MR (Mineralocorticoid Receptor) Induces Adipose Tissue Senescence and Mitochondrial Dysfunction Leading to Vascular Dysfunction in Obesity

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The mineralocorticoid receptor induces adipose tissue senescence and mitochondrial dysfunction leading to vascular dysfunction in obesity.

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Abstract

Adipose tissue (AT) senescence and mitochondrial dysfunction are associated with obesity. Studies in obese patients and animals demonstrate that the mineralocorticoid receptor (MR) contributes to obesity-associated cardiovascular complications through its specific role in AT. However, underlying mechanisms remain unclear. This study aims to elucidate whether MR regulates mitochondrial function in obesity, resulting in AT premature aging and vascular dysfunction.

Obese (db/db) and lean (db/+) mice were treated with an MR antagonist or a specific mitochondria-targeted antioxidant. Mitochondrial and vascular functions were determined by respirometry and myography, respectively. Molecular mechanisms were probed by western immunoblotting and real-time PCR in visceral AT and arteries and focused on senescence markers and redox-sensitive pathways.

Db/db mice displayed AT senescence with activation of the p53-p21 pathway and decreased sirtuins levels, as well as mitochondrial dysfunction. Furthermore, the beneficial anti-contractile effects of perivascular AT were lost in db/db via Rho kinase activation. MR blockade prevented these effects.

Thus, MR activation in obesity induces mitochondrial dysfunction and AT senescence and dysfunction, which consequently increases vascular contractility. In conclusion, our study identifies novel mechanistic insights involving MR, adipose mitochondria and vascular function that may be of importance to develop new therapeutic strategies to limit obesity-associated cardiovascular complications.

Keywords: mineralocorticoid receptor, mitochondrial dysfunction, senescence, obesity, vascular reactivity.

Non-standard Abbreviations and Acronyms

CVD	cardiovascular diseases
EVAT	epididymal visceral adipose tissue
GDP	guanosine diphosphate
MetS	metabolic syndrome
mtROS	mitochondrial ROS
MLC	myosin light chain
MR	mineralocorticoid receptor
MRA	MR antagonist
MT	mito-TEMPO
MYPT1	myosin light chain phosphatase subunit 1
NE	norepinephrine
PVAT	perivascular adipose tissue
ROCK	rho kinase
ROS	reactive oxygen species
SIRT	sirtuins
Spiro	spironolactone
UCP	uncoupling protein
WAT	white adipose tissue

Introduction

Obesity and its associated cardiovascular risk factors have reached epidemic proportions and are leading causes of morbidity and mortality worldwide. Pathophysiological processes linking obesity to cardiovascular diseases (CVD) still remain unclear, but aldosterone and its mineralocorticoid receptor (MR) may be of importance, as aldosterone plasma levels and MR expression in adipose tissue are increased in both animal models and subjects with obesity[1–3]. Under physiological conditions, aldosterone/MR modulates blood pressure by regulating water and sodium homeostasis in the kidney[4]. MR is also expressed in non-epithelial cells such as cardiomyocytes, vascular cells and adipocytes[5], and due to its activation, the mineralocorticoid system may play an important role in the pathogenesis of obesity, independently of the renal effects of aldosterone[3,5,6].

Several reports pointed out the association between adipose tissue premature aging and obesity[7–9]. Cellular senescence is characterized by stable growth arrest, implemented by the activation of cell cycle inhibitors such as the $p16^{INK4a}$/Rb and $p53/p21^{CIP1}$ tumor suppressor networks[10]. Studies have reported the activation of these pathways in the adipose tissue of obese animals[11,12]. Although it is unclear whether the aging-related changes in obesity result from senescence of the adipose tissue itself or from accumulated environmental stress, cell senescence could trigger adipose tissue and vascular dysfunctions. Moreover, mitochondrial dysfunction with increased production of mitochondrial reactive oxygen species (mtROS) is also observed in obesity[13] and may be linked to the process of adipose tissue aging[9,14].

The role of MR in adipocytes is especially interesting in the context of obesity-related metabolic diseases and CVD. Indeed, clinical[15,16] and experimental[6,17,18] studies, where the MR has been shown to be over-activated, in particular in the adipose tissue, have demonstrated its implication in CVD, obesity and metabolic syndrome (MetS). Obese

patients and obese rodents display high plasma levels of aldosterone, as well as increased aldosterone production by adipocytes with an impact on vascular signaling, as our team previously demonstrated[19,20]. These studies pointed out a possible important role of adipocytes-secreted aldosterone in obesity. However, the enzyme 11-beta-hydroxy-steroid dehydrogenase type 2 is not or weakly expressed in adipocytes. The role of this enzyme is to convert glucocorticoids into inactive metabolites, thus allowing the selectivity between aldosterone and MR, such as in the distal part of the nephron in kidney. Thus, glucocorticoids might be the primary ligands activating the MR in adipocytes.

Furthermore, a study reported the critical role of MR activation in mediating renal proximal tubular cells senescence in human[21]. In aged mice and rats, the MR is implicated in hypertension and age-related vascular stiffening and inflammation[22–24]. Studies have also linked mtROS production to MR activation in the myocardium and in kidney cells[25,26].

Our previous studies determined that functional MR over-activation in obesity is associated with adipose tissue and vascular dysfunctions[27–29]. We now question whether MR over-activation in obesity leads to adipose tissue senescence, possibly through modulation of the mitochondrial function, and that it ultimately results in vascular complications. To this end, obese db/db mice were treated with an MR antagonist (MRA) and we evaluated the effects on markers of senescence, mitochondrial respiration, ROS production in the epididymal visceral adipose tissue and pro-contractile signaling pathways in the vasculature. We used mito-TEMPO to treat *in vivo* db/db mice in order to delineate a causal mechanism for mitochondrial dysfunction on obesity-associated vascular injuries. Together, the data implicate MR in white adipose tissue (WAT) premature aging, mitochondrial dysfunction and alterations in vascular contractile responses.

Material and Methods

Detailed methodology is provided in the Online Data Supplement.

Animal model

All animal experiments were approved by the Animal Ethics Committees of the Cordeliers Research Centre in Paris and Uppsala University in Sweden and in accordance with the INSERM guidelines and European Community directives for the care and use of laboratory animals.

Male, 8 week-old, db/db mice, a genetic model of obesity-related type 2 diabetes mellitus, and age-matched heterozygote lean non-diabetic control db/+ mice were treated with vehicle (saline), potassium canrenoate for 4 weeks (MRA, 30 mg/kg body weight/day) or mitochondria-targeted antioxidant mito-TEMPO (0.7 mg/kg body weight/day) for 2 weeks, by daily subcutaneous injections. Mice were euthanized by retrieving the heart and tissues were collected. Mesenteric resistance arteries were used to assess vascular reactivity by wire myography. Mitochondria from epididymal visceral adipose tissue (EVAT) were isolated and used for high throughput respirometry. Molecular biology studies were performed in EVAT and in arteries.

Cell culture of mouse differentiated 3T3-L1 adipocytes

To interrogate the molecular mechanisms underlying mitochondrial dysfunction in db/db mice, we studied cultured 3T3-L1 adipocytes as previously described[20]. Differentiated 3T3-L1 adipocytes were stimulated with aldosterone (10^{-8} mol/L), the MRA spironolactone (spiro, 10^{-6} mol/L) or a combination of both for 24 hours (n=4 individual sets of experiments, vehicule=DMSO).

Mitochondrial function determination

Mitochondria isolation

Mitochondria were isolated from EVATs following a specific procedure detailed in Methods in the online supplemental data.

Measurement of in vitro oxygen consumption by high resolution respirometry

Oxygen consumption and H_2O_2 production in isolated mitochondria were measured with an Oroboros O_2K (Oroboros Instruments, Innsbruck, Austria). The procedure is detailed in the online supplemental data.

Mitochondrial respiration determination in 3T3-L1 adipocytes with Seahorse Assay

Bioenergetic profiles of 3T3-L1 adipocytes were determined using a Seahorse Bioscience XF24 Analyzer (Billerica, MA, USA), providing real-time measurement of the oxygen consumption rate (OCR) (Figure 3B). The effects of aldosterone and spiro on mitochondrial function were then evaluated using The Agilent Seahorse XF Cell Mito Stress Test, after addition of: oligomycin (inhibitor of ATP synthase), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, uncoupling agent) and rotenone/antimycin A (inhibitors of complexes I and III of the respiratory chain, respectively). For the calculations of the different parameters (basal, non-mitochondrial, ATP-linked, maximal respiration, proton leak and mitochondrial reserve capacity), see Methods in the online supplemental data.

Vascular studies

Second order branches of mesenteric arteries were isolated from db/db and db/+ mice, treated or not with MRA or mito-TEMPO and mounted with and without perivascular adipose tissue (PVAT) on a wire myograph (DMT620M Multi Myograph System, Danish Myotechnology) as previously described[29]. Concentration-response contraction curves to increasing doses of norepinephrine (NE, 10^{-9} to 3.10^{-5} mol/L) were performed in arteries with intact endothelium.

Western blot analysis

EVAT and aortas of db/+ and db/db mice were lysed for western blotting as previously described[28]. Antibodies are listed in Table S1.

Quantitative real time Polymerase Chain Reaction

Total RNA was extracted from EVAT of db/+ and db/db mice as previously described[28]. Primers sequences are listed in Table S2.

Data Analysis

Data are presented either as box plots with medians, max and min values or as means ± SE. Differences between groups were assessed with the two-way ANOVA test, followed by the Tukey multiple comparison test, as appropriate. Values of $p<0.05$ were considered significant.

Results

Body and tissue masses.

Body weights (BW) were increased in obese db/db compared to lean db/+ mice (Table S3). Treatment with the MRA K canrenoate had no effect on BW. EVAT weights (normalized to tibia length) were 3.5-fold higher in db/db vs db/+ mice, which is consistent with previous reports of adipose tissue hypertrophy in db/db mice. Treatment with K canrenoate had no effect on adipose tissue hypertrophy (Table S3).

MR activation is associated with senescence of adipose tissue in obese db/db mice.

Aging of the adipose tissue in obesity is a major risk factor in the development and progression of the disease and its associated cardiovascular complications[30]. To investigate whether MR activation in obesity is associated with senescence of the adipose tissue, we analyzed the levels of expression of markers of senescence. A general marker of DNA damage is the serine 139-phosphorylated histone (γ-H2A.X). In the EVAT of db/db, γ-H2A.X was 3-fold increased compared to db/+ mice (Figure 1A). In addition, the cell cycle inhibitors *p53*, *p21* and *p16* mRNA levels were significantly increased. K canrenoate abolished the increase in *p53* and *p21*, but not *p16* (Figure 1B), suggesting that MR activation regulates cell cycle mediators in adipose tissue. The p53-p21 pathway downstream target Serine 36-phosphorylated p66Shc was 3.5-fold increased in the EVAT of db/db vs db/+ mice and significantly reduced by the MRA treatment (p=0.0162) (Figure 1C). Sirtuins are deacetylases that play a pivotal role in obesity-associated premature aging[31]. We found a

significant decrease in SIRT1 and SIRT3 protein levels in the EVAT of db/db compared to db/+ mice. The MRA treatment restored the levels of both SIRT1 and SIRT3 (Figure 1D).

MR blockade improves obesity-associated mitochondrial dysfunction in the EVAT of db/db mice.

Aging is closely associated with oxidative stress. We and others previously showed that db/db display systemic and local oxidative stress with an increase in ROS generation, particularly by the adipose tissue[13,29,32,33]. We questioned whether senescence of adipose tissue is associated with mtROS production and dysfunction in EVAT with the hypothesis that adipose mitochondria play a major role in bioenergetics and oxygen consumption changes in db/db mice in an MR-dependent manner. We determined mitochondrial H_2O_2 levels and characterized mitochondrial function in the EVAT of db/db vs db/+ mice, treated or not by K canrenoate. Db/db mice displayed increased levels of mitochondrial H_2O_2 in EVAT compared to db/+, which was prevented by the MRA treatment (Figure 2A). Mitochondrial respiration parameters were then evaluated. Mitochondrial state 3 respiration was unchanged between groups (Figure 2B), whereas state 4 respiration (independent of ATP production) was significantly increased in db/db vs db/+ mice (Figure 2C) due to uncoupling protein (UCP) activation (Figure 2D), as indicated by the decrease in oxygen consumption following administration of the specific UCP inhibitor GDP. MRA treatment prevented these effects (Figure 2C and 2D).

MR activation regulates mitochondrial function in cultured 3T3-L1 adipocytes.

Our *in vivo* data in db/db mice suggest that MR activation can regulate mitochondrial function in adipocytes. We confirmed these results with *in vitro* experiments in differentiated 3T3-L1 adipocytes stimulated with aldosterone in the absence and presence of spiro, an MR antagonist. Stimulation with aldosterone led to an increase in oxygen consumption rate (OCR) as an indicator of mitochondrial respiration, which was prevented by spiro (Figure 3A

and 3B). Indeed, maximal mitochondrial respiration and reserve capacity were increased in 3T3-L1 stimulated by aldosterone, and these effects were blocked by spiro, whereas aldosterone did not change the parameters related to basal, ATP-linked, non-mitochondrial respiration or proton leak (Figure 3C). This was associated with an MR-dependent increase in H_2O_2 production by 3T3-L1 adipocytes (Figure S1)

Adipose tissue dysfunction mediates the loss of anti-contractile effects of PVAT in db/db obese mice.

To investigate how obesity-associated adipose tissue dysfunction impairs the modulation of resistance arteries contraction by the perivascular adipose tissue (PVAT), mesenteric arteries with and without PVAT were exposed to increasing doses of the vasoconstrictor norepinephrine (NE). Maximal responses to high KCl were identical between db/+ and db/db mice (Table S4). Control healthy PVAT displayed anti-contractile properties as indicated by the rightward shift of the NE log concentration-response curve and the decrease in the log(EC50) (inversely correlated to the sensitivity to the vasoconstrictor) in the presence of PVAT (Figure 4A, white circle vs black circle). In obesity these anti-contractile properties are lost as shown by the leftward shift of the NE log concentration-response curve and the increase in the log(EC50) in the presence of PVAT (Figure 4A, black square vs black circle).

We questioned the contribution of adipose MR regarding obesity-associated PVAT dysfunction. For this, we compared the effect of the MR antagonist treatment on vascular function in the absence and presence of PVAT. Interestingly, *in vivo* MR blockade exerted beneficial effects on sensitivity to NE in db/db mice arteries only in the presence of PVAT (Figure 4B).

We examined whether an increase in mtROS generation in PVAT of obese db/db mice mediated the effect of obesity-related anti-contractile effects of PVAT. *In vivo* inactivation of ROS by the mitochondria-targeted scavenger mito-TEMPO did not have the same beneficial

effect as MRA, since it partially shifted the NE concentration-response curve to the right but without significant modification of the log(EC50) (Figure 4C).

Mitochondrial ROS modulates the anti-contractile effects of PVAT in obesity via Rho kinase signaling in vascular smooth muscle.

Vascular Rho kinase signaling was over-activated in the arteries of obese db/db mice, since downstream targets of Rho kinase activation, MYPT1 and MLC phosphorylated forms, were significantly increased in db/db versus db/+ mice. MR blockade prevented MYPT1 and MLC phosphorylations as previously found[29] (Figure 5A and 5B). We next examined whether increased mtROS induced vascular Rho kinase signaling. We observed that treatment with the mitochondria-targeted scavenger mito-TEMPO only partially improved Rho kinase signaling, as mito-TEMPO partially decreased MYPT1 and MLC phosphorylations in db/db (Figure 5C and 5D), indicating a reduction in the Rho kinase pathway activation.

Discussion

We have summarized our findings in Figure 6. To our knowledge, the present study is the first to examine the regulation of adipose tissue mitochondrial respiration by MR in obesity. While previous studies in cultured renal cells[34] and cardiac fibroblasts[35] have demonstrated that MR activation can induce increased mtROS production, our *in vivo* and *in vitro* data show that MR activation regulates adipose tissue mitochondrial respiration. Indeed, while our results in db/db mice show an impact of systemic MR blockade on adipose tissue mitochondrial function, our *in vitro* studies in differentiated 3T3-L1 adipocytes stimulated with aldosterone lead us to the conclusion that adipocyte mitochondrial function is directly regulated by the MR, since OCR, used as an indicator of mitochondrial respiration, and H_2O_2 production are increased and these effects are abolished by MRA administration. Additionally, experiments with isolated mitochondria from the EVAT of db/db mice strongly suggest that the increased oxygen consumption is, at least in part, due to the activation of

UCPs, as oxygen consumption is increased independently of ATP-production and can be blocked by a specific UCP inhibitor. Importantly, mitochondrial uncoupling could be a compensatory mechanism related to the elevated mtROS production in the EVAT mitochondria of db/db mice[36]. Indeed, a mild uncoupling has been shown to lower the mitochondrial membrane potential and to limit ROS production in a variety of tissues (macrophages, pancreatic islet cells, muscle)[37]. Interestingly, previous reports demonstrated that MR can regulate uncoupling proteins expression in adipocytes, thus participating to energy expenditure control[38] and brown adipose tissue remodeling[39]. From the literature and our data, it is not clear whether the increased mtROS production is a cause or a consequence of WAT dysfunction. However, our data unambiguously show that mitochondria in the EVAT of db/db mice display hallmarks of mitochondrial dysfunction that are regulated in an MR-dependent manner. For practical reasons we studied mitochondrial function and senescence in the EVAT, however PVAT has also been shown to display oxidative stress from mitochondrial origin in mice fed with a high fat diet, suggesting that both EVAT and PVAT have mitochondrial dysfunction and oxidative stress in obesity[40,41]. The detailed cause and effect relationship between MR activation, mitochondrial dysfunction, AT senescence will be determined in future molecular studies.

In our study, we showed MR-dependent senescence of adipose tissue. Our results showed that MR regulates specifically the p53-p21 signaling pathway but not the p16-dependent pathway. These two pathways are distinct: the p53-p21 pathway is responsible for the induction of cell senescence and is reversible, whereas the $p16^{INK4a}$/Rb pathway is responsible of the maintenance of senescence and is irreversible[42,43]. This may be why the MRA treatment has an effect on *p53* and *p21* but not *p16*. Aldosterone/MR has previously been demonstrated to induce cellular senescence in vessels[22,23] and in kidney through a ROS/SIRT1/p53/p21-dependent pathway[21]. Indeed, aged mice deleted for MR gene

specifically in vascular smooth muscle cells are protected against aging-associated vascular dysfunctions including rise in blood pressure and vasoconstriction[22].

Sirtuins are highly conserved histone deacetylases that promote longevity[31]. In the literature, they have been reported to regulate aging process through down-regulation of oxidative stress-dependent mechanisms. For instance, sirtuins targets include p53[44]. Interestingly, sirtuins also regulate mitochondrial function[45]. In pancreatic beta cells, it has been reported that SIRT1 can decrease expression of the uncoupling protein UCP2 by directly binding to its promoter, impacting insulin secretion[46]. Several studies reported the decrease in sirtuins in obese animals[47] and humans[48,49]. One can interrogate whether the decrease in SIRT1 and SIRT3 protein levels in our studies occur upstream or downstream the mitochondrial dysfunction in obese db/db mice. We propose that in obesity there is a vicious cycle that involves sirtuins downregulation, mitochondrial dysfunction and aging-related processes. Further studies are needed to determine the exact order of the cascade of events.

PVAT is a dynamic endocrine organ capable of exerting an anti-contractile effect by releasing "adipocytokines" such as adiponectin, nitric oxide, or H_2O_2[50,51]. As obesity is associated with an increase in PVAT mass, it would be intuitive to expect an increased anti-contractile effect of the PVAT due to enhanced release of PVAT-derived factors. However, obesity leads to PVAT remodeling and dysfunction, resulting in the loss or attenuation of its anti-contractile effect[52]. Mechanisms involved may include increased oxidative stress[53], as well as hypoxia and inflammation, resulting in dysregulation of adipokines secretion[52,54].

Our studies elucidated whether adipose MR participates in adipose tissue dysfunction-associated vascular injuries through the modulation of PVAT mitochondrial function. By comparing the vascular function of arteries mounted with or without PVAT, we found that obese db/db mice lost the beneficial anti-contractile capacity of PVAT through an MR-dependent signaling pathway, possibly through activation of ROCK signaling, as we have

previously published the close interaction of ROCK signaling and adipose MR[28,29] in the modulation of the vascular tone. Mitochondrial oxidative stress seems to be only partially implicated in the obesity-associated loss of anti-contractile effect of PVAT since the treatment with mito-TEMPO did not fully restore the vascular function, in contrast to the MRA treatment. This may suggest that other mechanisms are involved which depend upon MR activation but not upon mtROS. Hence, the underlying mechanisms of MR-dependent PVAT dysfunction impacting the vascular function remain to be determined. Of note, our group already identified two potential mechanisms depending on excess of H_2O_2[28] and mtROS (present study) that triggered obesity-associated vascular dysfunction.

Additional work is needed to identify specific molecular mechanisms and targets by which MR modulates mitochondrial function. This may represent new therapeutic targets to limit the vascular complications occurring in obesity. However, some further studies will be necessary to identify the molecular mechanisms and targets by which MR is regulating mitochondrial function in adipose tissue and to study the contribution of other mechanisms such as the contribution of (new) adipokines which are dependent of adipocyte-MR activation. Moreover, it will be necessary to evaluate the potential side-effects of MR antagonist therapy, as MR antagonists may induce hyperkalemia in some patients. Besides, we are using K canrenoate, a nonselective MR antagonist, and the comparison with the effects of a more selective (eplerenone) or nonsteroidal (finerenone) MR antagonist may be of interest to overcome the adverse effects of MR antagonist of first generation such hormonal side-effects.

Perspectives

Besides its well-known physiological effects, MR activation induces injuries in the cardiovascular system that are part of the pathogenesis of various metabolic and

cardiovascular diseases including obesity, diabetes and hypertension. Therefore, the characterization of the mechanisms by which MR contributes to obesity-associated vascular complications will advance knowledge and is essential for the development of new effective therapies.

The results of the present study show that MR activation induces mitochondrial oxidative stress and mitochondrial respiratory dysfunction as well as premature aging in the adipose tissue of obese animals. These effects are reduced by MR blockade using K canrenoate.

The beneficial effects of MRA could further improve survival and quality of life in obese patients. However, adverse effects of these drugs, such as hyperkalemia, should be considered.

In conclusion, this is the first study that reports a direct link between MR activation with mitochondrial function and aging in adipose tissue. Mitochondrial function and integrity are necessary for adipose tissue homeostasis. Our data highlight once more the critical active role of MR on the regulation of vascular tone through mitochondrial metabolism-dependent mechanisms.

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Disclosures.

None.

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Novelty and Significance

What Is New?

- This study reports a direct link between mineralocorticoid receptor activation with mitochondrial function and aging in adipose tissue. These are new molecular mechanisms, whereby the mineralocorticoid receptor (MR) in adipose tissue influences vascular function.

What Is Relevant?

- We demonstrate that, in obesity, MR activation in the adipose tissue leads to mitochondrial dysfunction, oxidative stress and senescence, resulting in obesity-associated vascular dysfunction. We emphasized the functional importance of adipose tissue MR in the regulation of vascular tone.
- These findings identify novel players in the crosstalk between the adipose tissue and the vasculature including mitochondrial reactive oxygen species- and aging-dependent mechanisms in conditions where adipose tissue MR is hyper-activated. It highlights the critical role of adipose tissue MR which may trigger obesity-associated cardiovascular complications.

Summary

Increasing evidence indicates an important role for aldosterone/MR and perivascular adipose tissue (PVAT) dysfunction in obesity-associated cardiovascular complications. However the exact role of adipose tissue MR is unclear. Using a genetic model of obesity, the db/db mice, we demonstrated that adipose tissue MR participates to the PVAT dysfunction-associated vascular contractility alterations through the regulation of adipose tissue mitochondrial function and premature aging.

Figures legends

Figure 1: MRA treatment prevents senescence in EVAT of obese db/db mice. A: phosphorylation of H2A.X (γ-H2A.X), a marker of DNA damage, is significantly increased in db/db vs db/+ mice and corrected by the MRA treatment. B: mRNA levels of cell cycle inhibitors *p53*, *p21* and *p16* measured by real time PCR are increased in db/db mice. Only *p53* and *p21* levels are normalized by the MRA treatment. C: phosphorylation of p66Shc, a downstream effector of p53-p21 pathway, is significantly increased in db/db mice and attenuated by the MRA treatment. D: MRA treatment prevents the decrease in protein levels of deacetylases SIRT1 and SIRT3 in db/db vs db/+ mice. Data are presented in box plots as medians with max and min values. Two-way ANOVA and Tukey post-hoc test, n=6-8 mice per group. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$ vs db/+ V. † $p<0.05$, ††† $p<0.001$, †††† $p<0.0001$ vs db/db V. EVAT: epididymal visceral adipose tissue, H2A.X: phosphorylated form of histone H2A, MRA: mineralocorticoid receptor antagonist, SIRT: sirtuin, V: vehicle.

Figure 2: MRA treatment improves adipose mitochondrial function in the EVAT of obese db/db mice. The function of isolated mitochondria from EVAT was assessed by high resolution respirometry and by fluorometry (Oroboros O2k). We measured (A) mitochondrial H_2O_2 levels, as well as several respiratory parameters including (B) state 3 respiration corresponding to ATP-linked respiration, (C) state 4 respiration corresponding to uncoupled respiration and (D) GDP-induced O_2 consumption decrease (uncoupling protein (UCP)-linked oxygen consumption). All measurements were normalized by mitochondrial protein content. Data are presented in box plots as medians with max and min values or as means ± SE. Two-way ANOVA and Tukey post-hoc test, n=6-8 mice per group. **** $p<0.0001$ vs db/+ V. †††† $p<0.0001$ vs db/db V. ATP: adenosine triphosphate, GDP: guanosine

diphosphate, H_2O_2: hydrogen peroxide, MRA: mineralocorticoid receptor antagonist, O_2: oxygen, V: vehicle.

Figure 3: Aldosterone modulates mitochondrial respiration in differentiated 3T3-L1 adipocytes in a MR-dependent manner. Mitochondrial function in differentiated 3T3-L1 adipocytes was assessed using a Seahorse XFe24 Analyzer. A-C: Bioenergetic profile of 3T3-L1 adipocytes treated with aldo (10^{-8} mol/L) in the presence or absence of spiro (MRA, 10^{-6} mol/L) for 24 hours. Aldo induces increase in maximal respiration and in reserve capacity, which is corrected by spiro. No change among all stimulation conditions in the other parameters measured: basal, ATP-linked, non-mitochondrial respiration and proton leak. Data represent means ± SE. Two-way ANOVA and Tukey post-hoc test, n=4 individual sets of experiments. * $p<0.05$, vs V. †† $p<0.01$ vs Aldo. Aldo: aldosterone, AA/R: antimycin A/rotenone, F: FCCP, O: oligomycin, OCR: oxygen consumption rate, Spiro: spironolactone, V: vehicle = DMSO.

Figure 4: MRA but not Mito-TEMPO restores the anti-contractile capacity of PVAT from db/db mice. Mesenteric arteries were mounted in the presence (left panels) and in the absence (right panels) of PVAT and contractile responses to increasing doses of NE were assessed using wire myography. A: PVAT anti-contractility is impaired in db/db vs db/+ mice as shown by the right shift of the NE log concentration-response curve (black square vs black circle). B: MRA restores the anti-contractility of PVAT in db/db mice only in the presence of PVAT (left panel). C: Mito-TEMPO partially shifts the curve to the right in the presence of PVAT (black diamond vs black square, left panel) indicating partial decrease in sensitivity to NE (not significant). Data represent means ± SE. Two-way ANOVA and Tukey post-hoc test, n=6-8 mice per group. A: **** $p<0.0001$, db/+ no PVAT vs db/+ PVAT. ††† $p<0.001$, db/db no PVAT vs db/db PVAT. ## $p<0.01$, db/+ PVAT vs db/db PVAT. B: * $p<0.05$ vs db/+ V PVAT. † $p<0.05$, vs db/db V PVAT. C: ** $p<0.01$, vs db/+ V PVAT. Mito-

TEMPO: mitochondria-targeted antioxidant, MRA: mineralocorticoid receptor antagonist, NE: norepinephrine, PVAT: perivascular adipose tissue.

Figure 5. MR and mtROS modulate Rho kinase signaling in aorta of db/db mice. A-B: MRA treatment prevents (A) MYPT1 and (B) MLC phosphorylations in aorta of db/db mice. C-D: MT treatment partially prevents (C) MYPT1 and (D) MLC phosphorylations in aorta of db/db mice. Data are presented in box plots as medians with max and min values. Two-way ANOVA and Tukey post-hoc test, n=6-8 mice per group. ** $p<0.01$, **** $p<0.0001$ vs db/+ V. †††† $p<0.0001$ vs db/+ V. MLC: myosin light chain, MRA: mineralocorticoid receptor antagonist, MT: Mito-TEMPO, MYPT1: Myosin phosphatase target subunit 1, V: vehicle.

Figure 6. Scheme summarizing the regulation of mitochondrial function and adipose tissue senescence by MR in obesity, resulting in the loss of PVAT anti-contractile properties. In obesity, MR over-activation leads to alteration of mitochondrial function with increased mitochondrial ROS production and uncoupling (shown in Figure 2) and senescence of the adipose tissue with activation of the p53-p21-p66Shc pathway and decrease in sirtuins levels (shown in Figure 1), resulting into dysfunctional adipose tissue. As a consequence, it induces loss of PVAT anti-contractile properties, defined as "PVAT dysfunction", contributing to the increased vascular contractility (shown in Figure 4). This may involve up-regulation of ROCK signaling in arteries through MR-dependent but only partial mtROS-dependent mechanisms (shown in Figure 5). ROS, reactive oxygen species, mtROS: mitochondrial ROS.