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Adipocyte-specific mineralocorticoid receptor overexpression in mice is associated with metabolic syndrome and vascular dysfunction - role of redox-sensitive PKG-1 and Rho kinase.

Aurelie NGUYEN DINH CAT[1], Tayze T. ANTUNES[2], Glaucia E. CALLERA[2], Ana SANCHEZ[3], Sofia TSIROPOULOU[1], Maria G. DULAK-LIS[1], Aikaterini ANAGNOSTOPOULOU[1], Ying HE[2], Augusto C. MONTEZANO[1], Frederic JAISSER[4,5], Rhian M. TOUYZ[1,2].

[1] Cardiovascular Research and Medical Sciences Institute, University of Glasgow, Glasgow, United Kingdom; [2] Kidney Research Centre, Ottawa Hospital Research Institute, University of Ottawa, Ottawa, Canada; [3] Departamento de Fisiología, Facultad de Farmacia, Universidad Complutense, Madrid 28040, Spain; [4] Inserm Unit 1138 Team 1, Centre de Recherche des Cordeliers, University Pierre and Marie Curie, Paris, France; [5] INSERM, Clinical Investigation Centre 1430, APHP, Henri Mondor Hospital, Pole VERDI, Paris East University, Creteil, France.

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Correspondence:

Aurelie Nguyen Dinh Cat, PhD
Institute of Cardiovascular & Medical Sciences
BHF Glasgow Cardiovascular Research Centre
University of Glasgow
126 University Place
Glasgow G12 8TA
Tel: + 44 (0)141-330-8015
Fax: + 44 (0)141-330-3360
Email: cattuong.ndc@gmail.com

Rhian M Touyz MBBCh, PhD, FRCP, FRSE.
Institute of Cardiovascular & Medical Sciences
BHF Glasgow Cardiovascular Research Centre
University of Glasgow
126 University Place
Glasgow G12 8TA
Tel: + 44 (0)141 330 7775/7774
Fax: + 44 (0)141 330-3360
Email: Rhian.Touyz@glasgow.ac.uk

Abstract

Mineralocorticoid Receptor (MR) expression is increased in adipose tissue from obese individuals and animals. We previously demonstrated that adipocyte-MR over-activation in mice (Adipo-MROE) is associated with metabolic changes. Whether adipocyte-MR directly influences vascular function in these mice is unknown. We tested this hypothesis in resistant mesenteric arteries from Adipo-MROE using myography and in cultured adipocytes. Molecular mechanisms were probed in vessels/vascular smooth muscle cells and adipose tissue/adipocytes and focused on redox-sensitive pathways, Rho kinase activity and protein kinase G type-1 (PKG-1) signaling. Adipo-MROE versus Control-MR mice exhibited reduced vascular contractility, associated with increased generation of adipocyte-derived hydrogen peroxide, activation of vascular redox-sensitive PKG-1 and downregulation of Rho kinase activity. Associated with these vascular changes was increased elastin content in Adipo-MROE. Inhibition of PKG-1 with Rp-8-Br-PET-cGMPS normalized vascular contractility in Adipo-MROE. In the presence of adipocyte conditioned culture medium, anticontractile effects of the adipose tissue were lost in Adipo-MROE, but not in Control-MR mice. In conclusion, adipocyte-MR upregulation leads to impaired contractility with preserved endothelial function and normal blood pressure. Increased elasticity may contribute to hypocontractility. We also identify functional crosstalk between adipocyte-MR and arteries and describe novel mechanisms involving redox-sensitive PKG-1 and Rho kinase. Our results suggest that adipose tissue from Adipo-MROE secrete vasoactive factors that preferentially influence VSMCs rather than endothelial cells. Our findings may be important in obesity/adiposity where adipocyte-MR expression/signaling is amplified and vascular risk increased.

Keywords: Adipocyte, nuclear receptor, vascular reactivity, transgenic mice, oxidative stress, visceral fat, adiposity.

Non-standard Abbreviations and Acronyms

ACh	acetylcholine
ACM	adipocyte conditioned culture medium
Adipo-MROE	adipocyte MR overexpression
eNOS	endothelial nitric oxide synthase
ERM	ezrin-radixin-moesin proteins
EVAT	epididymal visceral adipose tissue
H_2O_2	hydrogen peroxide
IRS1	insulin receptor substrate 1
MLC	myosin light chain
MR	mineralocorticoid receptor
MYPT1	myosin light chain phosphatase subunit 1
Phe	phenylephrine
PKG-1	cGMP-dependent protein kinase G type 1
PVAT	perivascular adipose tissue (mesentery)
ROCK	rho kinase
ROS	reactive oxygen species
Sgk-1	serum and glucocorticoid regulated kinase-1
SNP	sodium nitroprusside
VSMCs	vascular smooth muscle cells

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Introduction

Aldosterone classically acts via the renal epithelial mineralocorticoid receptors (MR) leading to salt and volume homeostasis and consequently blood pressure regulation. MR is also expressed in non-epithelial cells including cardiomyocytes [1], vascular cells [2, 3] and macrophages [4, 5] and have been implicated in pathological conditions such as heart failure, endothelial dysfunction, hypertension, insulin resistance and obesity [6-10]. Recently, MR has been identified in adipocytes [11-14]. The (patho)-physiological significance of adipocyte MR is unclear, although experimental and human studies have demonstrated increased expression of adipocyte MR in obesity, metabolic syndrome and diabetes [6, 14, 15]. Adipose tissue has long been considered primarily as a simple fat storage of triglycerides to regulate several important functions such as energy balance and thermogenesis, but is now recognized as a metabolically active endocrine/paracrine/autocrine organ that synthesizes, stores, and secretes hormones, vasoactive factors and proteins, termed adipo(cyto)kines [16, 17]. MR mediates effects of aldosterone and glucocorticoids, which in adipocytes regulate adipogenesis, adipocyte maturation and adipokines production [12, 18-20]. We recently demonstrated that adipocytes possess functionally active MR and have the machinery to produce aldosterone, processes that are increased in obesity-associated diabetes and which influence vascular function [6, 12, 13].

To better understand the relevance of the adipocyte MR, we generated a conditional transgenic mouse model allowing restricted and inducible MR overexpression in an adipocyte-specific manner. These mice exhibit features of metabolic syndrome, including weight gain, insulin resistance and dyslipidemia, with increased activation of prostaglandin D2 synthase [14]. Considering the growing evidence that adipose tissue and adipokines influence vascular tone, and that the aldosterone/MR system is involved in vascular pathology associated with cardiovascular disease, we questioned whether upregulation of adipocyte MR in mice leads to vascular dysfunction.

Material and Methods

Generation of mice expressing human MR (hMR) in adipocytes

The study was approved by the Animal Ethics Committee of the University of Ottawa. All studies in animals were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures followed were in accordance with institutional guidelines. We generated mice with targeted overexpression of hMR in adipocytes using the conditional tetracycline-inducible system as we recently reported (14) and summarized in Supplementary Fig. 1A and B. Double-transgenic ('adipocyte MR over-expression', Adipo-MROE) mice were obtained by mating two strains: the mono-transgenic tetO-hMR mouse and the mono-transgenic transactivator aP2-rtTA mouse. In Adipo-MROE mice, hMR expression is switched on in adipocytes by administering doxycycline (Dox) (2 g/L for 4 weeks) (200 mg/kg Dox diet, Harlan Laboratories, Ontario, Canada). The litters consisting of Adipo-MROE, mono-transgenic tetO-hMR, mono-transgenic aP2-rtTA, and WT mice were born in the expected mendelian ratio (25% for each genotype). The last 3 groups, which displayed no molecular or functional differences between them, were considered as controls (Control-MR). Blood pressure was measured by the tail-cuff method in 2-3 month-old mice. Mice were sacrificed to recover mesenteric arteries and adipose tissues used for the subsequent manipulations of cell culture, histology or bio-molecular analysis.

Isolation and culture of mature adipocytes from mouse adipose tissue

Mouse mature adipocytes

Mouse abdominal epididymal visceral adipose tissue (EVAT) was obtained from 4 month-old male Adipo-MROE and their littermates Control-MR mice. Mature adipocytes fraction was isolated and cultured at 37°C for 24 hrs as previously described (12).

Conditioned culture medium

The conditioned culture medium, collected from mature adipocytes (ACM) 24 hrs after isolation (12,21), was concentrated in speed vacuum system (Vacufuge Eppendorf) and kept at -20°C for further aldosterone, corticosterone, angiotensin II and hydrogen peroxide levels determinations.

Vascular studies

Second order branches of mesenteric artery without perivascular fat (PVAT) were isolated from 3- 4 month-old Adipo-MROE and their littermates Control-MR mice and mounted on a wire myograph (DMT myograph, ADInstruments Ltd, Oxford, UK) as previously described (13). Endothelium-

dependent and -independent relaxations were assessed by measuring dilatory responses to acetylcholine (ACh, 10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (SNP, 10^{-9} to 10^{-5} mol/L), respectively, in arteries pre-contracted with phenylephrine (Phe) to similar degrees to achieve approximately 80% of maximal response. Dose-response curves for insulin-induced relaxation (10^{-10} to 10^{-5} mol/L) were also obtained in arteries with intact endothelium in absence or in presence of a general ROS scavenger, N-Acetyl-cysteine (10^{-6}M) or a H_2O_2 scavenger, PEG-catalase (100 U/mL) incubated 30 minutes prior to pre-contraction with Phe. Contraction curves to cumulative increasing doses of Phe, noradrenaline (NA) and serotonin (5-HT, 5-hydroxytryptamine) (10^{-9} to 10^{-5} mol/L) were performed in arteries with and without intact endothelium.

Healthy PVAT and other fat depots display a protective anticontractile effect on the vasculature that is lost in obesity. This anticontractile property remained with the transfer of the culture bath to vessels without adipose tissue and suggested the action of secreted relaxing substances. In that way, we investigated the effects of visceral fat using ACM (from EVAT) from Control-MR and Adipo-MROE mice on contractile responses to Phe. A small piece of EVAT is incubated in 10 mL of Krebs solution 30 min prior to concentration-response curve to Phe. It has been suggested that PVAT behaves similarly to visceral fat in terms of secretion of vasoactive factors (22). We also evaluated the effects of a specific inhibitor of Protein Kinase G type-1 (PKG-1) on vascular reactivity. Arteries were pre-treated with Rp-8-Br-PET-cGMPS (3×10^{-5} mol/L) or vehicle (DMSO) for 30 min before exposing vessels to Phe (endothelium-denuded mesenteric arteries) or hydrogen peroxide (H_2O_2, 10^{-6} to 10^{-3} mol/L) (endothelium-intact arteries).

Arterial stiffness was studied with a pressure myograph (Living Systems, Burlington, Vermont) as previously described (13). The artery was set to an internal pressure of 45 mmHg, pressure-fixed (30 min, 10% formalin, 37°C) and stored embedded in paraffin until histomorphometry studies. Vascular structure and mechanical parameters were calculated as previously described (13).

Elastin content assessment by Elastic Van Gieson

Deparaffinized Sections (5 μm) were rehydrated in sequential alcohol baths and washed in distilled water. For elastin content, tissue sections were stained with Miller's Elastin (VWR, Cat. #351154S) and Van Gieson solutions (Sigma Aldrich, Cat. # HT25A). Elastic fibers and mast cell granules are

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stained black/purple, collagen in red and cytoplasm and muscle in yellow. Mesenteric arteries were visualized under an Axio Observer-Z1 Zeiss microscope and captured with a colour camera under identical conditions of light intensity and exposure time settings. For all sections, total area and area of elastin content were measured on calibrated images at x40 magnification using Image J software. All data are presented as mean percentage of elastin content ± SE of 9 mice per group.

Cell culture of mouse vascular smooth muscle cells

To interrogate some molecular mechanisms underlying altered vascular function in Adipo-MROE mice, we also studied cultured VSMCs (passages 4 to 6) from control mice (C57/Bl6 mice), from mesenteric arteries as described in detail previously (12). VSMCs were stimulated with H_2O_2 (10^{-6} to 10^{-3} mol/L) for 24 hrs to recapitulate vascular oxidative stress observed in Adipo-MROE mice. In a second set of experiments, VSMCs were incubated with PEG-catalase (1000 U/mL) or the PKG-1 antagonist (DT-3, 10^{-8} mol/L) for 30 min and then stimulated with 10^{-3} mol/L H_2O_2 for 24 hrs. Concentrations of antagonists used, which effectively inhibited respective receptors, were based on preliminary dose-response studies (data not shown).

Both inhibitors DT-3 and Rp-8-Br-PET-cGMPS used in our studies are highly potent, permeable and selective for protein kinase G type-1α, and reduced cGMP-stimulated PKG activity (23).

Enzymatic immunoassays

Aldosterone, corticosterone and angiotensin II measurements

Aldosterone (#10004377), corticosterone (#500651) and angiotensin II (#A05880) concentrations were determined in plasma and ACM from cultured adipocytes from Adipo-MROE and Control-MR mice by enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer´s instructions. Standard curves were derived using non-conditioned medium and blank measurement of non-conditioned medium was subtracted from all samples. Blood samples were collected into heparinized tubes, centrifuged at 2000 g at 4ºC for 10 min and plasma was further stored at -80ºC until analysis.

Rho Kinase Activity

Enzymatic activity of Rho kinase was evaluated using a Rho-associated Protein Kinase (ROCK) Activity Assay Kit (Merck Millipore #CSA001, United Kingdom) and the experiments were

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performed in mesenteric arteries and aortic protein lysates, according to the manufacturer's instructions.

Amplex Red Assay

The Amplex Red assay (Invitrogen, Life technologies, Paisley, United Kingdom), involves measurement of H_2O_2 by the horseradish peroxidase–catalyzed oxidation of the colorless and non-fluorescent molecule N-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red) to resorufin which, when excited at 530 nm, strongly emits light at 590 nm. The assay was performed in vessels and adipose tissues from Adipo-MROE and Control-MR mice according to the manufacturer's instructions.

Phosphorylation of insulin receptor substrate 1 (IRS1)

Phosphorylation of IRS1 at the serine-307 was evaluated using an ELISA kit (Merck Millipore #17-459, United Kingdom) and the experiments were performed in mesenteric arteries protein lysates, according to the manufacturer's instructions. Serine-307 phosphorylation of insulin receptor substrate 1 (IRS1) has been shown to mediate insulin resistance (24-26).

Western blot analysis

Mesenteric arteries, aorta from Adipo-MROE and Control-MR mice and VSMCs were prepared and western blotting analysis was performed as previously described (12). Antibodies were as follows: anti-Phospho-MYPT1 (Thr-696) (1:1000, #sc-17556, Santa Cruz Biotechnology, CA, USA), anti-MYPT1 (H-130) (1:1000, #sc-25618), anti-Phospho-ezrin (Thr-567)-radixin (Thr-564)-moesin (Thr-558) (p-ERM, 1:1000, #3142, Cell Signaling, USA) and anti-ERM (1:1000, #3142), anti-Phospho-MLC 2 (Thr18/Ser19) (1:1000, #3674, Cell Signaling, USA) and anti-MLC (1:1000, #3672), anti-Phospho-eNOS (Ser-1177) (1:1000, #9571, Cell Signaling, USA), anti-eNOS (1:1000, #9572), anti-Rho kinase type 2 (ROCK2, 1:1000, #8236, Cell Signaling, USA), anti-RhoA (26C4) (1:500, #sc-418, Santa Cruz Biotechnology), anti-PKG-1 (1:1000, 3248, Cell Signaling, USA). Blots were analyzed densitometrically using the Image J software.

Quantitative real time Polymerase Chain Reaction

Total RNA was extracted from mature adipocytes, EVAT, PVAT and mesenteric arteries as previously described (13, 14). Real time PCR was carried out on a 7900HT Fast Real-Time PCR machine (Applied Biosystems) using gene-specific primers to quantify the relative abundance with

SYBR Green I as the fluorescent molecule. The primers were designed using the software Primer 3 and are listed in Supplementary Table 1. *Ubiquitin C* (*Ubc*) housekeeping gene was used as the reference gene for normalization. The relative copies number of the target genes were calculated with the $2^{(-\Delta\Delta Ct)}$ method, after assessment that PCR efficiency was 100%.

Drugs and solutions

ACh, SNP, Phe, IBMX, insulin, dexamethasone, hydrogen peroxide, N-Acetyl-cysteine and PEG-catalase were obtained from Sigma-Aldrich Ltd (Dorset, United Kingdom). DT-3 was obtained from Merck Millipore (Calbiochem Ltd, United Kingdom). Rp-8-Br-PET-cGMPS was obtained from Tocris (Canada). Dexamethasone was dissolved in 100% ethanol. IBMX was dissolved in 0.35N KOH. ACh, SNP, Phe, H_2O_2, N-Acetyl-cysteine and insulin were dissolved in distilled water. DT-3, PEG-catalase and Rp-8-Br-PET-cGMPS were dissolved in DMSO.

Data Analysis

Values reported are means ± SE. Differences between groups were assessed with the nonparametric Mann-Whitney test. Vascular reactivity results were assessed with one and two-ways ANOVA with repeated measures, followed by Bonferroni multiple comparison test, as appropriate. Morphometry analysis were assessed with one-way ANOVA. An independent two tailed t-test assuming equal variances was used for comparing the mean percentage of elastin content between the different groups. Values of $p < 0.05$ were considered significant.

Results

Conditional MR overexpression in adipocytes in mice.

As previously described (14), conditional MR overexpression for 4 weeks leads to a 3-4 fold increase in MR expression in mature adipocytes isolated from epididymal visceral adipose tissue (EVAT) from Adipo-MROE mice compared to adipocytes from littermate Control-MR mice (Supplementary Fig. 2A). MR expression is increased in whole EVAT, as well as in PVAT around the mesentery but not in mesenteric arteries (Supplementary Table 2). Gene expression of a known downstream target of aldosterone/MR activation, serum and glucocorticoid regulated kinase-1 (*Sgk1*) was increased 3-fold in adipocytes from Adipo-MROE versus Control-MR mice, whereas it is not changed in mesenteric arteries (Supplementary Fig. 2B) indicating that hMR is functionally active and its activation is

increased in Adipo-MROE mice. Supplementary Table 3 summarizes the general characteristics of the Control-MR and Adipo-MROE mice. Systolic blood pressure and heart rate were similar between Adipo-MROE and Control-MR mice. Body weight was increased by 21.4% in Adipo-MROE mice. Heart and kidney weights relative to tibia length were comparable between both groups, whereas EVAT weight relative to tibia length was increased by 21.7% in Adipo-MROE mice. Plasma levels of aldosterone, corticosterone, and angiotensin II were similar between Adipo-MROE and Control-MR mice. Levels of these hormones in adipocyte culture conditioned medium were also similar between both groups (Table 1).

Endothelial function and vascular contractility in Adipo-MROE mice.

Vasodilatory responses.

Acetylcholine (ACh)- and insulin- induced vasorelaxation was assessed in mesenteric arteries from Adipo-MROE and Control-MR mice. ACh-induced vasorelaxation, indicative of endothelium-dependent vasodilation, was similar between both groups, as well as for SNP-induced vasorelaxation (indicative of endothelium-independent vasodilation) (Supplementary Fig. 3A and B). However, the ability of insulin to cause dose-dependent vasorelaxation was significantly impaired in vascular segments from Adipo-MROE mice than those from Control-MR littermates ($15.7\pm4.4\%$ versus $30.3\pm8.3\%$ to 10^{-6} mol/L insulin; $p<0.05$), indicating an impaired insulin sensitivity in vessels from Adipo-MROE mice (Supplementary Fig. 3C). However, this was improved *ex vivo* by both inhibitors of reactive oxygen species (ROS), N-Acetyl-cysteine or PEG-catalase (Supplementary Fig. 3D). These results were confirmed with increased phosphorylation of insulin receptor substrate 1 (IRS1) at serine 307 (+154% from Control-MR) (Supplementary Fig. 3E). IRS1 phosphorylation at serine-307 has been highlighted as a molecular event that causes insulin resistance (25, 26). Thus, Adipo-MROE mice displayed vascular insulin resistance.

Contractile responses.

Maximum responses to high concentration of potassium chloride (KCl) are identical between Control-MR and Adipo-MROE mice (response to KCl: Control-MR, 2.2 ± 0.1 vs Adipo-MROE, 2.1 ± 0.2 mN/mm; n=10 mice per group, ns) (Supplementary Fig. 4A). Phe-induced contraction of arteries with intact endothelium was significantly reduced in Adipo-MROE mice versus control counterparts (Fig.

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1A). Similar contractile alterations were observed in Adipo-MROE mice in response to increasing doses of NA and 5-HT in which the endothelium had been mechanically denuded (Figure 1B and Supplementary Fig. 4B and C). The difference in contractile responses between the experimental and control groups was greater when the endothelium was denuded (Phe 10^{-5}M, mN/mm: Control-MR +endothelium, 2.5±0.2; Adipo-MROE +endothelium, 2.0±0.1, Δ =0.5; Control-MR -endothelium, 2.7±0.2; Adipo-MROE -endothelium, 1.7±0.3, Δ =1.0; n=8-10 mice per group, p<0.05).

Experimental evidences support a pathological effect of PVAT on the vasculature with increasing adiposity. To test if this is the case in our conditional model that displays visceral adiposity, we examined the effect of adipose tissue-secreted factors using mouse ACM (from EVAT) on contractility of mesenteric arteries of either Control-MR either Adipo-MROE mice. In both Control-MR and Adipo-MROE, ACM from Control-MR mice decreases contractile responses to Phe, but not the ACM from Adipo-MROE mice (Fig. 1C and D), suggesting that ACM from Adipo-MROE lose its anticontractile effect. Interestingly, ACM from Adipo-MROE mice increases contractility in arteries of Control-MR by secreting possibly vasoconstrictor factors which influence vascular tone in a paracrine way (Fig. 1C).

Adipocyte-specific MR over-expression is associated with down-regulation of vascular Rho kinase signaling.

Small GTPases of the Rho family influence vascular tone primarily by regulating VSMC contraction. Considering the significant alteration of vascular contractility in Adipo-MROE mice, we explored the possibility that RhoA/ROCK may be involved. ROCK activity was significantly reduced in mesenteric arteries from Adipo-MROE versus Control-MR mice (% Control-MR: 100±12.9 vs 41±7.5, n=7 mice per group, p<0.01). This was associated with decreased phosphorylation of downstream targets of ROCK signaling, specifically: the myosin phosphatase subunit 1 (MYPT1) (Fig. 2A), the ezrin-radixin-moesin (ERM) (proteins linking the actin cytoskeleton with the plasma membrane) (Fig. 2B) and the myosin light chain (MLC) (Fig. 2C).

Protein levels of RhoA and ROCK isoform 2 (ROCK2) were similar in experimental and control groups (Supplementary Fig. 5A). To evaluate whether contractile alterations relate to endothelial

nitric oxide synthase (eNOS), we assessed phosphorylation status of eNOS on the active site, Serine-1177, which was not significantly different in Adipo-MROE and control groups (Supplementary Fig. 5B).

Adipocyte-specific MR over-expression is associated with activation of cGMP-dependent protein kinase G type 1 (PKG-1) and increased levels of vascular reactive oxygen species production.

To investigate molecular mechanisms involved in decreased vascular ROCK activity in mice with adipocytes-targeted MR overexpression, we focused on cyclic GMP-dependent kinase G (PKG)-1, which is activated by ROS such as H_2O_2 in VSMCs, and which has been shown to attenuate vasoconstriction and promote vasodilation (27, 28). As shown in Fig. 3A, expression of PKG-1 was significantly higher in arteries from Adipo-MROE mice compared with controls. To explore potential molecular mechanisms whereby PKG-1 is activated, we focused on diffusible factors such as H_2O_2 that are involved in regulation of vascular tone. Production of H_2O_2 in ACM from mature adipocytes (EVAT) of Adipo-MROE mice showed a 2.8-fold increase versus Control-MR mice, as well as in extracts from EVAT (2.3 fold increase) (Fig. 3B). The accumulation of H_2O_2 in adipose tissue is supported by the 2.5 fold-decrease in *catalase* mRNA levels in EVAT from Adipo-MROE mice (Control-MR, 1.3 ± 0.2 vs Adipo-MROE, 0.52 ± 0.05, arbitrary units; n=6 mice per group, p<0.01), whereas *Sod* (superoxide dismutase) mRNA levels were unchanged (Control-MR, 0.9 ± 0.1 vs Adipo-MROE, 1.22 ± 0.2, arbitrary units; n=6 mice per group, ns). Moreover, we evaluated the mRNA levels of NADPH oxidase (Nox) isoforms 1, 2 and 4 to define the source of ROS. Interestingly *Nox-4* mRNA levels are increased whereas *Nox-1* and *Nox-2* are unchanged in adipose tissues from Adipo-MROE mice (Supplementary Fig. 6A and B). Thus, increased generation of H_2O_2 by adipocytes of Adipo-MROE mice may influence vascular contractility of Adipo-MROE mice and the RhoA/ROCK system through PKG-1 activation.

Exogenous hydrogen peroxide leads to down-regulation of ROCK activity.

To understand the mechanistic basis of the relationship between PKG-1 and ROCK activity in VSMCs exposed to oxidative stress conditions, we examined ROCK activity in primary cultured mouse VSMCs exposed to exogenous H_2O_2. H_2O_2 dose-dependently increased expression of PKG-1

(Fig. 4A). We next analyzed the effect of H_2O_2 on ROCK activity in the absence and presence of a specific inhibitor of PKG-1 (DT-3) or an antioxidant, PEG-catalase. Exogenous H_2O_2 induced a significant decrease in ROCK activity (Fig. 4B) and its downstream signaling (Fig. 4C). This response was reversed when cells were pre-treated with DT-3 and PEG-Catalase (Fig. 4B), indicating that H_2O_2 is a negative regulator of ROCK activation through processes that involve PKG-1.

PKG-1 inhibition restores normal contraction in Adipo-MROE mice.

To analyze whether modulating PKG-1 expression affects vascular functional responses, we analyzed contractile responses in the presence of Rp-8-Br-PET-cGMPS, a specific inhibitor of PKG-1. Phe-induced vasoconstriction in endothelium-denuded arteries from Adipo-MROE was comparable with Control-MR mice when PKG-1 was inhibited (Fig. 5A). Endothelium-dependent relaxation induced by H_2O_2 after PKG-1 inhibition was similar in arteries between Adipo-MROE and Control-MR mice (Fig. 5B).

Elasticity and elastin content are increased in mesenteric arteries from Adipo-MROE mice.

To evaluate potential factors contributing to vascular hypocontractility in Adipo-MROE mice, we examined elasticity and elastin status of mesenteric arteries from the different groups. As shown in Supplementary Fig. 7A, the stress-strain relationship curve was shifted to the right, indicating that the mesenteric arteries from Adipo-MROE mice are more elastic than the Control-MR mice.

Using Elastic Van Gieson stain we show that elastin content was higher, as assessed qualitatively and semi-quantitatively, in mesenteric arteries from Adipo-MROE mice compared to the Control-MR mice (Supplementary Fig. 7B, 7C). This is further supported by upregulation of *elastin* mRNA levels in mesenteric arteries from Adipo-MROE mice compared to the Control-MR mice (Supplementary Fig. 7D).

Upregulation of ACE2/Ang (1-7)/Mas system in Adipo-MROE mice.

Another molecular mechanism that could be involved in the hypocontractility is the counter-regulatory pathway of the renin-angiotensin-aldosterone system, the ACE2/Ang (1-7)/Mas receptor system, which has been reported to contribute to visceral obesity (29-31). *Ace2* and *Mrga* (Mas receptor) mRNA levels were upregulated in EVAT of Adipo-MROE mice by 5-fold and 2-fold

respectively. Moreover, *Mrga* transcripts levels were increased in mesenteric arteries of Adipo-MROE mice, but not *Ace2* (Supplementary Table 4).

Pro-inflammatory phenotype in adipose tissue from Adipo-MROE mice.

Insulin resistance and visceral obesity are commonly associated with chronic, low-grade inflammatory state. Thus the mechanisms whereby adipose tissue causes alterations in insulin sensitivity could include the secretion of pro-inflammatory factors by the adipose tissue. Moreover, these adipo(cyto)kines and chemokines would also represent candidates/mediators through which adipose tissue can modulate vascular function.

We evaluated by real time PCR mRNA levels of specific markers of macrophages: in EVAT and PVAT from Adipo-MROE mice vs Control-MR mice, levels of transcripts of *F4/80* and *Cd-68* are about 2 fold-increased suggesting that adipose tissues from Adipo-MROE contains more macrophages. We then assessed the two activation states of macrophages: M1 (pro-inflammatory, e.g IL-6, IL-12, MCP-1 (monocyte chemotactic protein-1), Tnf-α (tumor necrosis factor-α), Rantes) and M2 (anti-inflammatory e.g adiponectin, IL-10, Cd-206 (mannose receptor)). Interestingly, in EVAT and PVAT from Adipo-MROE some pro-inflammatory markers mRNA levels (*Il-6*, *Mcp-1* and *Rantes*) are increased, whereas mRNA levels of anti-inflammatory markers are decreased (*Adiponectin* and *Cd-206*). Results are summarized in Supplementary Tables 5.

Discussion

We previously demonstrated that MR over-activation specifically in adipocytes leads to significant metabolic abnormalities including increased visceral adiposity, insulin resistance, and dyslipidemia (14). Here, we examined the potential vascular comorbidities that might accompany these metabolic alterations. These studies were prompted by our previous findings where we demonstrated that increased expression of adipocyte MR in obese diabetic mice and humans was associated with vascular dysfunction (13) but a causal relationship was not established. Using mice that conditionally overexpress MR in an adipocyte-specific manner, we found that vascular contractility was markedly reduced, whereas endothelial function was normal. These phenomena were associated with increased H_2O_2 generation by adipocytes leading to upregulation of redox-sensitive PKG-1 and downregulation of vascular ROCK (Fig. 6). Adipose tissue from Adipo-MROE lost its

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anticontractile properties possibly by increasing secretion of contractile factors, as evidenced by our experiments with ACM on contractility of healthy arteries of Control-MR. Serine-307 phosphorylation of IRS1 has been highlighted as a molecular event that causes insulin resistance (24, 26). In our studies, the increase in phosphorylation IRS1 at serine-307 confirms the insulin resistant phenotype of the Adipo-MROE mice. Together these findings indicate that upregulation of adipocyte MR has significant impact, not only on metabolic parameters, but also on vascular function. Increasing evidence supports a role for adipocyte MR over-activation in pathophysiological conditions associated with metabolic disorders and cardiovascular diseases. For example, 1) there is increased expression and activation of MR in adipose tissue in experimental and human obesity, diabetes, and cardiovascular diseases (6, 14, 15, 32); 2) MR antagonism with eplerenone or spironolactone improves metabolic disorders and insulin resistance in obese and diabetic mice (9, 18, 19, 33); 3) Adipo-MROE mice exhibit features of metabolic syndrome (14); and 4) MR antagonism in obese mice improves vascular function (10,13). Here we further support this thesis by showing that increased activation of adipocyte-specific MR influences functional properties of arteries. The vascular phenotype identified in Adipo-MROE mice (which are obese and have features of metabolic syndrome but no hypertension) is unusual in that it shows no apparent endothelial dysfunction but exhibits hypocontractility of small arteries. Our transgenic mouse model has some special characteristics that differ to other models of obesity, such as the *db/db* mice. Our model did not display global obesity, did not have increased levels of aldosterone, and did not exhibit altered MR signaling in the vasculature. They did however show features of hyperelasticity and upregulation of the protective axis of the renin-angiotensin-system, namely Ang-(1-7)/Mas, which may explain, in part, why the Adipo-MROE mice do not have endothelial dysfunction associated with the weight gain and why the vessels are hypocontractile. Other models have also demonstrated hypocontractility of vessels in pathological conditions. For example, in portal hypertension, ROS (in particular H_2O_2) levels are increased and vascular contractility is reduced. In Ccl4-induced cirrhotic rats, H_2O_2 has been directly implicated in regulating mesenteric hypocontractility to noradrenaline through Rho kinase signaling pathway (34). Another study in splanchnic vessels of patients and rats with cirrhosis suggested a role for ACE2/Ang (1-7)/Mas axis to explain the vascular hypocontractility (35). In our

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study, *Ace2* and *Mrga* (Mas receptor) gene expression were upregulated in EVAT of Adipo-MROE mice, supporting other studies which demonstrated hypocontractility (35).

One of the major signaling pathways regulating vascular smooth muscle contraction is the RhoA/ROCK signaling pathway and its downstream targets (36). The contractile state of vascular smooth muscle is driven by phosphorylation of the regulatory protein, MLC and reflects the balance of the Ca^{2+}-calmodulin-dependent myosin light chain kinase and myosin light chain phosphatase (MLCP) activities (37). Phosphorylation of MYPT1 at Thr-696 plays a dominant role in MLCP inhibiton and has been shown to be increased in hypertension (38, 39). The ezrin-radixin-moesin (ERM) proteins are also downstream targets of RhoA and are involved in cytoskeletal organization. We found a significant decrease in ROCK activity and decreased activation of ROCK-dependent signaling proteins, including MLC, MYPT1 and ERM in vessels from Adipo-MROE mice. Downregulation of vascular RhoA/ROCK signaling may contribute to impaired contractility in an endothelium-independent manner as it was previously reported (40-42). This is unusual as well, since in the context of metabolic syndrome, obesity or diabetes, animals displayed increase in MR activity associated with increased ROCK activity (6, 43, 44). In addition, plasma aldosterone levels are increased in obese diabetic human and animals, whereas in Adipo-MROE mice, aldosterone plasma levels are unchanged and MR over-activation is restricted to the adipocytes.

Adipocyte MR over-activation leads to an increase in ROS production by adipocytes, which is also evident in adipose tissue from obese mice (45) and humans (46). Functionally, increased H_2O_2 has been associated with either vasoconstriction (47-49) or vasodilation (50-52) through activation of PKG-1. Activation of vascular PKG-1 was increased in Adipo-MROE mice, which may also impact on dysregulated VSMC contractility in these mice. Indeed, pharmacological inhibition of PGK-1 activity blunted mesenteric hypocontractility in Adipo-MROE mice. Hence, we demonstrated a direct role of MR-dependent ROS production by adipocytes on the vasculature. *In vitro* studies in cultured VSMCs indicated that exposure to H_2O_2 induced significant downregulation of ROCK and increased activation of PKG-1 pathways involved in VSMC contraction/dilation. The *ex vivo* studies recapitulated what we observed in Adipo-MROE mice, unravelling a key mechanism underlying the crosstalk between adipose tissue and vascular function.

Putative mechanisms whereby adipocyte MR-induced H_2O_2 generation regulates vascular function through PKG-1 likely involve cGMP, since PKG-1 regulates nitric oxide synthase (NOS) activation and NO production, which in turn activates guanylyl cyclase resulting in elevated cGMP, a potent regulator of vascular smooth muscle cell dilation (53). In addition to this classical pathway, recent evidence indicates that H_2O_2 can directly activate cGMP via compound I, which activates PKG-1 resulting in changes in activity or function of various serine/threonine proteins (54-56). Moreover, H_2O_2-dependant oxidation/activation of PKG-1 is associated with vasodilation (57). Accordingly, a possible link between adipocyte-derived H_2O_2 and PKG-1-associated changes in vascular function may be cGMP.

In summary, our *in vivo* study demonstrates that mice with conditional upregulation of adipocyte MR, which nave metabolic syndrome and obesity, exhibit impaired vascular contractility through processes that involve adipocyte-derived H_2O_2, which influences vascular redox-sensitive PKG-1 and Rho kinase pathways. Mechanisms associated with these vascular changes may relate to increased elastin content and upregulation of the protective axis of the renin-angiotensin system. Our study further highlights important crosstalk between adipocytes and vascular cells and indicates that activation of the adipocyte MR system leads to production of vasoactive adipocyte-derived factors, such as H_2O_2, which may impact on vascular dysfunction in conditions associated with metabolic disorders. These novel findings emphasize the functional importance of adipocyte MR in cardiovascular complications associated with obesity/adiposity, insulin resistance and metabolic syndrome.

Authors Contributions.

A.N.D.C. designed and performed experiments; analyzed data; interpreted results of experiments; prepared figures and tables; and drafted, edited, revised, and approved the final version of the manuscript. T.T.A., G.E.C., A.S. performed experiments; analyzed data; interpreted results of experiments; reviewed and approved the final version of the manuscript. S.T., M.G.D.L., A.A., Y.H. performed experiments; reviewed and approved the final version of the manuscript. A.C.M., F.J., R.M.T. provided guidance on experiments design; interpreted results of experiments; reviewed, and approved the final version of the manuscript. R.M.T. is the guarantor of this work and as such, had full access to all the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Parameters	Control-MR (7)	Adipo-MROE (10)
Plasma Aldosterone (pg/mL)	432 ± 45	388 ± 29
Plasma Corticosterone (ng/mL)	1064 ± 86	1105 ± 84
Plasma Angiotensin II (pg/mL)	476 ± 59	573.6 ± 70
Aldosterone in ACM (pg/mL/μg RNA)	272 ± 48	347 ± 96
Corticosterone in ACM (ng/mL/μg RNA)	2088 ± 378	2297 ± 403
Angiotensin II in ACM (pg/mL/μg RNA)	852 ± 295	705 ± 217

Table 1. Aldosterone, Corticosterone and Angiotensin II levels in plasma and adipocyte-conditioned culture media from EVAT from Adipo-MROE and littermate Control-MR mice.

Values are means ± SE. ACM, adipocyte culture conditioned medium; EVAT, Epididymal visceral adipose tissue.

Figures legends.

Figure 1. Effect of adipocyte conditioned medium on contractility of mesenteric arteries from Adipo-MROE and Control-MR mice.
A and B: Contractile responses to cumulative and increasing doses of phenylephrine (Phe, 10^{-9} to 10^{-5}M) in mesenteric arteries with and without intact endothelium were significantly decreased in Adipo-MROE vs Control-MR mice. C and D: Effect of adipocyte conditioned culture medium (ACM) on contractile responses to Phe of arteries from Control-MR mice and from Adipo-MROE mice. For A, B, C and D, two-way ANOVA test was performed, followed by Bonferroni test. Data were performed in repeated measures and presented as mean ± SE; n=8 to10 mice/group. * p<0.05 Adipo-MROE vs Control-MR.

Figure 2. ROCK signaling in mesenteric arteries from Adipo-MROE and control-MR mice.
A-C: Downregulation of downstream targets of RhoA/Rho kinase activation. The myosin phosphatase subunit 1 (MYPT1) (A), the ezrin, moesin, radixin (ERM) (B) and the myosin light chain (MLC_{20}) (C) protein phosphorylations were significantly decreased in mesenteric arteries from Adipo-MROE vs Control-MR mice. Western blotting was performed using Mypt1 and P-Mypt1 (Thr^{696}), ERM and P-ERM (Thr^{567}, Thr^{564}, Thr^{558}), MLC_{20} and P-MLC_{20} (Thr^{18}/ Ser^{19}) antibodies. Integrated intensities were obtained by the Image J software. For A-C: Mann-Whitney nonparametric test was performed. Data represent means ± SE; n=10 mice/group. * p<0.05, ** p<0.01, Adipo-MROE vs Control-MR.

Figure 3. Adipocyte-specific MR overexpression: increased vascular PKG-1 protein levels and H_2O_2 production by adipocytes. A: PKG-1 protein levels are upregulated in mesenteric arteries from Adipo-MROE vs Control-MR mice. B: Hydrogen peroxide levels in adipocytes culture medium, as well as in EVAT from Adipo-MROE versus Control-MR mice. For A and B: Mann-Whitney nonparametric test was performed. Data represent means ± SE; n= 6 to 7 mice/group. * p<0.05, ** p<0.01, *** p<0.001, Adipo-MROE vs Control-MR.

Figure 4. Exogenous H_2O_2 increases expression of PKG-1 and decreases activity of ROCK in mouse VSMCs. A: Dose-Response curve. VSMCs from mouse mesenteric arteries were stimulated with hydrogen peroxide 10^{-6}M to 10^{-3}M for 24 hours. B: VSMCs from mouse mesenteric arteries were stimulated with H_2O_2 10^{-3}M in the absence and presence of a specific PKG-1 inhibitor of or an antioxidant, for 24 hrs: ROCK kinase activity in H_2O_2-stimulated VSMCs. Values are expressed as means ± SE. n=8 individual experiments. C: ROCK signaling in VSMCs. Protein levels of a marker of ROCK activation, ERM phosphorylation is significantly decreased by hydrogen peroxide 10^{-3}M. For A, B and C: one-way ANOVA test was performed, followed by Bonferroni test. Data represent means ± SE; n=8 individual experiments. ** p<0.01 vs Vehicle, †† p<0.01 vs H_2O_2. Antioxidant: PEG-catalase, 1000 U/mL; PKG-1 inhibitor: DT-3, 10^{-8} M.

Figure 5. Effect of PKG-1 inhibition on vascular reactivity in Adipo-MR mice.
Contractile and vasodilatory responses of fat-free mesenteric resistant arteries, from Adipo-MROE mice and their Control-MR littermates were evaluated using a wire myography. A: Pre-incubation

with a specific PKG-1 inhibitor, 30 minutes prior to concentration responses curves to phenylephrine (Phe, 10^{-9} to 10^{-5}M), of endothelium-denuded mesenteric arteries from Adipo-MROE mice, restores the contractile responses to levels comparable with Control-MR mice. B: Pre-incubation with a specific PKG-1 inhibitor 30 minutes prior to concentration responses curves to H_2O_2 (10^{-6} to 10^{-3}M) of mesenteric arteries with intact endothelium has similar effects in relaxant responses between Adipo-MROE and Control-MR mice. For A and B: two-way ANOVA test was performed, followed by Bonferroni test. Data were performed in repeated measures and presented as mean $\pm$ SE; in brackets are indicated the number of animals per group. * $p<0.05$ Adipo-MROE vs Control-MR. PKG-1 inhibitor: Rp-8-Br-PET-cGMPS, 3×10^{-5} M.

Figure 6. Hypothetical scheme of the signaling mechanisms induced by aldosterone/MR over-activation in adipocytes. Adipocyte-specific MR over-activation leads to decrease in contractility of mesenteric arteries from Adipo-MROE vs Control-MR mice. This may involve down-regulation of Rho kinase activity and signaling in arteries through hydrogen peroxide (H_2O_2)-induced PKG-1 activation. ROS, reactive oxygen species; Sgk1, serum and glucocorticoid regulated kinase-1.