Original Article

Vascular Smooth Muscle Cell Alterations Triggered by Mice Adipocytes: Role of High-Fat Diet

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Aim: Inherent mechanisms leading to vascular smooth muscle cells (VSMC) alterations in obesity-linked type 2 diabetes (T2D) situation remain to be clarified. This study evaluates the impact of supernatant of adipocytes extracted from mice fed high-fat-diets (HFD) on the proliferation and apoptosis of VSMC.

Methods: Adipocytes were extracted from visceral white fat pads of male and female C57Bl6 mice showing different stages of metabolic alterations after 20 weeks of vegetal or animal HFD feeding. These cells were stimulated or not with insulin or glucose to condition VSMC media. After 24h of stimulation with adipocyte supernatants (AdS), VSMC proliferation and sustainability were assessed in the absence and presence of AdS. CD36 and insulin receptor mRNA levels were also evaluated.

Results: Proliferation and viability of VSMC were significantly modulated by the nature of the AdS used and the gender of mice from which adipocytes have been extracted. The most extensive effects on VSMC were triggered by adipocytes from males fed animal HFD and females fed vegetal HFD. These effects were concurrent with increased leptin concentration and decreased adiponectin levels in AdS. In addition, adipocytes of HFD-fed mice increased caspase-3 activity and apoptosis in VSMC. Significant up-regulation of CD36 mRNA was also found in these cells.

Conclusion: Adipocytes of HFD-fed mice induce VSMC alterations. These changes involved mouse gender, most probably correlated to the diet-induced adipocyte secretion profile. Greater sensitivity to AdS effects in VSMC raises concerns about the more frequent cardiovascular events associated with obesity in the presence of T2D, which impairs adipocyte activity.


Key words: Adipocytes, Adipokines, Atherosclerosis, Type 2 diabetes, Vascular smooth muscle cells

Introduction

The pathogenesis of atherosclerosis linked to obesity-related type 2 diabetes (T2D) involves several mechanisms related to metabolic alterations¹-³). Hyperglycemia increases oxidative stress and glycation, which release free radicals and advanced glycation end-products (AGE) in the systemic circulation, contributing to foam cell formation in the arterial wall. A lack of insulin sensitivity increases endothelial dysfunction, characterized by decreased NO release and increased inflammatory status⁴).

For their part, vascular smooth muscle cells (VSMC) undergo "phenotypic switching" showing less contractibility with increased proliferation and migration toward the intima, contributing to wall thickening and thrombosis⁵-⁶). Although VSMC accumulate in the intima, VSMC apoptosis is well established in atherosclerosis⁷). VSMC may also trans-differentiate into foam cells, contributing to atherogenesis⁸). These cells have a macrophage-like phenotype correlated with an increased level of oxLDL scavenger receptor CD36 and lipid accumulation⁹).

On the other hand, the impact of adipose tissue on VSMC alterations cannot be rejected. This tissue is a secretion organ that produces several hormones,
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Materials and Methods

Experimental Protocol

Adipocytes were obtained from C57BL/6J male and female mice fed with HFD for 20 weeks. Mice were fed one of two low cholesterol HFD (34.9% fat, 26.3% carbohydrate, cholesterol <0.03%; Research-Diet): a vegetal HFD (VD) composed of soy and cotton oil and an animal HFD (AD) composed of lard. The common standard diet (SD) was used as a control (6% fat, 57% carbohydrate; Harlan-Teklad). The animal protocol was approved by the Animal Care and Use Committee of Montreal Heart Institute.

VSMC Culture

VSMC were isolated from 10-week-old C57BL/6J mice as previously described. Briefly, the adventitia was removed from the dissected aorta, which was cut into 2 mm square pieces and then incubated in 1.5 mg/mL collagenase type II solution (37°C; 5% CO2; 5 h). Dissociated cells were suspended in 5 mL Dulbecco's modified Eagle's medium (DMEM) and centrifuged (300 g; 5 min). The cell pellet was suspended in 700 μL DMEM supplemented with 10% fetal bovine serum (FBS), transferred to a single well of a 48-well plate and left untouched for 5 days. VSMC purity was evaluated by confocal microscopy after 4 passages of amplification before their treatment with AdS (Supplementary Fig. 1). In order to eliminate gender interactions between factors secreted by adipocytes and VSMC, these latter were stimulated by AdS from adipocytes of the same gender. To separate the effect of insulin and glucose from the effect of adipocyte-secreted factors on VSMC in AdS, insulin or glucose stimulation of VSMC were used as controls for each experiment. Each condition was tested in duplicate for each experiment.

Adipocyte Culture

Mouse mature white adipocytes were isolated from visceral-abdominal adipose tissue obtained by dissection at sacrifice. Briefly, adipose tissue was finely minced and enzymatically digested in DMEM containing 2 mg/mL type II collagenase (37°C; 40 min). The digestion mixture tissue was then centrifuged (300 g; 7 min) and the top white supernatant was filtered through a 100 μm filter and washed twice in DMEM. The mature adipocytes were characterized by a rounded appearance with a large vacuole filling the majority of the cytoplasm containing lipids that suspend the cell at the top of the culture media (Supplementary Fig. 2).

Adipocytes (10⁴ cells/mL) were maintained in low glucose DMEM, without serum, supplemented or not with insulin (100 μU/mL) or glucose (25 mM) for 24 h. AdS were collected and immediately stored at −20°C. At the moment of experimentation, samples were thawed (37°C; 1 h) and added to the VSMC culture.

To simplify annotations, the supernatant of unstimulated adipocytes was noted as AdS-U, the supernatant of insulin-stimulated adipocytes as AdS-I and the supernatant of glucose-stimulated adipocytes as AdS-G.

To evaluate PPARγ2 action on adipokine secretion, adipocytes were stimulated with 6 μM Ciglitazone (symbolized by TZD), a PPARγ agonist, or with 2 nM of T0070907 (symbolized by T007), a PPARγ2 specific antagonist, for 24 h. These ligands were purchased from Tocris (Minneapolis, MN, USA).

Cell Proliferation Assays

VSMC were plated in 96-well plates at a density of 10⁴ cells per well in DMEM containing 10% FBS and allowed to attach and grow to sub-confluent density for 24 h before a 24 h synchronization step in...
DMEM containing 0.1% FBS. Proliferation of VSMC was assessed by manual counting and expressed as a percentage compared to the control.

**Quantification of mRNA Level**

Total RNA was isolated using Qiazol reagent according to the manufacturer’s instructions (Qiagen, Toronto, ON, Canada). Single-strand cDNA was synthesized according to the procedure in the iScript cDNA Synthesis Kit manual (Bio-Rad Laboratories, Montreal, QC, Canada). Q-PCR reactions were carried out using the Brilliant-II SYBR Green Master Mix (Stratagene, Mississauga, ON, Canada) and specific primers (Supplementary Table 1) for PPARγ2, nuclear receptor insulin receptor (InsR), Bax and fatty acid synthase (FAS). The mRNA levels were normalized to the cyclophilin-A expression level. The targeted and reference genes were amplified in duplicate in the same run using the Mx3000P Q-PCR System (Stratagene).

The relative quantification of target genes was determined using the MxProTM Q-PCR software version 3.00 (Stratagene). Briefly, Ct average of each duplicate was calculated for each gene and cyclophilin A and the ΔCT (CT, gene - CT, Cyclo-A) was determined. The control adipose tissue sample was chosen as a reference sample and set as 100% of gene quantity. Finally, the mRNA abundance of other samples to the mRNA abundance of control adipose tissue was calculated using the formula $2^{-ΔΔCT}$. Each reaction was performed in duplicate.

**Caspase-3 Activity**

Caspase-3 activity in VSMC was measured using the synthetic substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroalanilide (Ac-DEVD-pNA) (Invitrogen, Burlington, ON, Canada) diluted to 40 μM in reaction buffer composed of 100 mM Hepes, pH 7.3, 300 mM sucrose, 10 mM DTT and 0.1% Nonidet P-40. VSMC were homogenized in RIPA lysis buffer and 100 μg protein of each extract was incubated (37°C; 1 h) in 0.2 mL of reaction buffer-substrate mix. The reaction used Ac-DEVD-pNA as a substrate hydrolyzed by caspase-3 to generate Ac-DEVD + p-nitroalanilide. The amount of hydrolyzed substrate (p-nitroalanilide), expressed in μM, was then measured at 405 nm. This amount directly correlated with caspase-3 activity. As a positive control of caspase-3 activity, VSMC were irradiated for 5 min with a short-wave UV lamp, washed once and incubated in DMEM/10% FBS for 24 h. Each experiment was performed in triplicate.

**Biochemical Assay**

The concentration of adiponectin and leptin in AdS was measured using mouse ELISA kits according to the manufacturer’s protocol (ALPCO). Prior to analysis, samples were thawed on ice and used rapidly. Each condition was evaluated in duplicate.

**Statistical Analysis**

All statistical analyses were performed separately for males and females. Data are presented as the mean ± standard deviation for continuous variables.

Repeated measures analysis of variance (ANOVA) models were used to compare the adiponectin and leptin levels in adipocyte supernatant among groups (SD, VD and AD groups). The cell proliferation and mRNA levels were compared among groups (SD, VD and AD groups) using an ANOVA model.

In addition, the relationships among adiponectin and leptin levels, mRNA levels and proliferation parameters were investigated using Pearson or Spearman correlations according to the nature of the data distribution.

All analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and conducted at the 0.05 significance level.

**Results**

We have previously shown that HFD-triggered obesity in mice correlated with increased leptin levels in the systemic circulation [19]. The blood profile of adipokines was shaped by the diet, which also impacted abdominal visceral white adipose tissue gene expres-
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Biochemical Characteristics

PPARγ Ligand Effect

To evaluate if a reduced PPARγ2 mRNA expression level in HFD-fed mice-derived adipocytes was due to resistance to FA stimulation leading to impaired regulation of its activity, we evaluated their leptin and adiponectin secretion response to a PPARγ agonist (TZD) or antagonist (T007). In males, comparable hyper-leptinemia was obtained in AdS-U from VD (1.20 ± 0.09 ng/mL) and AD (1.42 ± 0.09 ng/mL) groups compared to SD group (0.33 ± 0.03 ng/mL) (Fig. 2A). In females, basal leptin in SD was markedly lower than in male cell supernatants. AdS-U contained 5 times more leptin in VD group (0.34 ± 0.03 ng/mL) and 3 times more in AD group (0.20 ± 0.03 ng/mL) than the control (0.06 ± 0.004 ng/mL) (Fig. 2B). While T007 induced comparable inhibition of leptin secre-
of all groups; however, this effect was more marked in male adipocytes, where adiponectin levels increased by up to 500% (Fig. 2C), compared to female adipocytes, where adiponectin secretion amplification was 20% in SD, 40% in VD and 200% in AD group (Fig. 2D). T007, for its part, significantly decreased adiponectin levels in all male groups by 20% compared to AdS-U; however, in female groups, T007 decreased adiponectin secretion only in adipocytes from VD (20%) and AD (53%) groups. Finally, in all cases, the adiponectin secretion profile was lower in HFD groups than in control.

**Insulin/Glucose Effect**

We have previously shown that HFD induced various degrees of metabolic alterations in our mouse groups. PPARγ agonist increased leptin concentration in AdS by 250% in SD male group compared to AdS-U while it had less impact in HFD groups (154% for VD and 111% for AD). In contrast, in female TZD, leptin secretion increased by only 78% in SD group and by more than 7 times in HFD groups compared to AdS-U.

In parallel, adiponectin decreased in AdS-U of adipocytes from males under VD (0.75 ± 0.11 ng/mL) and AD (1.00 ± 0.22 ng/mL) compared to SD group (2.75 ± 0.46 ng/mL) (Fig. 2C). In female groups, adiponectin in AdS-U decreased more in AD group (2.77 ± 0.17 ng/mL) than in SD (8.20 ± 0.18 ng/mL) and VD (6.30 ± 0.15 ng/mL) groups (Fig. 2D). As expected, PPARγ agonist increased adiponectin in AdS of all groups; however, this effect was more marked in male adipocytes, where adiponectin levels increased by up to 500% (Fig. 2C), compared to female adipocytes, where adiponectin secretion amplification was 20% in SD, 40% in VD and 200% in AD group (Fig. 2D).

![Fig. 3. AdS concentrations of leptin (A, B) and adiponectin (C, D) after 24 h of stimulation with insulin or glucose in standard diet (SD), vegetal HFD (VD) and animal HFD (AD) -fed mice groups. *p < 0.05 vs AdS-U; #p < 0.05 vs. SD; $p < 0.05 vs. VD.](image-url)
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was slightly increased in AdS-I but more markedly amplified in AdS-G (96%) compared to AdS-U in SD male group (Fig. 3C). Insulin had no effect on adiponectin secretion in both HFD groups; however, glucose strongly promoted it (350% for VD and 222% for AD) compared to the level observed with unstimulated fat cells. Despite these differences, adiponectin concentration in HFD-derived AdS remains 30 to 70% lower than in the corresponding SD group.

For its part, insulin treatment significantly increased adiponectin secretion in AdS-I by 5, 7 and 21% in female SD, VD and AD groups, respectively

AdS-U hyper-leptinemia observed in HFD groups was further increased in response to insulin or glucose stimulation compared to unstimulated cells with the maximum level reached with AdS-G (Fig. 3A). In females, insulin or glucose stimulation had no effect on the leptin level in AdS of SD group but increased it by 3 to 6 times in AdS of HFD groups compared to unstimulated cells (Fig. 3B).

On the other hand, adiponectin concentration was slightly increased in AdS-I but more markedly amplified in AdS-G (96%) compared to AdS-U in SD male group (Fig. 3C). Insulin had no effect on adiponectin secretion in both HFD groups; however, glucose strongly promoted it (350% for VD and 222% for AD) compared to the level observed with unstimulated fat cells. Despite these differences, adiponectin concentration in HFD-derived AdS remains 30 to 70% lower than in the corresponding SD group.

For its part, insulin treatment significantly increased adiponectin secretion in AdS-I by 5, 7 and 21% in female SD, VD and AD groups, respectively.

**Fig. 4.** Incubation of VSMC with insulin or glucose for 24 hours was used as a control (A). These results are expressed as a percentage of the proliferation rate of unstimulated cells (Ø). The effect of leptin on VSMC proliferation was also evaluated (B) for 2 concentrations of 50 ng/mL (Leptin 50) and 100 ng/mL (Leptin 100). $p<0.05$ vs unstimulated cells (Ø). VSMC proliferation rate after 24 h of treatment with AdS of male (C) and female (D) mice fed a standard diet (SD), vegetal HFD (VD) or animal HFD (AD). $p<0.05$ vs AdS-U; $p<0.05$ vs SD. 

- **A**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **B**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **C**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **D**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **A**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **B**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **C**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD

- **D**
  - VSMC proliferation (% of control)
  - SD
  - VD
  - AD
(Fig. 3D). After glucose treatment, adipocytes from SD and VD groups increased their adiponectin secretion by 25% while it increased by 130% in the supernatants of AD-fed mouse-derived adipocytes. Finally, adiponectinemia in females remained up to 8 times higher than in corresponding male groups.

**Effects of AdS on VSMC Proliferation**

Insulin or glucose by themselves significantly increased VSMC proliferation by up to 37% compared to the basal rate set as 100% (Fig. 4A). We also showed that purified leptin increased the VSMC proliferation rate in a concentration-dependent manner (Fig. 4B). To examine how adipocyte-conditioned media modulated this parameter, VSMC were incubated for 24 h with AdS of adipocytes stimulated or not with insulin or glucose. Results are expressed as a percentage of the proliferation of VSMC exposed to AdS compared to VSMC not exposed to AdS, in both cases stimulated or not with insulin or glucose.

Secreted factors from unstimulated adipocytes of SD-fed males decreased the proliferation of VSMC by 30% compared to cells unexposed to AdS (set as reference value 100%) (Fig. 4C). This inhibition was highlighted with adipocytes from AD group that decreased VSMC proliferation by 60% (42.74 ± 0.12%) compared to SD (68.18 ± 0.06%) and VD (67.10 ± 0.09%) groups. AdS-I maintained the same inhibitory effect on VSMC proliferation in VD (65.60 ± 0.07%) and AD (61.50 ± 0.09%) groups; however, insulin treatment of adipocytes in SD group increased their anti-proliferative effect (48.10 ± 0.02%) compared to AdS-U. Stimulation of adipocytes with glucose abrogates the capacity of adipocytes to reduce the VSMC proliferation rate and this occurred in all diet groups.

In females, AdS-U inhibited VSMC proliferation in SD (58.50 ± 0.03%) and VD (79.20 ± 0.08%) groups with no significant effect on AD group (91.80 ± 0.16%) compared to the control (Fig. 4D); a major difference from the male AD group (42.74 ± 0.12%). Insulin treatment completely inhibited the anti-proliferative effect of VD adipocytes (98.66 ± 0.09%) but had no impact on SD. Finally, VSMC proliferation was partially restored under AdS-G in SD group (83.71 ± 0.03%) compared to AdS-U from SD group (58.50 ± 0.03%) without any significant effect in VD (103.61 ± 0.11%) group. On the other hand AdS-G showed a pro-proliferative effect in AD (114.31 ± 0.15%) group.

**VSMC Apoptosis Induction by AdS Stimulation**

VSMC apoptosis is one of the triggering events of atherogenesis. To evaluate the impact of AdS on this parameter, the basal level of FAS and Bax mRNA expression or caspase-3 activity in VSMC maintained 24 h in DMEM culture media was set at zero as a reference value. In males, AdS-U from AD group significantly increased caspase-3 activity in VSMC (3.65 ± 0.62 μM) compared to SD (2.58 ± 0.32 μM) and VD (2.48 ± 0.31 μM) groups (Fig. 5A). This diet effect in AD group was furthermore amplified with AdS-I (7.09 ± 0.79 μM) and AdS-G (6.90 ± 0.64 μM) compared to unstimulated cells (AdS-U). Insulin- or glucose-treated fat cells from SD and VD groups did not show any modulation of VSMC caspase-3 activity compared to AdS-U. The pro-apoptotic effect of AD-derived AdS was confirmed using Bax and FAS mRNA levels; both were increased in VSMC exposed to AD-derived AdS (Table 1).

In females, AdS-U from AD group also increased...
VSMC caspase-3 activity (3.33 ± 0.26 μM) compared to SD (2.56 ± 0.34 μM) and VD (2.00 ± 0.18 μM) groups (Fig. 5B). However, in contrast with male supernatants, AdS-I from VD group (4.32 ± 0.54 μM) preferentially increased caspase-3 activity compared to SD (1.84 ± 0.20 μM) and AD (2.59 ± 0.19 μM) groups and the effect was reflected at the level of FAS mRNA (Table 1). Among untreated adipocytes, only the AD female group showed increased FAS mRNA (5.81 ± 0.70) in VSMC compared to SD (3.91 ± 1.10) and VD (2.51 ± 0.29).

**CD36 and InsR Gene Expression**

The impact of AdS on VSMC could be linked to FA released by adipocytes. Modulation of FA receptor expression may be an indicator of their implication in VSMC alteration. We evaluated the CD36 mRNA expression level in VSMC, a FA scavenger receptor recognized for its role in the atherogenesis process9. In males, when VSMC are exposed to AdS-U from HFD groups, CD36 mRNA is significantly increased (0.48 ± 0.05 for VD; 0.88 ± 0.55 for AD) compared to SD group, which had no effect on the basal expression level (0.02 ± 0.13) (Fig. 6A). Insulin stimulation increases CD36 mRNA expression by 2-fold in VSMC compared to unstimulated and glucose stimulated cells. In addition, glucose alone increases CD36 mRNA by 30% (Fig. 6E). AdS-I of all diet groups increased CD36 mRNA expression in VSMC with still higher levels in VD (1.84 ± 0.09) and AD (2.10 ± 0.02) groups compared to SD group (1.03 ± 0.11). AdS-G increased scavenger receptor mRNA expression in VSMC in SD (0.43 ± 0.15), VD (0.90 ± 0.03) and AD (1.07 ± 0.02) groups, following a similar pattern but to a lesser extent than AdS-I.

In female groups, among unstimulated adipocytes, only AdS-U of AD group triggered a marginal increase in CD36 mRNA in VSMC (0.12 ± 0.03) and the effect was reflected at the level of FAS mRNA (Table 1). Among untreated adipocytes, only the AD female group showed increased FAS mRNA (5.81 ± 0.70) in VSMC compared to SD (3.91 ± 1.10) and VD (2.51 ± 0.29).

**Table 1.** Bax and FAS mRNA expression levels in AdS-treated VSMC in male and female groups

<table>
<thead>
<tr>
<th>Sex groups</th>
<th>Diet groups</th>
<th>Adipocytes’ supernatants</th>
<th>Bax mRNA level ± SEM</th>
<th>FAS mRNA level ± SEM</th>
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<tbody>
<tr>
<td>Males</td>
<td>Standard Diet</td>
<td>AdS-U</td>
<td>0.77 ± 0.20</td>
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<td></td>
<td></td>
<td>AdS-I</td>
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<td></td>
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<td>AdS-G</td>
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<td></td>
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<td>Animal HFD</td>
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<td>0.91 ± 0.22</td>
<td>1.32 ± 0.26**</td>
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<td></td>
<td></td>
<td>AdS-I</td>
<td>0.95 ± 0.09*</td>
<td>3.05 ± 0.06**</td>
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<tr>
<td></td>
<td></td>
<td>AdS-G</td>
<td>2.87 ± 0.53* **</td>
<td>2.27 ± 0.16**</td>
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</table>

Females

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<th>Sex groups</th>
<th>Diet groups</th>
<th>Adipocytes’ supernatants</th>
<th>Bax mRNA level ± SEM</th>
<th>FAS mRNA level ± SEM</th>
</tr>
</thead>
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<td>Females</td>
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<td>AdS-I</td>
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<td>AdS-G</td>
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<td>AdS-I</td>
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<td>AdS-I</td>
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<td>AdS-G</td>
<td>1.90 ± 0.40</td>
<td>2.87 ± 0.12**</td>
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</table>

*p < 0.05 vs AdS-U; *#p < 0.05 vs. standard diet mouse group; #p < 0.05 vs vegetal HFD mouse group.
Fig. 6. CD36 and InsR mRNA expression levels in VSMC after treatment with AdS of stimulated adipocytes from male (A, C) and female (B, D) mice fed a standard diet (SD), vegetal HFD (VD) or animal HFD (AD). *p < 0.05 vs AdS-U; ^p < 0.05 vs. SD; $p < 0.05 vs. VD. The basic mRNA expression level (Ø) in VSMC (E, F), reported as 100%, was compared to its level after glucose and insulin stimulation. +p < 0.05 vs unstimulated cells (Ø).
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group (0.58 ± 0.09), and increased it in SD (1.47 ± 0.12) and AD (1.43 ± 0.12) groups compared to the control (Fig. 6D). In response to insulin or glucose stimulation, adipocytes from the male SD group significantly decreased the InsR expression by 30% and 75%, respectively in VSMC compared to unstimulated cells. In contrast, AdS-I and AdS-G from male VD and AD groups had no influence on InsR gene expression in VSMC compared to the unstimulated condition; however, AD-derived adipocytes significantly increased the AdS-I and AdS-G potential to increase VSMC InsR expression levels compared to SD and VD groups.

In females, adipocytes from SD and AD groups did not affect InsR gene expression in VSMC under any stimulation condition. In contrast, adipocytes from VD group significantly increased InsR mRNA in SMC compared to unstimulated cells in response to insulin (1.53 ± 0.14) stimulation. Glucose stimulation showed a diet effect in the female VD group that decreased InsR mRNA (0.72 ± 0.13) compared to SD and AD (Fig. 6D).

Discussion

Adipokines have become widely accepted as key regulators of VSMC proliferation and viability, two important cellular processes in atherogenesis. Most research so far has focused on the direct roles of these substances in VSMC physiology; however, the physiological regulation and role of HFD in mediating the unhealthy effects of adipocytes on VSMC have not been fully elucidated.

The novelty of this study is the use of mature adipocytes isolated from obesity-linked diabetic mice to condition VSMC culture media. Thus, we were able to evaluate the effect of the adipocyte secretion mixture on VSMC.

In order to investigate the potential mechanisms in HFD-induced adipokine modulations in our mouse model, the mRNA level of the specific nuclear receptor of adipose tissue PPARγ2 was calculated. Adipokine level in AdS in response to PPARγ ligand stimulation was also explored. Since PPARγ expression has been found to be modulated in various metabolic diseases, many studies have confirmed the anti-diabetic properties of PPARγ agonists. Based on these results, PPARγ activation by glitazones has been proven as an effective anti-atherosclerotic regimen. The results of the present study showed that TZD treatment increased leptin in AdS and that T007 decreased it. TZD had a different effect on adipocytes extracted from male or female mice with a higher response of adipocytes from SD group in male adipocytes and a more marked effect of TZD in HFD group in females. This could be due to the lower expression of PPARγ2 in HFD-fed male adipocytes compared to female mice. It was reflected by decreased adiponectin in AdS of adipocytes from HFD-fed male mice after TZD treatment. All together, these data showed that HFD in vivo alterations were transposed in the modulation of adipocytes activity.

To further investigate adipocyte activity, we evaluated leptin and adiponectin levels in unstimulated and insulin- or glucose-stimulated cell supernatants. Insulin and glucose increased adipokine secretions in AdS. Adiponectin was supposed to inhibit VSMC proliferation and leptin to promote it. The unbalance of these two adipokines in AdS had an unusual effect on VSMC proliferation. In SD groups, VSMC proliferation reduced concordantly with increased adiponectin and decreased leptin levels. On the other hand, VSMC proliferation was reduced by AdS of males under HFD in spite of increased leptin and decreased adiponectin compared to the control. These findings show the modulated effect of adipokines depending on the mixture present in AdS. In fact, adipocytes used in this protocol were conditioned by diet FA during 20 weeks. Male mice fed HFD were diabetic, a status associated with elevated FA production. AdS FA concentration was not evaluated, but we expect that its release was increased in HFD-origin adipocytes concordantly with increased CD36 expression levels in VSMC. In fact, two distinctive phenotypes of adipocytes were found in isolated abdominal white fat (Supplementary Fig.2): hyperplasic with various stages of hypertrophy in VD-fed mice while mainly severely hypertrophic in AD-fed male mice. Dysfunctional hypertrophic adipocytes are associated with the release of free fatty acids.

Increased FA release also triggers VSMC apoptosis. In AD group, AdS reduced the number of VMSC by a pro-apoptotic effect, reflected by the increased mRNA expression of apoptotic factors (Bax and FAS) and caspase-3 activity in these cells. Activation of the pro-apoptotic pathways was also noticed in VSMC treated with AdS from AD-fed females, a contradictory effect to the increased proliferation rate of VSMC compared to SD group. These results suggest that increased cell mortality is counterbalanced by enhanced cell proliferation sustained by increased leptin secretion and decreased adiponectin levels in AdS from AD-fed mice, especially as these mice do not have a diabetic status. Moreover, increased FA concentration in AdS of VD and AD groups can
increase PPARγ activity in VSMC\textsuperscript{12}). Activation of PPARγ in these cells is well established to inhibit their proliferation\textsuperscript{20}. Thus, elevated FA in AdS of HFD groups could have contributed to the inhibition of VSMC proliferation. Finally, ERα, known to improve insulin sensitivity and glucose tolerance\textsuperscript{25}), showed decreased mRNA in adipocytes of HFD-mice with a 4 times higher rate in females than in males\textsuperscript{19}). In females, therefore, the higher ERα expression level in adipocytes may have contributed to maintaining better sensitivity to insulin and glucose stimulation, which inhibits lipolysis and reduces FA release.

Released FA could also affect glucose cell homeostasis and the response to insulin. In the presence of AdS treatment, InsR mRNA increased in AdS-I and AdS-G from the male AD group. Saturated FA present in AD as well as leptin present in AdS are known to impair insulin activation\textsuperscript{26, 27}). To counteract these effects, which reduce glucose uptake, VSMC increased their InsR expression. In contrast, AdS-U and AdS-G from VD female group decreased InsR mRNA. This corresponds with the effect of the MUFA-like oleic acid present in VD and known to improve insulin sensitivity\textsuperscript{28}).

Male and female mice from HFD groups had an increased serum concentration of FA\textsuperscript{19}). FA rich AdS from HFD-mice can stimulate PPARγ in VSMC\textsuperscript{12}), triggering CD36 gene expression\textsuperscript{9}). High leptin concentrations also increased CD36 expression in VSMC\textsuperscript{29}). In our study, CD36 expression was increased in hyperleptinemic AdS from HFD groups. CD36 expression increased FA accumulation in VSMC and induced apoptosis, as shown in the male AD group. In the female AD group, the less advanced adipocyte hypertrophy combined with higher adiponectin, known to prevent FA accumulation\textsuperscript{30}), may have contributed to limit apoptosis in VSMC exposed to AdS.

Collectively, these data indicate that the development of adipocyte-induced VSMC alterations is sex related and linked to diet FA composition and the degree of mouse metabolic alterations. Such alterations modulate free FA and secrete a cocktail of adipokines from adipocytes and define VSMC proliferation and sensitivity to apoptosis as well as their profile of response to insulin and glucose stimulation.

These results may contribute to better understanding of the complex interplay between abdominal white fat and distal vascular sites that lead to increased atherosclerotic events in obesity-linked metabolic syndrome.

Conflicts of Interest

None of the authors has any potential benefits or conflicts of interest to disclose.

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Adipocytes Influence Vascular Alterations


Adipocytes from Male and Female Mice Fed High-Fat Diets Differently Alter Vascular Smooth Muscle Cell Phenotype (Supplementary Data)

**Supplementary Results to be Replaced by the New Version of Supplemental Data**

**Immunocytochemical Characterization of VSMC**

VSMC were cultured and allowed to grow for 12 hr on 8 mm glass lamella. Following two washes in culture media DMEM, the cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were then washed twice with PBS BSA 1%, and incubated for 1 hr in 2% normal goat serum to block unspecific protein. Cells were then washed twice and incubated with anti-α smooth muscle actin antibody (NeoMarker, Fremont, CA, USA) overnight at 4°C. A control lamella was incubated with the corresponding IgG to reveal unspecific labeling. To evaluate any endothelial cell contamination in our culture, a control lamella was incubated with anti-von Willebrand antibody (Invitrogen, Burlington, ON, USA) for the same time.

After two washes in PBS BSA 1% and further incubation with affinity purified Alexa 488 conjugated anti-rabbit antibody (Invitrogen) for one hour at room temperature, fluorescent specimens were visualized under a microscope and photographed (Supplementary Fig. 1). As a control for unspecific labeling of the secondary antibody, VSMC were treated with Alexa 488 for one hour at room temperature.

**Supplementary Fig. 1.** Immunocytochemical characterization of VSMC. VSMC fluorescent specimens were visualized under a microscope and photographed after incubation with anti-α smooth muscle actin antibody (A) or the corresponding IgG (B) and then Alexa 488 conjugated anti-rabbit antibody. To evaluate any endothelial cell contamination in our culture, a control lamella was incubated with anti-von Willebrand antibody (C) and then with Alexa 488 conjugated anti-rabbit antibody. A final control for unspecific labeling of the secondary antibody was performed by incubating VSMC with Alexa 488 conjugated IgGs (D).
**Supplementary Table 1.** Specific primer sequence

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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>PPARγ2</td>
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<td>5’-GGT-GGA-GAT-GCA-GGT-TCT-AC-3’</td>
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<tr>
<td>CD36</td>
<td>5’-GCC AAG CTA TTG CGA CAT GA-3’</td>
<td>5’-AAG GCA TTG GCT GGA AGA AC-3’</td>
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<td>Insulin receptor</td>
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<td>5’-TGG AGA GGT AGA TGA GCC GC-3’</td>
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<td>Bax</td>
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<tr>
<td>FAS</td>
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**Supplementary Fig. 2.** Mature adipocytes in culture.

Mature adipocytes were observed under a microscope after extraction from the visceral adipose tissue of corresponding mouse groups.