Adipocyte-Derived Factors Regulate Vascular Smooth Muscle Cells Through Mineralocorticoid and Glucocorticoid Receptors

Aurelie Nguyen Dinh Cat, Ana M. Briones, Glaucia E. Callera, Alvaro Yogi, Ying He, Augusto C. Montezano, Rhian M. Touyz

Abstract—Adipose tissue influences vascular function through adipocyte-derived factors, including components of the renin-angiotensin-aldosterone system. Molecular mechanisms underlying these phenomena are elusive. We investigated the role of adipocyte-derived factors on mitogen-activated protein kinases (MAPKs), proinflammatory status, apoptosis, and mitogenic signaling in vascular smooth muscle cells (VSMCs) and questioned whether these effects involve mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and angiotensin II type 1 receptor (AT1R). Cultured mouse VSMCs were exposed to adipocyte-conditioned medium (ACM) from differentiated 3T3-L1 adipocytes. ACM induced phosphorylation of stress-activated protein kinase/c-Jun N-terminal kinase, p38MAPK, and extracellular signal–regulated kinase 1/2 and increased expression of proinflammatory and proliferative markers in VSMCs. Eplerenone (MR antagonist), mifepristone (GR antagonist), and candesartan (AT1R antagonist) inhibited ACM-induced effects on extracellular signal–regulated kinase 1/2, p38MAPK, and proliferating cell nuclear antigen, without influencing apoptosis (Bax, Bcl, and caspase 3). Stress-activated protein kinase/c-Jun N-terminal kinase phosphorylation was inhibited by mifepristone and candesartan but not by eplerenone. ACM-induced increase of fibronectin, vascular cell adhesion molecule 1, and plasminogen activator inhibitor 1 expression was blocked by MR and AT1R antagonism but not by GR inhibition. ACM has no effect on GR, MR, and AT1R expression. Our data show that adipocyte-derived factors influence MAPK signaling, leading to VSMC proinflammatory and profibrotic responses through distinct pathways. Although ACM stimulates p38MAPK and extracellular signal–regulated kinase 1/2 phosphorylation through MR, GR, and AT1R, activation of stress-activated protein kinase/c-Jun N-terminal kinase involves GR and AT1R. These findings suggest that adipocyte-derived factors regulate VSMC function through specific MAPKs linked to MR, GR, and AT1R, a posttranslational phenomenon, because ACM did not influence receptor expression. Such cross-talk between adipocytes and VSMCs may provide a potential molecular mechanism linking renin-angiotensin-aldosterone system, adipocytes, and vascular function. (Hypertension. 2011;58:479-488.) ● Online Data Supplement

Key Words: adipocyte-secreted factors ▪ vascular smooth muscle cells ▪ inflammation ▪ corticosteroid receptors

The classic perception of adipose tissue as a passive storage organ for fatty acids has been replaced by the notion that adipose tissue serves as a highly active metabolic and endocrine organ by regulating lipid and glucose metabolism and by producing numerous humoral mediators, collectively called “adipokines.” Adipokines are involved in many physiological and pathological processes, such as weight homeostasis, energy metabolism, and local and systemic inflammation.1-3 Obesity/adiposity is accompanied by an overproduction of proinflammatory adipokines, which may impact on the cardiovascular system, leading to vascular injury and target-organ damage. Adipocytes also possess all of the components of the renin-angiotensin (Ang) system, and blockade with Ang II type 1 receptor (AT1R) antagonists exert beneficial effects on survival,4 blood pressure elevation,5 and cardiovascular risk6 in obese animal models. In addition, adipocytes produce mineralocorticoid-releasing factors that directly stimulate aldosterone secretion by adrenocortical cells in models of obesity, and this is associated with end organ damage.7-10

Increasing evidence indicates that adipokines and adipocyte-derived hormones, including Ang II and mineralocorticoid-releasing factors, modulate vascular function, inflammation, and hypertrophy through a paracrine mechanism.11 Physiologically, adipocytes produce undefined adipocyte-derived relaxing factor(s) that activate K+ channels and inhibit vasoconstriction, and, pathologically, adipose tissue produces factors that stimulate vascular smooth muscle cell
(VSMC) growth and inflammation. Pathways through which adipocytes signal in VSMCs remain unclear, but endothelial NO synthase, AMP-activated protein kinase, and mammalian target of rapamycin have been implicated. Activation of mitogen-activated protein kinases (MAPKs) has been shown to be important in adipocytokine (visfatin)-mediated VSMC proliferation. Considering the importance of the renin-Ang system in vascular (patho)biology and the fact that adipocytes exhibit all of the components of this system, as well as mineralocorticoid-releasing factor, we questioned whether adipocytes release signaling in VSMCs through mineralocorticoid receptors (MRs), glucocorticoid receptors (GRs), and AT1Rs. Aldosterone and glucocorticoids can activate MRs and GRs, both of which are expressed in vascular cells, as are AT1Rs. To assess the signaling between adipocytes and VSMCs, we used an in vitro approach by exposing cultured VSMCs to adipocyte-conditioned medium (ACM) from differentiated 3T3-L1 adipocytes or mouse mature adipocytes and evaluated the effects on specific signaling pathways associated with growth, apoptosis, fibrosis, and inflammation.

### Materials and Methods

#### Cell Culture

**Mouse VSMCs**

The study was approved by the University of Ottawa Animal Ethics Committee and performed according to the recommendations of the Canadian Council for Animal Care. Mesenteric VSMCs derived from C57BL/6J mice were isolated and characterized as described.

**3T3-L1 Cells**

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were differentiated into adipocytes in DMEM containing dexamethasone and insulin. Differentiated adipocytes were maintained in serum-free DMEM (without dexamethasone) for 24 hours. ACM was collected. Nondifferentiated 3T3-L1 cells were cultured in parallel, and the medium was collected (nondifferentiated 3T3-L1 cell-conditioned medium [NDM]). Growth medium not exposed to cells was used as control. We also collected medium from differentiated 3T3-L1 cells, treated with the specific inhibitor of aldosterone synthase, FAD286 (10⁻⁵ mol/L, 24 hours), to inhibit putative adipocyte-derived aldosterone production (ACM+FAD). All of the conditioned media were frozen at −20°C until further use.

**Mouse Mature Adipocytes**

Mouse abdominal adipose tissue was obtained from 16-week-old male C57BL/6J mice. Mature adipocytes were obtained by collagenase type I digestion and cultured for 24 hours. The mature adipocyte medium was collected and frozen until further use.

**Protocols for VSMC Stimulation With ACM**

VSMCs were exposed to control, NDM, or ACM for 1, 5, 10, 30, and 60 minutes (short term) or 2, 8, and 24 hours (long term). In a second set of experiments, VSMCs were incubated with the MR antagonist (eplerenone, 10⁻⁵ mol/L), the GR antagonist (mifepristone, 10⁻⁶ mol/L), or the AT1R antagonist (candesartan, 10⁻⁶ mol/L) for 30 minutes and then exposed to ACM for 5, 60, or 120 minutes or 24 hours. In some experiments, VSMCs were also exposed to mature adipocyte medium and to medium obtained from FAD286-treated
differentiated adipocytes (ACM+FAD). Concentrations of antagonists used, which effectively inhibited respective receptors, were based on preliminary dose-response studies and previous reports.17,18

Western Blot Analysis
VSMCs were lysed and proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were incubated overnight at 4°C with the following primary antibodies (1:1000); anti-p38MAPK (Thr180/Tyr182), anti-p38MAPK, anti-extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204), anti-ERK1/2, anti-stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK; Thr183/Tyr185), anti-SAPK/JNK, anti-ERK1/2, anti-stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK; Thr183/Tyr185), and anti-AT1R. Washed membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Immuneoreactive proteins were detected by chemiluminescence. Signals were revealed by chemiluminescence, visualized by autoradiography, and quantified densitometrically. For quantification of MAPK phosphorylation, the content of phosphorylated:total protein ratio was calculated. GAPDH expression was also used as a housekeeping protein.

Aldosterone and Corticosterone Measurements
Concentrations of aldosterone, corticosterone, and Ang II were determined in culture media by ELISA (Cayman Chemical, Ann Arbor, MI; SPI-BIO, Montigny le Bretonneux, France). Please see the online Data Supplement at http://hyper.ahajournals.org for the expanded Methods section.

Data Analysis
Results are presented as mean±SEM. Comparisons were performed by Student t test or 1-way ANOVA followed by a Newman-Keuls test. A value of P<0.05 was considered statistically significant.
Results

Effects of ACM on MAPK Phosphorylation in VSMCs
To evaluate whether adipocyte-releasing products stimulate VSMC signaling, we analyzed effects of ACM on MAPK activation. As shown in Figure 1A, ACM induces a rapid increase in SAPK/JNK phosphorylation (1 to 10 minutes). ACM stimulation increased p38MAPK phosphorylation at 30 and 60 minutes (Figure 1B). In VSMCs, ACM long-term stimulation (2 to 24 hours) did not influence phosphorylation of SAPK/JNK or p38MAPK (data not shown). ERK1/2 phosphorylation levels were rapidly increased (5 to 10 minutes), returning to baseline after 30 minutes of ACM stimulation (Figure 1C). ACM induced a second peak of ERK1/2 activation at 2 hours (Figure 1D). The time point at which the greatest increase in each MAPKs was observed was used for further experiments.

MR, GR, and AT1R Antagonists Differentially Inhibit ACM-Induced MAPK Phosphorylation in VSMCs
We next examined the contribution of MR, GR, and AT1R on ACM-mediated VSMC signaling. The increase in SAPK/JNK phosphorylation at 5 minutes was inhibited by the GR and AT1R antagonists (Figure 2A). Increased phosphorylation of p38MAPK at 60 minutes (Figure 2B) and ERK1/2 at 2 hours (Figure 2C) was inhibited by MR, GR, and AT1R antagonists.

ACM Has No Effect on VSMC Expression of MR, GR, and AT1R
We assessed the presence of MR, GR, and AT1R in mouse cultured VSMCs and evaluated whether adipocyte-releasing products interfere with the expression of these receptors. In basal conditions, MR, GR, and AT1R are detected in VSMCs, and incubation with ACM (2 to 24 hours) had no effect on expression levels (Figure S1, available in the online Data Supplement).
Profibrotic, and Proliferative Markers in VSMCs

Figure 5. Effect of adipocyte-conditioned medium (ACM) from FAD286-treated differentiated 3T3–L1 adipocytes-synthes (ACM+FAD) on mitogen-activated protein kinase (MAPK) activation. Top, Representative immunoblots of p38 MAPK and extracellular signal-regulated kinase (ERK)1/2 phosphorylation in vascular smooth muscle cells (VSMCs). Bottom, Corresponding bar graphs show densitometric data for phosphorylation of p38 MAPK at 60 minutes (A) and ERK1/2 at 5 minutes and 2 hours (B) in VSMCs exposed to control (CTRL), ACM, or ACM+FAD. Data are expressed as the phosphorylated:total protein content, with control taken as 1.0. Results are presented as mean±SEM; n=6 experiments. ***P<0.001 vs CTRL; †P<0.05 ACM vs ACM+FAD.

Contribution of MR, GR, and AT1R on ACM-Induced Expression of Proinflammatory, Profibrotic, and Proliferative Markers in VSMCs

To investigate the biological significance of adipocyte-derived factors in VSMC signaling, we evaluated the expression of proinflammatory (VCAM-1 and PAI-1), mitogenic (PCNA), profibrotic (fibronectin), and proapoptotic (Bax, Bcl-2, and cleaved caspase 3) molecular markers in ACM-treated VSMCs. ACM stimulation (2 hours) induced a significant increase in expression of fibronectin, VCAM-1, PAI-1, and PCNA (Figure 3). ACM had no effect on Bax/Bcl-2 and cleaved caspase 3/caspase 3 (Figure S2).

Increased expression of fibronectin (Figure 3A), VCAM-1 (Figure 3B), and PAI-1 (Figure 3C) was inhibited by the MR and AT1R antagonists but not by the GR antagonist. However, increased PCNA expression was inhibited by MR, GR,
and AT1R antagonists (Figure 3D). MR, GR, and AT1R antagonists had no effect on Bax/Bcl-2 and cleaved caspase 3/caspase 3 (Figure S2A and S2B and data not shown).

Effects of ACM From Adipocytes Treated With Aldosterone Synthase Inhibitor in VSMCs

To investigate the contribution of aldosterone to ACM effects, VSMCs were exposed to conditioned medium from adipocytes treated with the aldosterone synthase inhibitor FAD286 (ACM+FAD). In VSMCs stimulated with ACM+FAD, phosphorylation of p38MAPK (Figure 4A) and ERK1/2 (Figure 4B) was partially reduced compared with ACM effects. Expression of VCAM-1 and PAI-1 (Figure 5B and 5C) but not of fibronectin or PCNA (Figure 5A and 5D) was lower in ACM+FAD-stimulated VSMCs compared with ACM.

Effect of Nondifferentiated 3T3-L1 Cell Conditioned Medium (NDM) on MAPK Signaling and Proinflammatory, Profibrotic, and Proliferative Markers in VSMCs

NDM did not affect phosphorylation of SAPK/JNK and p38MAPK nor the expression of the proinflammatory, profibrotic, and proliferation markers in VSMCs (Figure S3A and S3B).
However, phosphorylation of ERK1/2 was transiently decreased by NDM at 5 minutes (Figure S3A).

**Effect of Conditioned Medium From Mouse Mature Adipocytes on VSMCs**

To confirm that ACM effects are not specific to 3T3-L1 cells, we also examined the effects of conditioned medium from primary cultures of mature adipocytes (mature adipocyte medium) from C57BL/6J mice on VSMCs. As shown in Figures 6 and 7, phosphorylation of MAPKs and expression of proinflammatory, profibrotic, and proliferative markers are increased in VSMCs exposed to mature adipocyte medium. These responses are similar to those observed with ACM.

**ACM Contains Aldosterone, Corticosterone, and Ang II**

We described previously the presence of aldosterone in NDM from 3T3-L1 cells, and higher levels were detected in ACM.19 Here, we confirm this and show that corticosterone and Ang II are also detected. In NDM from 3T3-L1 cells, the concentrations of aldosterone, corticosterone, and Ang II are, respectively, 1.3±0.2, 3.5±0.8, and 0.23±0.03 pg/mL per microgram. Levels of aldosterone (4.3±1.1 pg/mL per microgram), corticosterone (17.3±5.6 pg/mL per microgram), and Ang II (0.88±0.15 pg/mL per microgram) were significantly increased in ACM compared with NDM (P<0.05; n=4 to 8 per group). In ACM+FAD, the concentration of aldosterone was significantly reduced to 1.4±0.1 pg/mL per microgram.

**Discussion**

Major findings from our study demonstrate the following: (1) ACM induces MAPK activation in VSMCs through multiple receptors, including MR, GR, and AT1R, and whereas adipocytes influence ERK1/2 and p38MAPK through MR, GR, and AT1R, activation of SAPK/JNK involves GR and AT1R in VSMCs; (2) VSMC inflammatory and profibrotic responses by adipocytes engage MR but not GR, and growth responses involve all 3 receptors; (3) ACM-induced activation of p38MAPK and ERK1/2 and expression of VCAM-1, PAI-1, or PCNA relative to that of GAPDH, with control taken as 1.0. Results are presented as mean±SEM; n=6 to 8 experiments. *P<0.05, **P<0.01 vs control (CTRL).
p38MAPK are rapidly activated (within minutes) by ACM, possibly through nongenomic MR/GR signaling, ERK1/2 activation is sustained for ≤2 hours. Moreover, the effects of ACM appear to be highly regulated and specific, because ACM influenced VSMC proinflammatory fibrotic and proliferative responses but not apoptosis. Our findings highlight the complex cross-talk between adipocytes and VSMCs, an event that probably occurs at the posttranslational level, because ACM did not significantly modify expression of MR, GR, or AT1R in VSMCs.

Here we show that the effects of adipocyte-derived factors on MAPK activation are mediated through MR and GR and that these effects on ERK1/2, p38MAPK, and SAPK/JNK phosphorylation are differentially regulated. Our findings suggest that ACM contains mineralocorticoids, such asaldosterone, and glucocorticoids that signal through their respective VSMC receptors. To confirm this, we demonstrated that adipocytes produce aldosterone and corticosteroids and that the levels of these hormones are detectable in ACM. Although others have shown that adipocytes produce and secrete glucocorticoids,20 we present the novel findings that adipocytes have the capacity to synthesize aldosterone. We reported recently that adipocytes possess aldosterone synthase and the synthetic machinery for aldosterone production21 and that adipocyte-derived aldosterone is biologically active. In addition to MR and GR, AT1R seems to be important for ACM effects, because candesartan attenuated ACM-mediated VSMC signaling. Together with aldosterone and corticosterone, we detected Ang II in ACM, supporting a role for adipocyte-derived Ang II in the activation of VSMC AT1R.21 It is also possible that Ang II/AT1R interacts directly with MR in VSMCs and/or that AT1R are activated in a ligand-independent manner, as demonstrated previously.22–24

MRs and GRs belong to the nuclear receptor superfamily and share high sequence homology.25 Both receptors bind glucocorticoids (cortisol in humans and corticosterone in rodents) with high affinity, and both have been identified in VSMCs.26–27 Aldosterone binds to the MR with high affinity, whereas its affinity for the GR is much lower.28 The cortisol/MR complex seems to be less stable than the aldosterone/MR complex because of a different receptor conforma-
tion, leading to the dissociation of cortisol from the MR faster than aldosterone from the MR.29 The rapid VSMC signaling events induced by ACM involved both MR and GR. It is also feasible that the acute responses by these hormones occur through receptors other than MR and GR. Gros et al30 showed that aldosterone at low concentrations can act rapidly through nongenomic mechanisms via classic MRs, as well as through GPR30 (originally an “orphan” G protein–coupled receptor, subsequently a membrane estrogen receptor). Because VSMCs possess functionally active GPR30,30 we cannot exclude the possibility that some of the aldosterone-mediated responses observed in our study may occur through GPR30.

Acute VSMC signaling by ACM, which engaged both MR and GR, seems to be mediated primarily by glucocorticoids, because effects with aldosterone-deficient ACM (ACM+FAD) partially inhibited the ACM-increased p38MAPK and ERK1/2 phosphorylation. However, long-term proinflammatory signaling, (ERK1/2, VCAM-1, and PAI-1) occurs via an aldosterone/MR-dependent mechanism, because both epler-
enone and FAD286 blocked ACM-induced effects. Hop
mann et al31 reported that MR and GR induce divergent inflammatory responses. Although selective MR stimulation with aldosterone promotes the expression of interleukin 6, PAI-1, and leptin, selective GR stimulation with dexamethasone inhibits interleukin 6, tumor necrosis factor-α, monocyte chemoattractant protein 1, and leptin expression.31–35 Although we did not find a role for GR in the increase of VSMC inflammatory markers by ACM, at least in the time period studied, it is possible that GR may be important in settings of chronic stimulation. This awaits further confirmation.

To further evaluate the potential biological function of ACM vascular responses, markers of VSMC proliferation (PCNA) and apoptosis (Bax, Bcl, and cleaved caspase 3) were examined. Although ACM induced promitogenic responses (through MR, GR, and AT1R), it had no effect on molecular processes associated with apoptosis. These findings indicate that adipocyte-derived factors regulate VSMC function in a highly regulated and specific manner and that these effects are not generalized phenomena.

In summary, our data demonstrate that adipocyte-derived factors, possibly aldosterone, corticosterone, and/or Ang II, stimulate VSMC proinflammatory, proliferative, and profibrotic, but not apoptotic, pathways through specific MR-, GR-, and AT1R-dependent processes (Figure 8). Although ACM-induced ERK1/2 and p38MAPK activation involves MR/GR and AT1R, JNK activation occurs via GR and AT1R. MAPKs are master signaling regulators of cell function and play a central, albeit complex, role in vascular inflammatory responses, as well as in cardiovascular remodeling and fibrosis.36–38 Individual members of the MAPK family are multifunctional, with the final functional cellular response determined, in part, by the specific receptors being activated and by the kinetics of MAPK activation. In VSMCs, rapid MAPK signaling through MR/GR appears to involve primarily glucocorticoids, whereas long-term proinflammatory signaling seems to be mediated via aldosterone/GR. Our findings highlight the complexities relating to interactions among vascular MR, GR, and AT1R and suggest that adipocyte-derived factors may regulate vascular function through steroid receptors and/or G protein–coupled receptors, effects that occur at the postreceptor level (Figure 5). The exact adipocyte-derived mediators remain unclear and warrant further examination, although mineralocorticoids, glucocorticoids, and Ang II are putative candidates.

**Perspectives**

Results from the present study elucidate important cross-talk between adipocytes and VSMCs through MR/GR and AT1R. Such networking may link aldosterone/MR, Ang II/AT1R, adipocytes and vascular function, and inflammation and fibrosis, phenomena associated with vascular injury in diabetes mellitus, obesity, atherosclerosis, and hypertension. Moreover, from a therapeutic viewpoint, targeting both MR and AT1R will inhibit not only classic damaging effects of aldosterone and Ang II but also proinflammatory and proliferative vascular actions of adipocytes. Such strategies may
protect against vascular injury, especially in the context of obesity/adiposity.

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**Disclosures**

None.

**References**


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Adipocyte-derived factors regulate vascular smooth muscle cells through mineralocorticoid and glucocorticoid receptors.

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Material and Methods

The study was approved by the Animal Ethics Committee of the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care.

Cell culture

*Mouse vascular smooth muscle cells.*

Mesenteric vascular smooth muscle cells (VSMCs) derived from C57BL/6J mice (8 to 10 weeks old) were isolated and characterized as described in detail previously. Briefly, mesenteric beds were cleaned of adipose and connective tissue; VSMCs were dissociated by enzymatic digestion of vascular arcades for 30 minutes at 37°C. Cell suspension was centrifuged and resuspended in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum, 2 mmol/l glutamine, 20 mmol/L HEPES (pH 7.4), and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin). At subconfluence, the culture medium was replaced with serum-free medium for 24 hours to render the cells quiescent. Low-passage cells (passages 4 to 7) were studied.

*3T3-L1 cells.*

3T3-L1 cells (ATCC, Manassas, VA) were grown in monolayer culture in DMEM supplemented with 10% calf serum and antibiotics (5% CO₂, 37°C). Two days post confluence, 3T3-L1 fibroblasts were differentiated into adipocytes in DMEM containing 10% FBS, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 0.25 µmol/L dexamethasone, and 1µmol/L insulin for 2 days. The medium was then replaced with DMEM supplemented with 10% FBS and 1µM insulin for additional 2 days followed by DMEM containing 10% FBS for 4 days. At the end of this period the cells exhibited characteristic adipocyte morphology. Adipocytes were maintained in DMEM serum-free medium for 24 hours and adipocyte-conditioned medium (ACM) was collected. Non differentiated 3T3-L1 cells were cultured in parallel and the medium was collected (NDM. Non differentiated 3T3-L1 cells conditioned medium). Growth medium not exposed to cells was used as control (CTRL). We also collected medium from differentiated 3T3-L1 cells, exposed to the specific inhibitor of aldosterone synthase, FAD286 (10⁻⁵ mol/L, 24 hours), to inhibit putative adipocyte-derived aldosterone production (ACM+FAD). All media were frozen at -20°C until further use.

*Mouse mature adipocytes*

Mouse abdominal adipose tissue was obtained from 16 week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Mature adipocytes were isolated by collagenase type I digestion. After digestion, the mixture was filtered through a 200 µm filter mesh and centrifuged. The floating adipocytes were washed and centrifuged twice. Mature adipocytes were placed on α-MEM containing 15 mmol/L HEPES, 17 mmol/L insulin and 1% FBS and incubated at 37°C during 24h. The mature adipocyte medium (MAM) was collected and frozen at -80°C until further use.

Stimulation of VSMCs with adipocyte-conditioned medium (ACM).

VSMCs were exposed to 1 mL control medium or 1 mL ACM from differentiated 3T3-L1 cells for 1, 5, 10, 30, 60 min (short-term) or 2, 8 and 24 hr (long-term). In a second set of experiments,
VSMCs were incubated with the MR antagonist (eplerenone, 10^{-5} \text{ mol/L}), the GR antagonist (mifepristone, 10^{-6} \text{ mol/L}), or the AT\(_1\)R antagonist (candesartan, 10^{-6} \text{ mol/L}) for 30 min and then exposed to ACM for 5, 60 or 120 minutes. Concentrations of antagonists used, which effectively inhibited respective receptors, were based on preliminary dose-response studies and previous reports.\(^2,^3\) In a third set of experiments, VSMCs were exposed to 500 \mu\text{L} of mouse mature adipocyte-conditioned medium (MAM).

**Western blot analysis**

VSMCs were lysed for western blotting with lysis buffer containing 50 \text{ mmol/L} Tris/HCl, pH 7.4; 5 \text{ mmol/L} EGTA and 2 \text{ mmol/l} EDTA, 5\% Triton 100, 0.1 \text{ mmol/L} PMSF, 1 \text{ mmol/L} pepstatin A, 1 \text{ mmol/L} leupeptin, and 1 \text{ mmol/L} aprotinin. Equal amounts of proteins (40 \mu\text{g}) were separated by electrophoresis on a 10\% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Boehinger, Mannheim, Laval, Quebec, Canada). Non-specific binding sites were blocked with a 5\% w/v non-fat dry milk blocking buffer, containing Tris-buffered saline and 0.1\% Tween-20 (TBS-T) (1h, room temperature). Membranes were incubated overnight at 4\,^\circ\text{C} with the following primary antibodies : anti-p38MAPK (Thr180/Tyr182), anti-p38MAPK, anti-extracellular signal regulated kinase (ERK)1/2 (Thr202/Tyr204), anti-ERK1/2, anti-stress activated protein kinase (SAPK)/\text{c-Jun N-terminal kinase (JNK; Thr183/Tyr185)}, anti-SAPK/JNK, anti-Bax, anti-cleaved caspase-3, anti-caspase-3 (1:1000; Cell Signaling, New England Biolabs Ltd, Pickering, ON, Canada), anti-vascular cell adhesion molecule (VCAM)-1, anti-plasminogen activator inhibitor (PAI)-1, anti-proliferating cell nuclear antigen (PCNA), anti-Bcl-2, anti-MR (1:500), anti-GR (1:1000) and anti-AT\(_1\)R (1:500) (Santa Cruz Biotechnology, CA, USA), anti-fibronectin (1:4000) (Sigma-Aldrich, Oakville, ON, Canada). Washed membranes were incubated with horseradish peroxidase-conjugated second antibody. Immunoreactive proteins were detected by chemiluminescence. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. After incubation with secondary antibodies, signals were revealed by chemiluminescence and visualized by autoradiography and quantified densitometrically using the Scion Image software. For ERK1/2 and SAPK/JNK, where 2-3 bands (isoforms) were visible, combined density of the 2-3 bands was evaluated. For MAP kinases phosphorylation quantification, phosphorylated:total protein content ratio was calculated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was also used as a housekeeping protein.

**Aldosterone, corticosterone and angiotensin II measurements**

Concentrations of aldosterone, corticosterone and angiotensin II were determined in culture media by enzyme immunoassay (ELISA) (Cayman Chemical, Ann Arbor, MI, USA, SPI-BIO, Montigny le Bretonneux, France).

**Drugs and solutions**

IBMX, insulin and dexamethasone were from Sigma Chemical, Co. Eplerenone and Mifepristone were from Sigma-Aldrich. Candesartan was from AstraZeneca (Mississauga, ON, Canada). Drug solutions were made in bidistilled water except for FAD286, candesartan and eplerenone which were dissolved in DMSO. Stock solutions were kept at -20\,^\circ\text{C}, and appropriate dilutions with distilled water were made on the day of the experiment.
References


Figure S1.

Effects of adipocyte-conditioned medium (ACM) on phosphorylation of MAP kinases in VSMCs. Top panels, representative immunoblots of MAP kinases phosphorylation in VSMCs. Bottom panels, corresponding bar graphs show densitometric data for phosphorylation of SAPK/JNK (A), p38MAPK (B) and ERK1/2 (C and D) in VSMCs exposed to control (CTRL) and to adipocyte-conditioned medium (ACM) for different times. Data are expressed as the phosphorylated:total protein content, with control taken as 1.0. Results are presented as means ± SEM, n= 6 experiments. *P<0.05 vs CTRL; *** P<0.001 vs CTRL.
Figure S2.

Expression of MR, GR and AT₁R in VSMCs.
Representative immunoblots of MR, GR, AT₁R and GAPDH in VSMCs. Corresponding bar graphs show MR (A), GR (B) and AT₁R (C) expression in VSMCs exposed to control medium (CTRL) or adipocyte-conditioned medium (ACM) for 2-24 hours. Results are presented as mean ± SEM of the receptor expression relative to that of GAPDH, with control taken as 1.0, n= 5-8 experiments.
Figure S3.

Effects of ACM on expression of pro-apoptotic molecules in VSMCs in the presence and absence of MR, GR and AT1R antagonists.

Representative immunoblots for Bax, Bcl-2, cleaved Caspase-3 and Caspase-3 expression in VSMCs. Corresponding bar graphs show: (A) the time-course effect of adipocytes-conditioned medium (ACM) on Bax/Bcl-2 expression ratio; (B) the effect of adipocyte-conditioned medium (ACM) for 2 hours on Bax/Bcl-2 expression ratio, in the presence (+) or absence (-) of the MR antagonist, eplerenone (EPLE, 10^{-5} mol/L), GR antagonist, mifepristone (MIFE, 10^{-6} mol/L) or AT1R antagonist (CAND, 10^{-6} mol/L); (C) the effect of adipocytes-conditioned medium (ACM) or non-differentiated 3T3-L1 cells conditioned medium on cleaved Caspase-3/Caspase-3 expression ratio. CTRL, control medium. Results are presented as mean ± SEM of Bax/Bcl-2 expression ratio and cleaved Caspase-3/Caspase-3 expression ratio, with control taken as 1.0., n=6 experiments.
Figure S4.
Effect of ACM from FAD286-treated differentiated 3T3-L1 adipocytes-synthase (ACM+FAD) on MAP kinases activation.

Top panels, representative immunoblots of p38MAPK and ERK1/2 phosphorylation in VSMCs. Bottom panels, corresponding bar graphs show densitometric data for phosphorylation of p38MAPK at 60 minutes (A) and ERK1/2 at 5 minutes and 2 hours (B) in VSMCs exposed to CTRL, ACM or ACM+FAD. Data are expressed as the phosphorylated:total protein content, with control taken as 1.0. Results are presented as means±SEM, n= 6 experiments. **P<0.01 vs CTRL, †P<0.05 ACM vs ACM+FAD.
Figure S5.

Effect of ACM from FAD286-treated differentiated 3T3-L1 adipocytes-synthase (ACM+FAD) on increased expression of pro-fibrotic, pro-inflammatory and proliferative markers in VSMCs.

Top panels, representative immunoblots of fibronectin, VCAM-1, PAI-1, PCNA and GAPDH in VSMCs. Bottom panels, corresponding bar graphs show densitometric data for expression of fibronectin (A), VCAM-1 (B), PAI-1 (C), PCNA (D) expression in VSMCs exposed to CTRL, ACM and ACM+FAD for 2 hours. Data are presented as the expression of fibronectin, VCAM-1, PAI-1 or PCNA relative to that of GAPDH, with control taken as 1.0. Results are presented as means ± SEM, n= 6 experiments. *P<0.05, **P<0.01, ***P<0.001 vs CTRL, †P<0.05, †† P<0.01 vs ACM.
Figure S6.
Effects of non-differentiated 3T3-L1 cell conditioned medium (NDM) on VSMC signaling. Representative immunoblots of MAP kinase phosphorylation and VCAM-1, fibronectin, PAI-1, PCNA and GAPDH expression in VSMCs. Corresponding bar graphs show: (A) the effect of control medium (CTRL) and NDM on phosphorylation of ERK1/2 (5 and 120 min), p38MAPK (60 min) and SAPK/JNK (5 min) in VSMCs; (B) long term (24 hr) effect of CTRL and NDM on VCAM-1, fibronectin, PAI-1 and PCNA in VSMCs. Results are presented as mean ± SEM of MAP kinases phosphorylated:total protein content and expression of VCAM-1, fibronectin, PAI-1 or PCNA relative to that of GAPDH, with control taken as 1.0, n= 6-8 experiments. *P<0.05 vs CTRL.