

SUPPLEMENTARY DATA

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

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Short title: Adipocyte MR induces vascular dysfunction.

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SUPPLEMENTARY DATA

Supplementary Table 1. Primers for real-time PCR analysis.

| Genes | Forward Primer | Reverse Primer |
|-----------------------|---------------------------|------------------------|
| m- <i>Ubc</i> | GGTCAAACAGGAAGACAGACGTA | CACACCCAAGAACAAGCACA |
| m- <i>Nr3c2</i> | TCACATTTTTTAACATGTGACGGC | TCCTTTTCACCAGCAAGCT |
| t- <i>Nr3c2</i> | GGCTACCACAGTCTCCCTGA | CGTTGACAATCTCCATGT |
| m- <i>Sgk1</i> | GATGGGCCTGAACGATTTTA | GGACCCAGGTTGATTTGTTG |
| m- <i>Ace2</i> | CTACAGGCCCTTCAGCAAAG | TGTCGCCATTATTTTCATCCA |
| m- <i>Mrga</i> | TTCTCCACCATCAACAGCAG | CCTGGGTTGCATTTTCATCTT |
| m- <i>Il-6</i> | TCTAATTCATATCTTCAACCAAGAG | TGGTCCTTAGCCACTCCTTC |
| m- <i>Il-12</i> | TGGTTTGCCATCGTTTTGCTG | ACAGGTGAGGTTCACTGTTTCT |
| m- <i>Mcp-1</i> | CCCACTCACCTGCTGCTACT | TCTGGACCCATTCTTCTTG |
| m- <i>Adiponectin</i> | TTGCAAGCTCTCCTGTTCTT | ATCCAACCTGCACAAGTTCC |
| m- <i>Il-10</i> | CAGAGCCACATGCTCCTAGA | TGTCCAGCTGGTCTTTGTT |
| m- <i>Cd-206</i> | GATATGAAGCCATGTACTCCTTACT | GGCAGAGGTGCAGTCTGCAT |
| m- <i>F4/80</i> | CTTTGGCTATGGGCTTCCAGTC | GCAAGGAGGACAGAGTTTATCG |

Ubc, ubiquitin C; *Nr3c2*, nuclear receptor subfamily 3, group C member 2 (MR, mineralocorticoid receptor); *Sgk1*, serum glucocorticoid-induced kinase 1; *Ace2*, angiotensin I converting enzyme 2; *Mrga*, G-coupled receptor Mas; *Mcp-1*, monocyte chemoattractant protein-1; *Il*, interleukine; m, mouse; t, total: endogenous and exogenous MR.

SUPPLEMENTARY DATA

Supplementary Table 2. MR gene expression in epididymal visceral and perivascular adipose tissues and mesenteric arteries.

| Genes | EVAT | | PVAT | | Mesenteric Arteries | |
|-----------------|------------|-------------|------------|-------------|---------------------|------------|
| | Control-MR | Adipo-MROE | Control-MR | Adipo-MROE | Control-MR | Adipo-MROE |
| m- <i>Nr3c2</i> | 1.02±0.23 | 1.12±0.37 | 1.14±0.28 | 1.11±0.28 | 1.11±0.16 | 0.92 ±0.25 |
| t- <i>Nr3c2</i> | 1.14±0.28 | 3.52±0.44** | 1.08±0.24 | 3.95±0.38** | 1.09±0.15 | 0.99±0.13 |

Values are means ± SE. n = 10 mice per group. Mann-Whitney test, ** p<0.01, Control-MR vs. Adipo-MROE. EVAT, Epididymal visceral adipose tissue; PVAT, Perivascular adipose tissue.

Supplementary Table 3. Characteristics of Adipo-MROE and Control-MR mice after 4 weeks of transgene induction.

| Parameters | Control-MR (11) | Adipo-MROE (11) |
|-------------------------------------|-----------------|-----------------|
| Blood Pressure (mmHg) | 108 ± 3 | 114 ± 3 |
| Heart Rate (bpm) | 692 ± 24 | 718 ± 4 |
| Body Weight (g) | 29.0 ± 3.0 | 35.2 ± 1.6* |
| Heart Weight/Tibia Length (mg/mm) | 10.6 ± 0.2 | 10.8 ± 0.5 |
| Kidney Weight/ Tibia Length (mg/mm) | 32.5± 0.8 | 33.5 ± 1.2 |
| EVAT Weight/ Tibia Length (mg/mm) | 31.3± 1.3 | 38.1 ± 1.8* |

Values are expressed as means ± SE. Mann-Whitney test, * p < 0.05, Control-MR vs. Adipo-MROE. Number of mice are indicated in the buckets. Bpm, beats per minute; EVAT, epididymal visceral adipose tissue.

SUPPLEMENTARY DATA

Supplementary Table 4. *Ace2* and G-coupled receptor *Mas* gene expressions in epididymal visceral adipose tissue and mesenteric arteries.

| Genes | EVAT | | Mesenteric Arteries | |
|-------------|------------|--------------|---------------------|------------|
| | Control-MR | Adipo-MROE | Control-MR | Adipo-MROE |
| <i>Ace2</i> | 1.01±0.06 | 4.65±0.47*** | 1.09±0.14 | 1 ±0.15 |
| <i>Mrga</i> | 1.09±0.16 | 1.89±0.27* | 1.09±0.15 | 1.99±0.39* |

Values are means ± SE. For EVAT, n=6 mice per group and for mesenteric arteries, n = 10 mice per group. Mann-Whitney test, * p<0.05, *** p<0.001, Control-MR vs. Adipo-MROE. EVAT, Epididymal visceral adipose tissue; *Ace2*, angiotensin I converting enzyme 2; *Mrga*, G-coupled receptor *Mas*.

Supplementary Table 5. Pro- and anti- inflammatory markers expressions in epididymal visceral and perivascular adipose tissues.

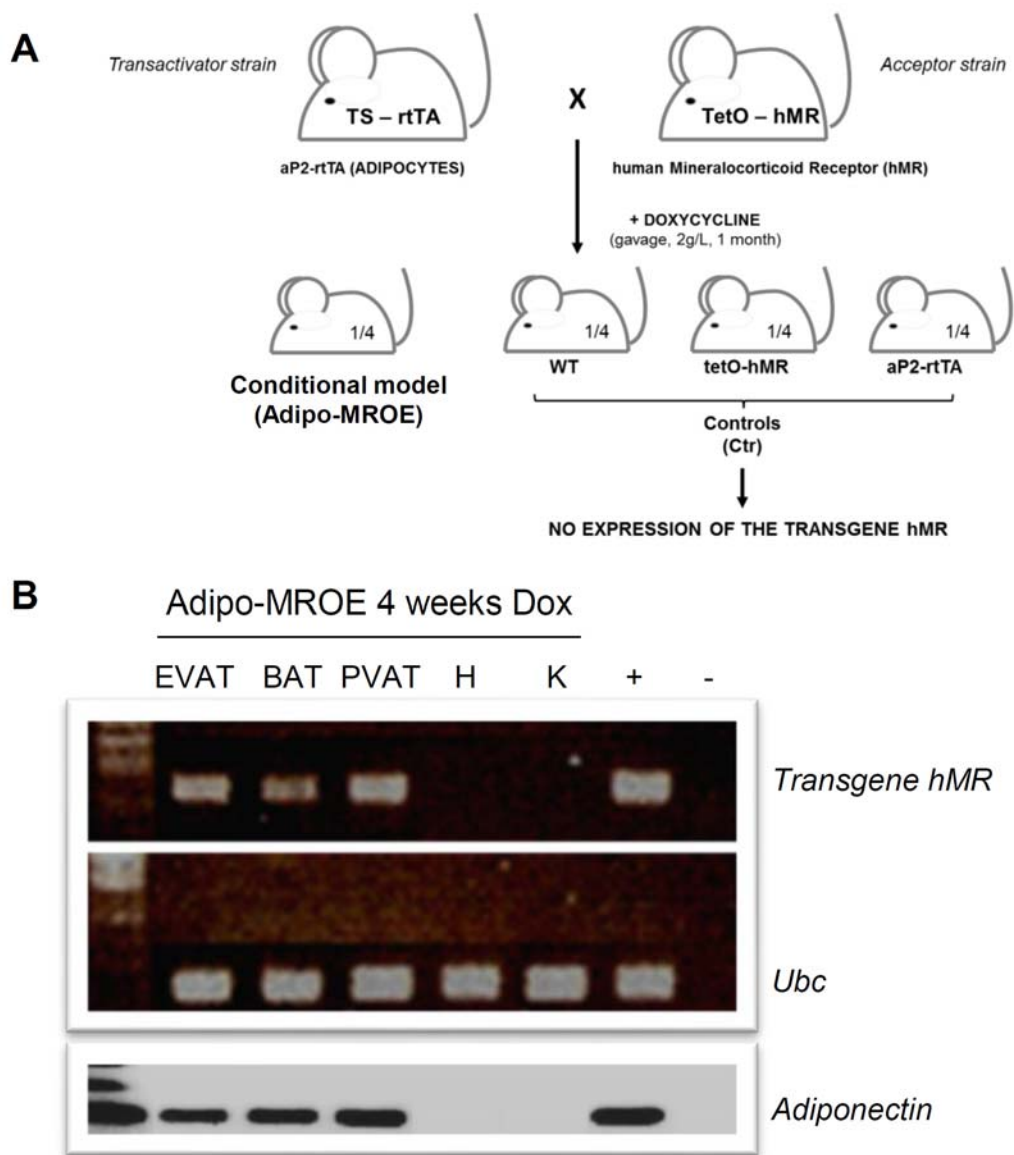
| Genes | EVAT | | PVAT | |
|--------------------|------------|-------------|------------|--------------|
| | Control-MR | Adipo-MROE | Control-MR | Adipo-MROE |
| <i>F4/80</i> | 1.01±0.08 | 1.82±0.3** | 1.17±0.31 | 2.45±0.23** |
| <i>Cd-68</i> | 1.21±0.22 | 2.12±0.15** | 1.06±0.11 | 1.88±0.35* |
| <i>Il-6</i> | 1.04±0.13 | 2.78±0.52** | 1.21±0.24 | 4.22 ±0.45** |
| <i>Il-12</i> | 1.11±0.21 | 1.22±0.11 | 1.14±0.30 | 1.32±0.29 |
| <i>Mcp-1</i> | 1.12±0.09 | 1.61±0.27* | 1.05±0.21 | 1.52±0.23 |
| <i>Rantes</i> | 1.15±0.21 | 2.31±0.11* | 1.2±0.21 | 2.72±0.41* |
| <i>Tnf-α</i> | 1.05±0.1 | 1.11±0.15 | 1.15±0.08 | 1.12±0.13 |
| <i>Adiponectin</i> | 1.07±0.21 | 0.41±0.07** | 1.15±0.08 | 0.34±0.1** |
| <i>Il-10</i> | 1.12±0.3 | 1.72±0.17* | 1.15±0.1 | 1.85±0.13* |
| <i>Cd-206</i> | 1.02±0.11 | 0.72±0.04* | 1.2±0.16 | 0.67±0.1* |

Values are means ± SE. n=6 mice per group. Mann-Whitney test, * p<0.05, ** p<0.01, Control-MR vs. Adipo-MROE. EVAT, Epididymal visceral adipose tissue; PVAT, perivascular mesenteric adipose tissue; *Il*, interleukine; *Mcp-1*, monocyte chemotactic protein-1; *Tnf-α*, tumor necrosis factor alpha

SUPPLEMENTARY DATA

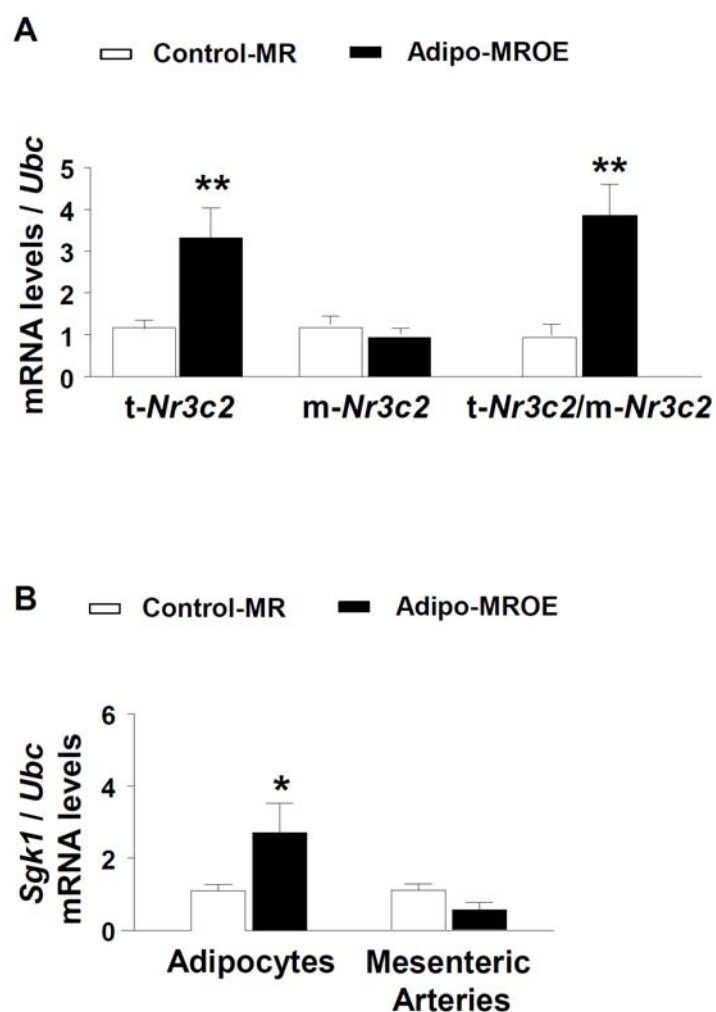
Supplementary Figure 1. Generation of transgenic mice over-expressing the human MR only in adipocytes.

A: Schematic representation of conditional adipocyte-specific animal model (Adipo-MROE mice): Tetracycline system. The conditional model is obtained by crossing two murine mono-transgenic strains, on one hand the "acceptor strain" that possesses the transgene of interest, human MR gene under the control of an inducible minimal promoter, tetO (tet Operator) and on the other hand, the "transactivator strain", which possesses the transactivator rtTA under the control of the tissue-specific promoter aP2 that allows the targeting in adipocytes. Thus, in the presence of doxycycline, the protein rtTA binds to the promoter tetO, and activates constitutively the expression of the transgene (hMR) in aP2 expressing cells. Therefore, by treating or not the mice with Dox, we can choose to activate or inactivate the transgene expression in the targeted tissue (adipocytes). B: Expression of the reporter gene *lacZ*. In presence of Dox, the reporter gene *LacZ* (blue) is only express in adipose tissue from Adipo-MROE mice whereas there is no expression of the reporter gene in the heart of Adipo-MROE mice treated with Dox. Expression of the transgene hMR only in adipose tissue from Adipo-MROE mice treated with Dox for 3 weeks by semi-quantitative RT-PCR. Human mature adipocytes were used as positive (+) control and RNase free water as negative (-) control. n=6 mice per group. Dox, doxycycline; BAT, periaortic brown adipose tissue; EVAT, Epididymal Visceral Adipose Tissue; PVAT, Perivascular Adipose Tissue (mesentery); H, Heart; Dox, doxycycline.



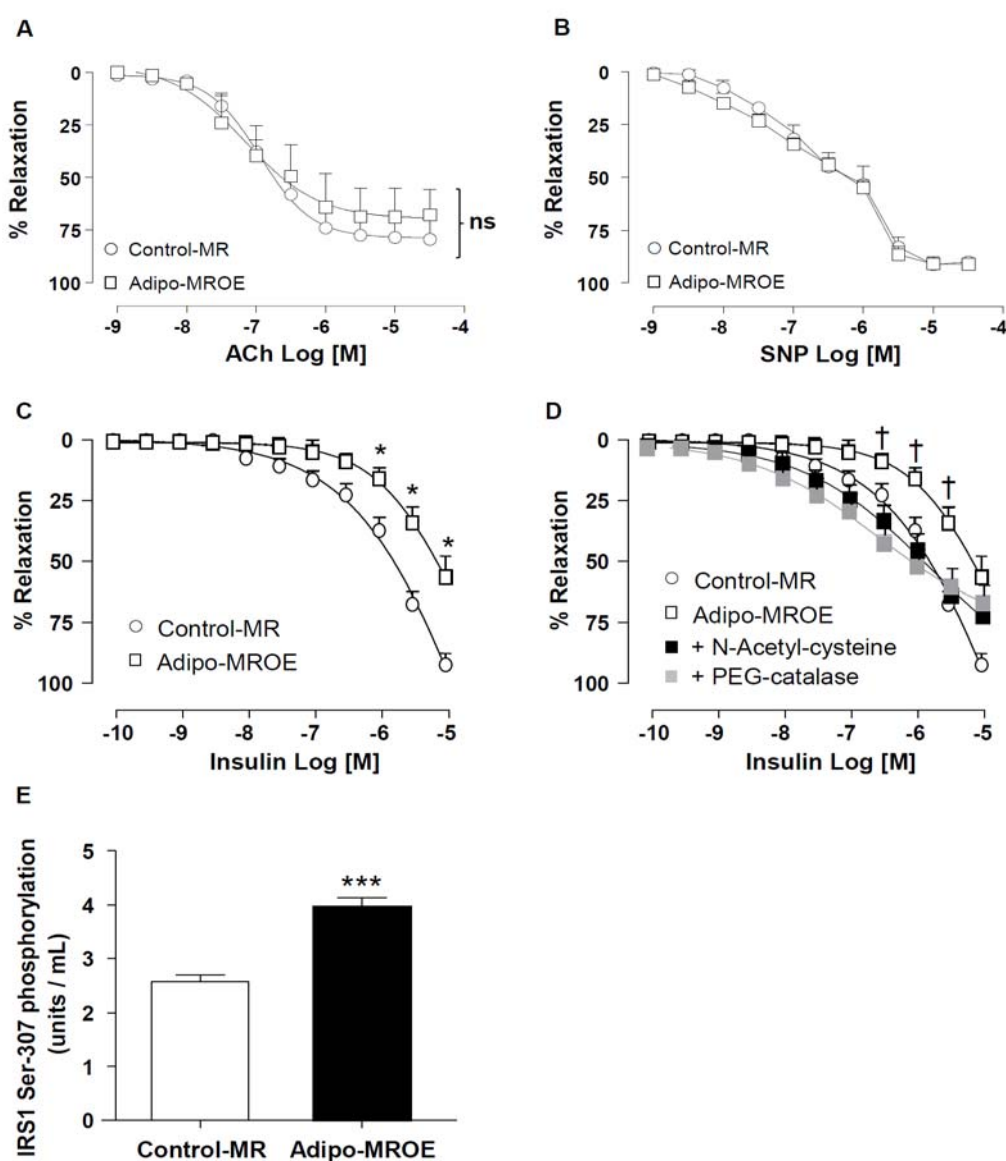
SUPPLEMENTARY DATA

Supplementary Figure 2. Adipocyte-specific MR overexpression. A: Total MR (t-*Nr3c2*) (endogenous+exogenous) and endogenous MR (m-*Nr3c2*) mRNA expression levels were assessed by real time PCR in mature adipocytes isolated from EVAT of Adipo-MROE mice and their Control-MR littermates. B: *Sgk-1* mRNA levels in mature adipocytes and mesenteric arteries. For A and B: Mann-Whitney nonparametric test was performed. Data represent means \pm SE; n=6 mice/group. * p<0.05, ** p<0.01, Adipo-MROE vs Control-MR. *Nr3c2*: nuclear receptor subfamily 3 group C member 2; *Sgk1*: serum and glucocorticoid-regulated kinase 1.



SUPPLEMENTARY DATA

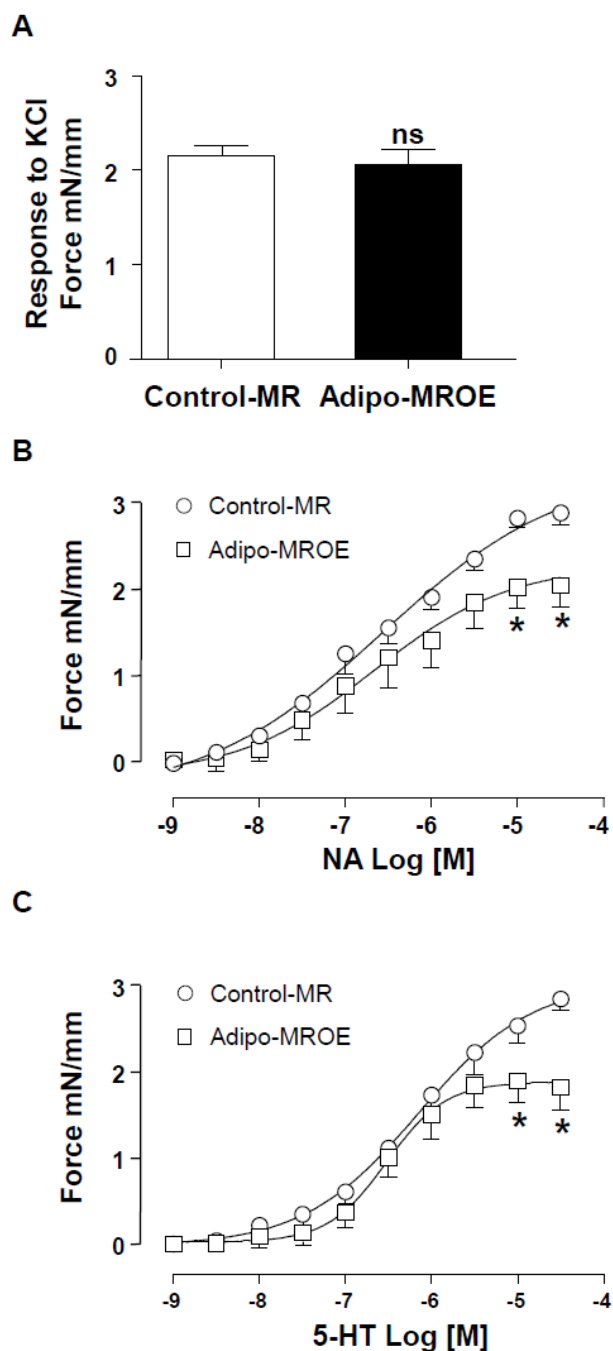
Supplementary Figure 3. Adipocyte-specific MR overexpression: vasodilatory responses studies. Relaxation responses of fat-free mesenteric resistant arteries from Adipo-MROE and Control-MR mice were evaluated using a wire myography. A: Endothelium-dependent relaxation to cumulative and increasing doses of acetylcholine (ACh, 10^{-9} to 10^{-5} M). There is no difference between Adipo-MROE and their Control-MR littermates, as well as for the endothelium-independent relaxation (B) to increasing doses of a donor of nitric oxide, the sodium nitroprusside (SNP, 10^{-9} to 10^{-5} M). C: Dose-response curves for insulin-induced relaxation were obtained from mesenteric arteries of Control-MR and Adipo-MROE mice. D: Effects of N-Acetyl-cysteine (general antioxidant, 10^{-6} M) and PEG-catalase (H_2O_2 scavenger, 100 U/mL) on insulin-induced relaxation. E: Serine-307 Phosphorylation of IRS1 was increased in mesenteric arteries from Adipo-MROE vs control-MR mice. For A-D: two-way ANOVA test was performed, followed by Bonferroni test. Data were performed in repeated measures, expressed as percentage of contraction to Phe and presented as mean \pm SE; n=5 to 7 mice/ group. Data represent means \pm SE. ns: not significant, * $p < 0.05$ Adipo-MROE vs Control-MR. For E: Mann-Whitney nonparametric test was performed. Data are expressed mean \pm SE; n=6 mice/group. *** $p < 0.001$ vs Control-MR.



SUPPLEMENTARY DATA

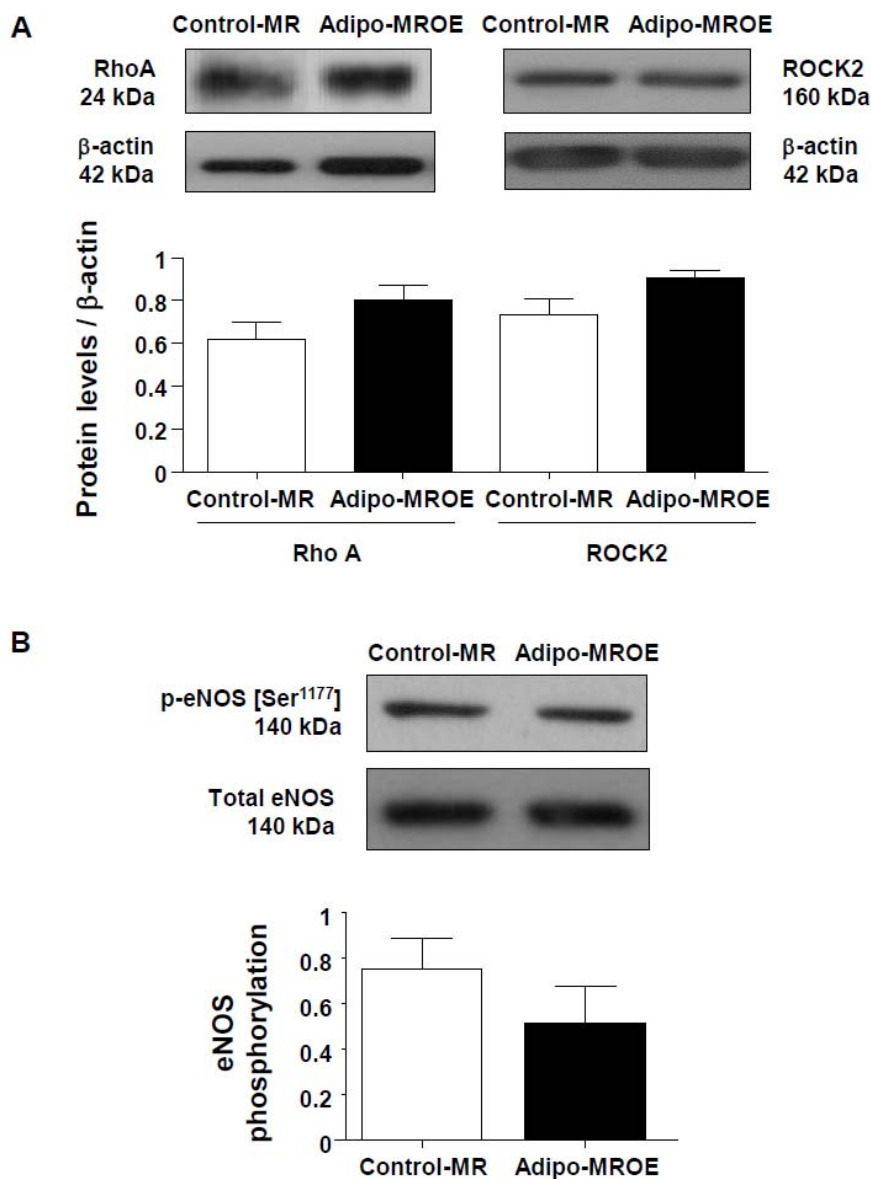
Supplementary Figure 4. Contractile responses to other vasoconstrictors in mesenteric arteries from Adipo-MROE and control-MR mice.

A: Responses to high concentrations to potassium chloride. Contractile responses to cumulative and increasing doses (10^{-9} to 10^{-5} M) of (B) noradrenaline (NA), (C) serotonin (5-HT, 5-hydroxytryptamine) in mesenteric arteries without intact endothelium, were significantly decreased in Adipo-MROE vs Control-MR mice. For A: Mann-Whitney nonparametric test was performed. Data are expressed mean \pm SE; n=6 mice/group. ns: not significant. For B and C: two-way ANOVA test was performed, followed by Bonferroni test. Data were performed in repeated measures, expressed as percentage of contraction to Phe and presented as mean \pm SE; n=6 mice/group. Data represent means \pm SE. * $p < 0.05$ Adipo-MROE vs Control-MR.



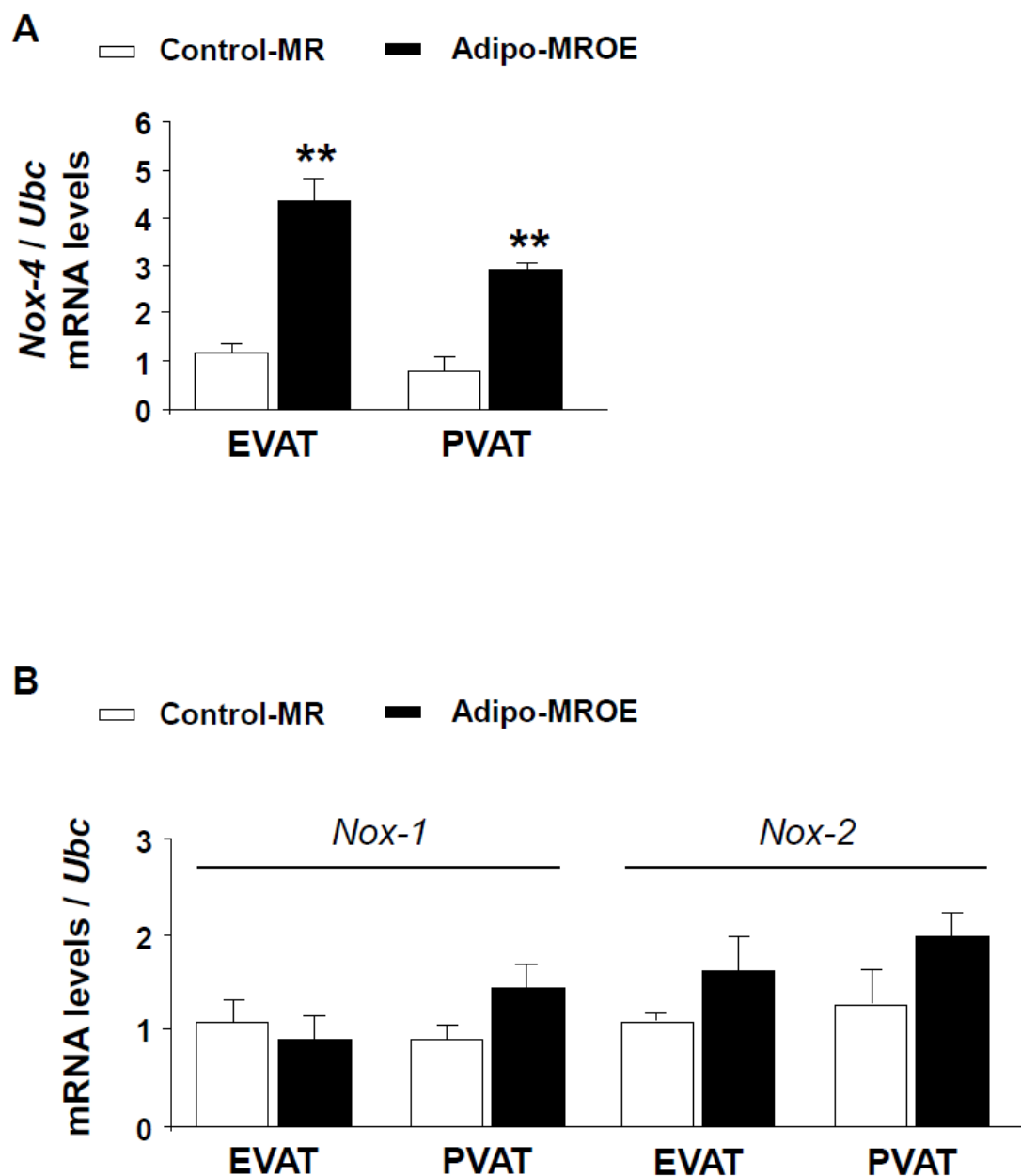
SUPPLEMENTARY DATA

Supplementary Figure 5. Increase in elasticity and elastin content in mesenteric arteries from Adipo-MROE mice. A: Stress-strain relationship was assessed and calculated using pressurized myography. The curve is shifted to the right, indicating that the mesenteric arteries from Adipo-MROE mice are more elastic than arteries from Control-MR mice. n=6-10 mice/group. Values are expressed as means \pm SE. ANOVA two ways, followed by Newman-Keuls multiple comparison test, * $p < 0.05$ Adipo-MROE vs Control-MR. B: Histological assessment of elastin content in the mesenteric arteries of Control-MR and Adipo-MROE mice. Representative images of Mesenteric arteries sections from Control-MR (a,c) and Adipo-MROE mice (b,d) stained with Elastic van Gieson stain for elastin. Images of all sections were taken under transmitted light at the same light intensity and exposure time settings. S. Bar = 150 mm (a, b) and S. Bar = 75 mm. (c and d). C: The percentage of elastin content (area of elastin content/total area of tissue \times 100) was assessed semi-quantitatively by using Image J software analysis. D: *Elastin* mRNA levels are increased in mesenteric arteries from Adipo-MROE mice compared to Control-MR. n=9 mice/group. Bars represent means \pm SE. Mann-Whitney nonparametric test, * $p < 0.05$ Adipo-MROE vs Control-MR.



SUPPLEMENTARY DATA

Supplementary Figure 6. NADPH oxidases mRNA levels in adipose tissues from Control-MR and Adipo-MROE mice. A and B: *Nox-4* mRNA levels and *Nox-1* and *Nox-2* mRNA levels were evaluated by real time PCR. For A and B: Mann-Whitney nonparametric test was performed. Data represent means \pm SE; n=6 mice/group. ** $p<0.01$, Adipo-MROE vs Control-MR. EVAT, epididymal visceral adipose tissue; PVAT, perivascular adipose tissue (mesenteric); *Nox*, NADPH oxidase; *Ubc*, ubiquitin C.



SUPPLEMENTARY DATA

Supplementary Figure 7. RhoA and ROCK protein levels and eNOS phosphorylation in arteries from Control-MR and Adipo-MROE mice. A: Rho A and ROCK2 protein levels in mesenteric arteries are not modified in Adipo-MROE versus Control-MR mice. B: eNOS phosphorylation at the active Serine-1177 residue in mesenteric arteries is similar between Adipo-MROE and Control-MR mice. Western blotting was performed using RhoA, ROCK2, β -actin, eNOS and P-eNOS (Ser¹¹⁷⁷) antibodies. Integrated intensities were obtained by the Image J software. For A and B: Mann-Whitney nonparametric test was performed. Data are expressed mean \pm SE; n=5 to 7 mice/group. ns: not significant.

