM versus control medium; ††, $P < 0.01$; †, $P < 0.05$ for CM only versus CM + inhibitors; ‡‡, $P < 0.05$ for miR-143 or miR-145 versus control pre-miR. C/D. Impact of silencing ORP expression in primary human vascular smooth muscle cells. Lysates from cells transduced with lentiviruses coding for control shRNA or ORP8 shRNA were analyzed for ORP8 protein abundance (C) and insulin-mediated phosphorylation of Akt-Ser473 (D) under untreated conditions (−) or following stimulation with insulin (10 min; 100 nM). Signals were normalized for β-actin (C) or Akt (D) respectively, and expressed as mean ± standard error of the mean of 8 independent experiments using cells from two different donors and 4 distinct shRNA constructs. ### indicates $P < 0.001$; ##, $P < 0.01$ for the effect of insulin versus untreated cells; §§, $P < 0.01$ for the effect of ORP8 shRNA versus control shRNA.

and causing plaque rupture [3]. Finally, insulin-stimulated eNOS-derived NO production has important anti-inflammatory and anti-thrombotic properties through inhibition of leucocyte adhesion, and limiting platelet adhesion and aggregation, and reduced expression of plasminogen activator inhibitor-1 (PAI-1), a prothrombotic protein [21]. Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells [10,25,26]. Therefore, impaired insulin signaling with reduced bioavailable NO may predispose vasculature to hyper-inflammatory and thrombotic states.

The present study shows that CM directly impairs insulin action in hVSMC via induction of miR-143 and subsequent downregulation of ORP8. Although the mechanism via which ORP8 regulates insulin action remains unclear, the induction of miR-143 is closely associated with obesity and insulin resistance. Feeding mice with a high-fat diet increased the levels of miR-143 in adipose tissue [31], while both miR-143 and miR-145 are upregulated in the liver of mice fed also with a high-fat diet [18]. Moreover in the liver, heart, skeletal muscle and pancreas from *db*/*db*-mice, the expression of miR-143 was increased as compared to tissues isolated from wild type control animals. Liver specific knock out of the miR-143/145-cluster protects against high-fat diet induced insulin resistance and hepatic Akt-inhibition [18]. The authors of that study further identified ORP8 as a direct miR-143 target, and showed that a decrease in ORP8 protein abundance is responsible for the abrogation of insulin action in the liver [18]. Finally, in cardiomyocytes we recently reported that the induction of miR-143 by activin A secreted from epicardial adipose tissue promotes insulin resistance via ORP8, and that silencing miR-143 expression protects cardiomyocytes against the induction of insulin resistance [1].

The strong association of miR-143 with insulin resistance in multiple tissues seems in contrast to the function ascribed to this miRNA in SMC. In SMC, an anti-proliferative function has been reported for the miR-143/145-cluster [8,22], and especially miR-145 has been found to promote the differentiation to the quiescent contractile phenotype of SMC [22]. Here, CM also induced the expression of miR-145 in quiescent differentiated hVSMC. Accordingly, this was accompanied by the induction of SMA, and TAGLN. In mice, miR-145 overexpression reduces neo-intima formation after vascular injury, but paradoxically miR-145 deficient mice show similar effects [6,37]. In humans, levels of miR-145 are elevated in atherosclerotic plaques [7,28], and were found to correlate with an unstable plaque phenotype [7]. Collectively these findings not only point toward an important dual role for the miR-143/145 in SMC homeostasis, but also indicate that further studies toward the underlying mechanism(s) are clearly needed, such as comparing the impact of CM on differentiated versus undifferentiated SMC and elucidation of the targets regulated by the miR-143/145-cluster.

A limitation of the present study is that we could not identify the factor in CM responsible for the induction of the miR-143/145-cluster. In line with previous reports, the induction of miR-143/145 was sensitive to inhibition of Alk4/5/7- and p38-signaling [1,22]. Previous studies identified TGFβ and activin A as inducers of miR-143/145. However, the levels of these factors as determined by enzyme-linked immunosorbent assay were below the limit of detection in the CM used in the present study. Consequently, one may speculate that other members of the TGFβ superfamily, which consists of at least 23 members [32], elicit the effects observed here. Alternatively, CM itself may induce the production of factors promoting the induction of the miR-143/145-cluster in an autocrine fashion. In this context, we could demonstrate that CM enhance the expression of activin A in differentiated hVSMC by 1.7-fold (supplementary Fig. 2). Yet, it remains to be investigated whether this is associated by the release of biologically relevant amounts of activin A from hVSMC. Another limitation is that the CM used in the present study was prepared from adipocytes isolated from subcutaneous adipose tissue biopsies collected from healthy young females. In previous studies, we reported that the adipose tissue secretory profile is affected by type 2 diabetes and different among various adipose tissue depots [1,11,29]. Therefore, one may speculate that conditioned media generated from adipocytes from donors with obesity or type 2 diabetes may exert a more detrimental impact on the determinants of smooth muscle cell function examined in the present study.

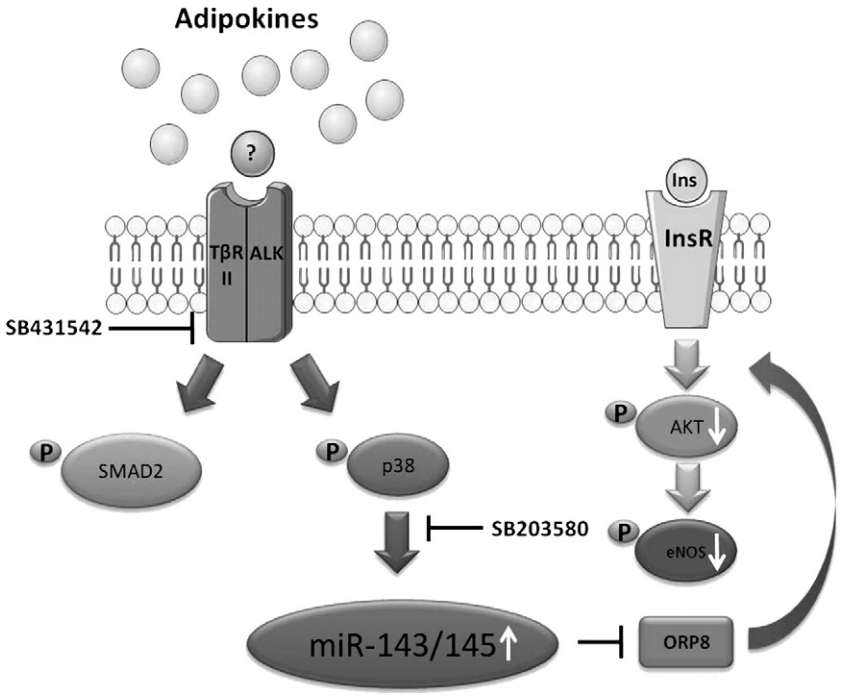

Fig. 7. Possible mechanism for CM-induced insulin resistance in hVSMC. Adipocyte-derived factors belonging to the TGFβ-superfamily and binding to ALK induce SMAD2 and p38 phosphorylation. Especially the SMAD-independent Pathway through p38 leads to an upregulation of the miRNA 143/145 cluster. miR-143 is able to reduce the expression of ORP8 resulting in impaired insulin-stimulated Akt and eNOS phosphorylation. ALK: activin receptor-like kinases, Ins: insulin, InsR: insulin receptor, TβR II: transforming growth factor β receptor 2, ORP8: oxysterol-binding protein-related protein 8.

5. Conclusion

This study shows for the first time that adipocyte-derived factors impair insulin signaling in hVSMC. The inhibition of insulin signaling can be ascribed to the Alk4/5/7- and p38-dependent induction of miR-143 (Fig. 7). This miRNA plays a pivotal role in the CM-induced impairment of insulin-induced Akt/eNOS-signaling via downregulation of ORP8. These data further highlight the involvement of adipokines in the pathogenesis of cardiovascular complications in type 2 diabetes.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.12.001.

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