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Purinergic signaling modulates human visceral adipose inflammatory responses: implications in metabolically unhealthy obesity

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ABSTRACT

Obesity is accompanied by chronic inflammation of VAT, which promotes metabolic changes, and purinergic signaling has a key role in a wide range of inflammatory diseases. Therefore, we addressed whether fat inflammation could be differentially modulated by this signaling pathway in the MUO and in individuals who remain MHO. Our results show that the necrotized VAT of both groups released greater levels of ATP compared with lean donors. Interestingly, MUO tissue SVCs showed up-regulation and engagement of the purinergic P2X7R. The extracellular ATP concentration is regulated by an enzymatic process, in which CD39 converts ATP and ADP into AMP, and CD73 converts AMP into adenosine. In VAT, the CD73 ectoenzyme was widely distributed in immune and nonimmune cells, whereas CD39 expression was restricted to immune CD45PAN$^+$ SVCs. Although the MUO group expressed the highest levels of both ectoenzymes, no difference in ATP hydrolysis capacity was found between the groups. As expected, MUO exhibited the highest NLRP3 inflammasome expression and IL-1β production. MUO SVCs also displayed up-regulation of the A2AR, allowing extracellular adenosine to increase IL-1β local secretion. Additionally, we demonstrate that metabolic parameters and BMI are positively correlated with purinergic components in VAT. These findings indicate that purinergic signaling is a novel mechanism involved in the chronic inflammation of VAT underlying the metabolic changes in obesity. Finally, our study reveals a proinflammatory role for adenosine in sustaining IL-1β production in this tissue. *J. Leukoc. Biol.* 97: 941–949; 2015.

Introduction

Obesity is a major health threat in developing and developed countries and represents a significant risk factor for type II diabetes, cardiovascular diseases, and cancer [1, 2]. However, a proportion of obese individuals might not be at an increased risk for metabolic complications, and therefore, their phenotype can be referred to as MHO; conversely, those with metabolic abnormalities are referred to as MUO [3]. As no universally accepted criteria exist to define MHO, many questions have been raised regarding the biologic basis of metabolic changes in the MUO phenotype [4]. Within this context, it remains unclear how obesity promotes insulin resistance; nonetheless, results from clinical, epidemiologic, and molecular studies have converged to highlight the role of inflammation [5].

The progression of obesity results in a state of chronic, low-grade system inflammation [6] that includes fat deposition [7], particularly VAT [8–10]. This tissue releases soluble factors that contribute to the maintenance of local homeostasis and/or the development of inflammatory responses [11]. In fact, NLRP3 inflammasome activation and IL-1β secretion by VAT have been associated with the metabolic disorders related to obesity [12–14].

In their physiologic state, mammalian cells contain high concentrations of ATP. Under pathologic conditions, ATP is released from intracellular stores to the extracellular space where it acts as a danger- associated molecular pattern [15]. Among several functions, this nucleotide is a potent inflammasome-activating signal in vitro and in vivo [16]. With regard to the different purinergic receptors capable of sensing ATP, the P2X7R is unique in its inflammasome-activation ability [17]. Indeed, the role of purinergic signaling as an important regulatory mechanism has been described in a wide range of inflammatory diseases, such as allergen-driven lung inflammation [18, 19], diabetes [20], inflammatory bowel disease [21, 22], arthritis [23], multiple sclerosis [24], and graft-versus-host disease [25].

The extracellular ATP concentration is regulated by a two-step enzymatic process. In the first step, ATP and ADP are converted

Abbreviations: BDS = base-deactivated silica, BMI = body mass index, BP = blood preassure, CD54PAN = CD45 pan leukocyte antigen, FSK = forskolin, MHO = metabolically healthy obese, MS = metabolic syndrome score, MUO = metabolically unhealthy obese, NLRP3 = nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3, qRT-PCR = quantitative RT-PCR, SVC = stromal vascular cell, VAT = visceral adipose tissue

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to AMP through CD39; in the second step, the ecto-5′-nucleotidase CD73 converts AMP into adenosine. In humans, polymorphisms in both ectoenzymes lead to an increased susceptibility to inflammatory diseases [26–28].

In contrast to the proinflammatory role of ATP, adenosine is known to limit overwhelming inflammation and tissue damage [29]. However, adenosine can also increase IL-1β production through the A2AR [30, 31].

Although ATP and adenosine levels increase in the extracellular space in inflammatory states [15, 18, 22, 25], no studies have examined their contribution to obesity-associated inflammation. Our results show that the purinergic signaling pathway in MUO VAT can sustain chronic local inflammation through adenosine production. Although tissue inflammation was found in all obese patients, the higher levels of purinergic receptors and inflammasome activation in the MUO group suggest that this pathway is involved in the development of the metabolic disorders related to obesity.

MATERIALS AND METHODS

Subjects and tissue sampling

The study recruited 55 surgical patients who were included into 3 groups, as defined by World Health Organization criteria for obesity and by the definition for MS [32]. We studied 40 obese patients (BMI $>$ 30 kg/m^2; 30 MUO and 10 MHO) undergoing laparoscopic bariatric surgery and 15 lean donors (BMI $<$ 24.9 kg/m^2) undergoing laparoscopic abdominal surgery (e.g., fundoplication for reflux disease, cholecystectomy for gallstone disease, appendectomy or endoscopic repair of hernias).

Participants with 3 or more of the following criteria were considered to be MUO: 1) waist circumference $\geq$ 102 cm in men and $\geq$ 88 cm in women; 2) triacylglycerol level $\geq$ 150 mg/dl; 3) HDL-cholesterol level $<$ 40 mg/dl in men and $<$ 50 mg/dl in women; 4) systolic BP $\geq$ 130 mmHg and/or diastolic BP $\geq$ 85 mmHg; 5) fasting glucose level $\geq$ 100 mg/dl.

A MS (from 0 to 5) was calculated as a function of the number of criteria described above, shown by each patient. The MHO and MUO phenotypes were defined as the absence (MS $\leq$ 2) or presence (MS $\geq$ 2), respectively, of MS. The MUO group contained 5 patients with type 2 diabetes, all of whom were treated with insulin. Patients with inflammatory and malignant diseases or those taking oral glucose-lowering drugs were excluded. Lean donors were age and sex matched with obese patients. This study was approved by the Ethics Committee at the Hospital de Clínicas "José de San Martín."

Explant preparation and SVC isolation

With the use of a mini-knife, we prepared small pieces (200 mg) of tissue (explants) composed of adipocytes and SVCs.

To separate SVCs from adipocytes, the tissue was minced and digested with collagenase type II (Gibco, Grand Island, NY, USA) for 1 h. The digested material was filtered through a 100 μm nylon cell strainer, and SVCs were isolated through a Ficoll-Hypaque gradient. The cells were washed and suspended in PBS.

ATP measurement in VAT

To measure the release of ATP from VAT, necrotized explants (200 mg fat tissue) were subjected to 1 cycle of freezing/thawing (−70 to 37°C), whereas viable explants were not subjected to any stressful stimulus. Afterward, both the necrotized and viable explants were washed with 200 μl RPMI, and the levels ATP levels were quantified by use of the CellTiter-Glo reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. Light units were measured by use of an optimal luminometer (VICTOR X; Perkin Elmer, Waltham, MA, USA). To calculate the ATP concentration, a standard curve was plotted, and a regression analysis was applied.

VAT culture

Explants (200 mg fat tissue) or SVCs (5×10^5) were cultured in RPMI-1640 medium (Gibco), supplemented with 10% heat-inactivated FBS at 37°C. To test P2X7R mRNA modulation, SVCs were exposed to ATP (25 μM) for 12 h. The cells were then resuspended in Trizol (Life Technologies, Carlsbad, CA, USA) and stored at −70°C until use. To test for IL-1β secretion, explants or SVCs were primed with LPS overnight (1 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) and then stimulated with ATP (last 5 h of culture; 25 μM; Sigma-Aldrich) and/or adenosine (last h of culture; 100 μM; Sigma-Aldrich). In some experiments, the cells were also exposed to FSK, an adenylate cyclase activator (100 μM; Sigma-Aldrich). The supernatants were collected and stored at −70°C until use.

qRT-PCR

Total RNA was extracted by use of Trizol and subjected to reverse transcription by use of Improm-II RT (Promega). A PCR analysis for P2X7R, A2AR, NLRP3, and pro-IL-1β was performed with a real-time PCR detection system (Mx3000P; Stratagene, Agilent Technologies, Santa Clara, CA, USA) by use of SYBR Green as a fluorescent DNA-binding dye. The primer sets used for amplification were as follows: P2X7R, forward 5′-tccagtaactgctgtcgctc-3′, and P2X7R, reverse 5′-tggactcgcacttcttcctg-3′; pro-IL-1β, forward 5′-agctacgaatctccgaccac-3′, and pro-IL-1β, reverse 5′-cgttatcccatgtgtcgaagaa-3′; NLRP3, forward 5′-ccacaagatccgtgagaaaaccc-3′, and NLRP3, reverse 5′-cggtcctatgtgctcgtca-3′; A2AR, forward 5′-tcactttcttctgccccgac-3′, and A2AR, reverse 5′-cgtggctgcgaatgatcttg-3′; GAPDH, forward 5′-cgaccactttgtcaagctca-3′, and GAPDH, reverse 5′-ttactccttggaggccatgt-3′. All primer sets yielded a single product of the correct size. The relative mRNA expression levels were calculated by use of $2^{-\Delta \text{comparative threshold}}$ [33], and the data were normalized according to GAPDH mRNA levels. The results are presented as a value relative to the control value.

Flow cytometry

SVCs were stained with anti-CD45PAN (BioLegend, San Diego, CA, USA), anti-CD39 (BD PharMingen, San Diego, CA, USA), and anti-CD73 (BD PharMingen) antibodies. Isotype-matched mAb were used as controls. Data were acquired by use of a FACSAria II (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software. Statistical analyses were based on at least 100,000 events gated on the population of interest.

ATP hydrolysis assay

SVCs (1×10^5/well) were incubated in the presence of 25 μM ATP for 15 min at room temperature. The CellTiter-Glo reagent was added, per the manufacturer's instructions. ATPase activity was quantified by a reduction in luminescence. ATPase inhibitor ARL67156 (100 μM; Sigma-Aldrich) or anti-human CD39 (10 μg/ml; BioLegend) was added to the culture, as indicated. The percentage of ATP hydrolysis was determined as follows: 100-[(luminescence SVC/25 μM ATP luminescence) $\times$ 100].

Adenosine determination

SVCs (1×10^5/well) were incubated in the presence of 25 μM ATP for 40 min at 37°C. Adenosine production was determined by HPLC (Agilent 1100; Agilent Technologies, Wilmington, DE, USA), equipped with a UV detector, quaternary pump, and a degasser. Separation was achieved by use of a BDS Hypersil-C18 column (100 $\times$ 4.6 mm, 5 μM) and a BDS Hypersil-C18 guard column (10 $\times$ 4.6 mm, 5 μM). The flow rate was 0.8 ml/min, and the column temperature was 40°C during the run. The mobile phase was 0.4% phosphoric acid:methanol (95:5). The injection volume was 10 μl, and detection was at 257 nm.

Cytokine quantification

The release of IL-1β (17 kD) by explants (200 mg fat tissue) or SVCs (5×10^5) was analyzed in culture supernatants by use of a commercially available IL-1β ELISA kit (BD Biosciences). The assays were performed in duplicate, and the results are expressed as the mean ± SEM.

TABLE 1. Demographic and clinical characteristics of the study groups

	Lean	MHO	MUO
Age	40 ± 2.9	41 ± 3.5	43 ± 1.4
Men/women	3/12	3/7	8/22
BMI (kg/m^2)	22 ± 0.6	34 ± 2.6[a]	40 ± 1.3[a]
Waist circumference[b]	0/15	10/10	30/30
Triacylglycerol level[c]	0/15	0/10	25/30
HDL-cholesterol level[d]	0/15	3/10	24/30
BP[e]	0/15	0/10	21/30
Fasting glucose level[f]	0/15	5/10	17/30
Metabolic score[g]			
0	15/15	0/10	0/30
1	0/15	2/10	0/30
2	0/15	8/10	0/30
3	0/15	0/10	11/30
4	0/15	0/10	11/30
5	0/15	0/10	8/30

[a]$P < 0.001$ versus lean. [b]Waist circumference: ≥102 cm in men; ≥88 cm in women. [c]Triacylglycerol level: ≥150 mg/dl. [d]HDL-cholesterol level: <40 mg/dl in men; <50 mg/dl in women. [e]Systolic BP: ≥130 mmHg and/or diastolic BP: ≥85 mmHg. [f]Fasting glucose level: ≥100 mg/dl. [g]Metabolic score: 0–5; number of positive criteria ([b–f]) shown by each patient.

Immunohistochemistry

Explants were fixed in 10% formaldehyde, embedded in paraffin (Biopack, Buenos Aires, Argentina), and sectioned by the Department of Pathology at the Hospital de Clínicas "José de San Martin." Sections (4 μm thick) were cut by use of a sliding microtome and stained with H&E or processed as described. The slides were deparaffinized in xylene, rinsed in ethanol, and rehydrated. Endogenous peroxidase activity was quenched, and induced epitope retrieval was performed. The slides were incubated with an anti-CD39 antibody (1:100; BioLegend) and detected with the Vectastain Universal Elite ABC kit (PK-6200; Vector Laboratories, Rodovre, Denmark). Human pancreatic cryosections were used as the positive control. Images were acquired by use of a Leica ICC50 high-definition microscope system (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Statistical analyses were performed by use of GraphPad Prism 5.0 software. Data normality was evaluated by the Shapiro-Wilk test. For comparisons between groups, the paired *t*-test, repeated-measures ANOVA, one-way ANOVA, or Kruskal-Wallis test was used, as appropriate. Correlations were assessed by use of the Spearman correlation test. $P < 0.05$ was considered statistically significant.

RESULTS

Patients' anthropometric and clinical characteristics

The clinical details are summarized in **Table 1**. As expected, the BMI of the MHO (35 ± 3.7, $P < 0.001$) and MUO (40 ± 1.3, $P < 0.001$) groups was significantly higher than that of the lean donors (22 ± 0.6). MUO showed a higher BMI compared with MHO, even when no significant differences were found. Waist circumference was increased in all MHO and MUO individuals. The prevalence of elevated BP was similar among lean donors (0/15) and MHO (0/10). Additionally, some MHO individuals showed low HDL values (3/10) or elevated fasting glucose levels (5/10). In contrast, most of the MUO individuals exhibited high triacylglycerol levels (25/30), low HDL levels (24/30), elevated BP (21/30), and increased fasting glucose levels (17/30). When evaluating the criteria for MS, all lean donors showed a score of 0 (0/15), and 2 patients in the MHO group had a score of 1 (2/10), with 8 patients receiving a score of 2 (8/10); 11 patients in the MUO group had a score of 3 (11/30) or 4 (11/30); and 8 patients had a score of 5 (8/30).

ATP released by VAT modulates the P2X7R

To test whether VAT is capable of releasing ATP in the presence of a stressful signal, explants were necrotized, and the ATP concentration was measured ($n = 6$ from each group).

Figure 1A shows that VAT from MHO (53 μM ± 8) and MUO (61 μM ± 5.4) individuals was able to release higher levels of ATP compared with lean donors (37 μM ± 4.9). However, we only found a significant difference between the MUO and lean groups ($P < 0.05$). ATP levels were barely detectable when viable VAT was tested (0.02 μM ± 0.01, $n = 6$) but increased when it was necrotized, ranging between 20 and 80 μM.

We then evaluated the capacity of extracellular ATP to modulate P2X7R, one of ATP receptors expressed in human VAT [34]. As shown in Fig. 1B, after 12 h of culture, ATP exposure significantly increased P2X7R mRNA levels in the SVCs from lean donors ($n = 7$, $P < 0.05$). In addition, we quantified the constitutive expression of this ATP receptor in SVCs from the lean ($n = 8$), MHO ($n = 6$), and MUO ($n = 12$) groups. Interestingly, a significant increase of P2X7R mRNA levels was found in MUO SVCs compared with lean ($p < 0.05$) and MHO ($p < 0.001$; Fig. 1C) SVCs.

Higher expression of CD39/CD73 ectoenzymes in MUO SVCs

We next analyzed the expression of CD39 and CD73 in SVCs from the 3 groups of patients. The strategy of gating is summarized in **Fig. 2A**.

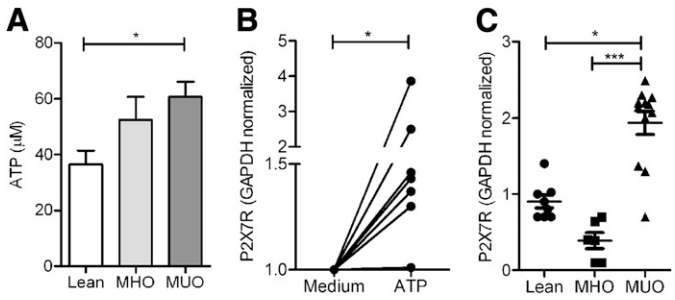

Figure 1. ATP released by VAT modulates P2X7R expression in MUO SVC. (A) Explants (200 mg fat tissue) from lean donors ($n = 6$), MHO ($n = 6$), and MUO ($n = 6$) patients were necrotized by freezing/thawing, and ATP levels were measured in the supernatant by luminometry. (B) P2X7R expression in the SVC from lean donors ($n = 7$), cultured for 12 h in the presence of ATP or medium alone as qRT-PCR. (C) Basal P2X7R expression in lean donor ($n = 8$), MHO ($n = 6$), and MUO ($n = 12$) SVC as qRT-PCR. The data represent the mean ± SEM. *$P < 0.05$; ***$P < 0.001$.

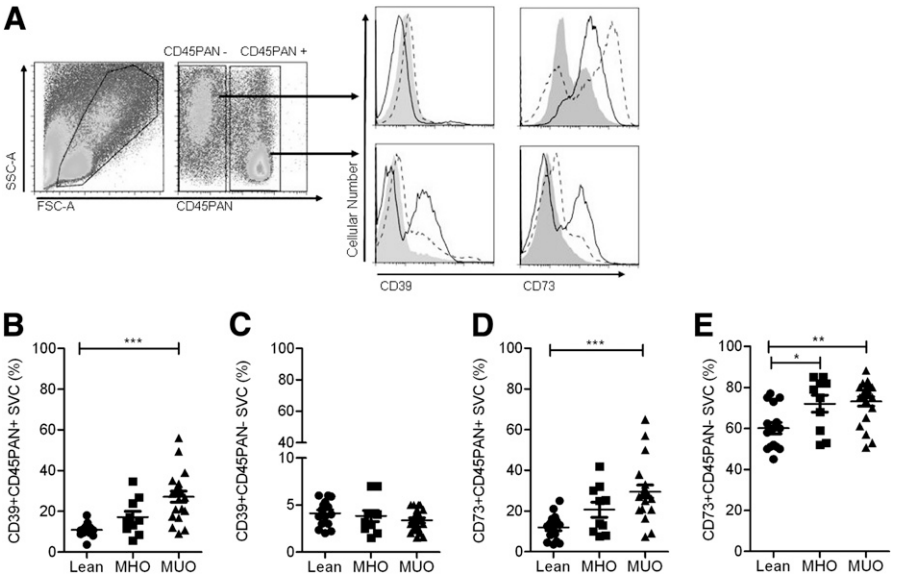

Figure 2. The expression of CD39 and CD73 is increased in SVC from obese patients. SVCs, isolated from lean donor ($n = 15$), MHO ($n = 10$), and MUO ($n = 20$) VAT, were labeled with anti-CD45PAN, -CD39, and -CD73 antibodies and analyzed by flow cytometry. (A) Representative FACS profile showing the gating strategy of CD39 and CD73 expression in the lean donor (filled histogram), MHO (discontinuous line), and MUO (continuous line) groups. SSC-A, Side-scatter-area; FSC-A, forward-scatter-area. (B and C) The frequency of $CD39^+$ SVC in $CD45PAN^+$ (B) and $CD45PAN^-$ (C) subsets. (D and E) The frequency of $CD73^+$ SVC in $CD45PAN^+$ (D) and $CD45PAN^-$ (E) subsets. The data represent the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

In VAT, the CD39/CD73 ectoenzymes were differentially expressed among immune ($CD45PAN^+$) and nonimmune ($CD45PAN^-$) SVCs. For instance, CD73 was widely distributed in SVCs, but CD39 was only expressed in $CD45PAN^+$ cells.

CD39 showed increased expression in $CD45PAN^+$ MHO (17% ± 2.8, $n = 10$) and MUO (27% ± 2.7, $n = 20$) SVCs compared with lean donors (11% ± 0.8, $n = 15$), a difference that was statistically significant between the MUO and lean groups ($P < 0.001$; Fig. 2B). In contrast, CD39 expression did not show any difference among lean (4% ± 0.4), MHO (4% ± 0.6), and MUO (3% ± 0.3) $CD45PAN^-$ SVCs (Fig. 2C).

CD73 expression was found to be increased in $CD45PAN^+$ MHO (21% ± 3.7) and MUO (30% ± 3.2) SVCs when compared with cells from lean donors (12% ± 1.7), but the difference was only statistically significant between the MUO and lean groups ($P < 0.001$; Fig. 2D). Unlike CD39 expression, CD73 was also increased in $CD45PAN^-$ MHO (72% ± 4.1) and MUO (73% ± 2.3) SVCs when compared with cells from lean donors (60% ± 2.7, $P < 0.05$, and $P < 0.01$ for MHO and MUO, respectively; Fig. 2E).

VAT from lean, MHO, and MUO individuals generates extracellular adenosine

We next investigated whether the increased expression of CD39 and CD73 in MUO SVCs had functional implications. Upon the addition of exogenous ATP, we detected that SVCs from lean, MHO, and MUO ($n = 6$ from each group) individuals showed ATPase activity, as assessed by luminometry. In the presence of unspecific (ARL67156) or specific (anti-CD39-blocking) ATPase inhibitors, the percentage of hydrolysis decreased significantly ($P < 0.05$), indicating that the CD39 ectoenzyme plays a key role in ATP catabolism in VAT (**Fig. 3A**).

Despite MUO showing the highest levels of CD39, we found no difference in the hydrolysis capacity among the 3 groups. To

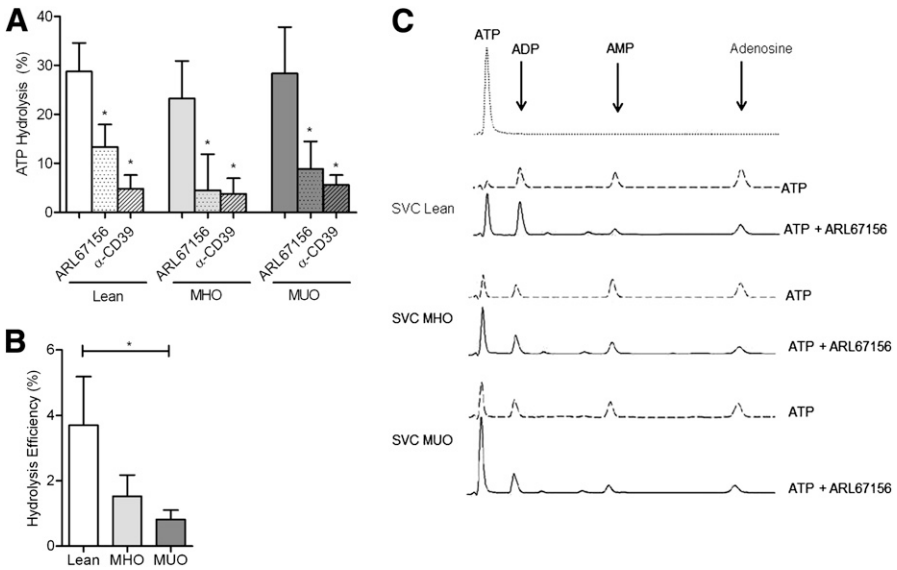

Figure 3. SVCs from lean donors and MHO and MUO patients produce extracellular adenosine. (A) SVCs (10×10^5), from lean donors and MHO and MUO ($n = 6$ in each group) patients, were incubated in the presence of ATP, ATP plus ARL67156, or ATP plus anti-human CD39 for 15 min. The bars show the percent of ATP hydrolysis quantified by luminometry. (B) The hydrolysis efficiency of ATP by SVCs from the 3 groups of donors, calculated as follows: % ATP hydrolysis/% $CD39^+CD45PAN^+$ SVC. (C) Representative HPLC chromatograms showing the ATP breakdown products ADP, AMP, and adenosine by SVCs ($n = 2$ in each group), incubated with ARL67156 or medium alone. The data represent the mean ± SEM. $^*P < 0.05$.

clarify this issue, the hydrolysis efficiency was calculated as follows: % ATP hydrolysis/% $CD39^+CD45PAN^+$ SVC (the subset that expresses this ectoenzyme) in each group of donors. Figure 3B shows that the MHO (1.5% ± 0.7) and MUO (0.8% ± 0.3) groups had a lower hydrolysis efficiency compared with the lean donors (3.7% ± 1.5). However, it was only statistically significant between the MUO and lean groups ($P < 0.05$), with no functional correlation between the percentage of cells expressing CD39 and the capacity of ATP catabolism.

Finally, adenosine production by SVC was studied by HPLC. This assay showed that SVC can generate ADP, AMP, and adenosine in the presence of extracellular ATP, with CD39 activity partially inhibited by the addition of ARL67156 (Fig. 3C).

Inflammasome components and IL-1β secretion are increased in MUO VAT

Several proinflammatory cytokines, such as IL-1β, are implicated in disrupting insulin signaling [13, 14]. Moreover, the inflammasome NLRP3 component is known to be the key post-translational regulator of IL-1β [12].

To confirm that VAT inflammation is related to obesity, we tested the mRNA levels of pro-IL-1β in lean ($n = 8$), MHO ($n = 6$), and MUO ($n = 12$) SVCs. Compared with the lean donors, pro-IL-1β mRNA expression was increased in the MHO ($P < 0.001$) and MUO ($P < 0.05$; **Fig. 4A**) groups. However, the levels of NLRP3 mRNA were only up-regulated in MUO compared with lean ($P < 0.01$) and MHO ($P < 0.01$; Fig. 4B) individuals.

To investigate whether the observed differences in NLRP3 expression in MUO were related to differences in the IL-1β secretion capacity, explants and SVCs from donors were exposed to LPS/ATP for 18 h, and the levels of this cytokine were measured in the culture supernatant ($n = 6$ from each group). As shown in Fig. 4C, explants from MUO individuals (367 pg/ml ± 47) secreted higher levels of IL-1β compared with the MHO (285 pg/ml ± 47) and lean (152 pg/ml ± 98) groups. Moreover, IL-1β production by SVC was also increased in MUO (731 pg/ml ± 102) compared with the MHO (301 pg/ml ± 11, $P < 0.05$) and lean (222 pg/ml ± 47, $P < 0.05$; Fig. 4D) groups, reaching statistical significance. This finding suggests that the metabolic changes in obesity can increase inflammasome chronic activity in VAT, enhancing the maturation of pro-IL-1β to IL-1β in MUO.

Adenosine increases IL-1β secretion in VAT

In mice, it was found that adenosine sustains inflammasome activation by acting through A2AR [31]. Thus, we analyzed the expression of this receptor in SVCs. As shown in **Fig. 5A**, a significant increase was observed in the MUO ($n = 12$) compared with the MHO ($P < 0.001$, $n = 6$) and lean ($P < 0.05$, $n = 8$) groups.

We next tested whether the addition of adenosine could increase IL-1β secretion above levels induced by inflammasome signals 1 (LPS) and 2 (ATP) in the VAT of lean donors. As expected, the combination of LPS and ATP resulted in high levels of IL-1β production in explant (317 pg/ml ± 77) and SVC (352 pg/ml ± 92) cultures. When adenosine was added to LPS and ATP, a significant increase in production was quantified in the explant supernatants (554 pg/ml ± 88, $P < 0.05$; Fig. 5B).

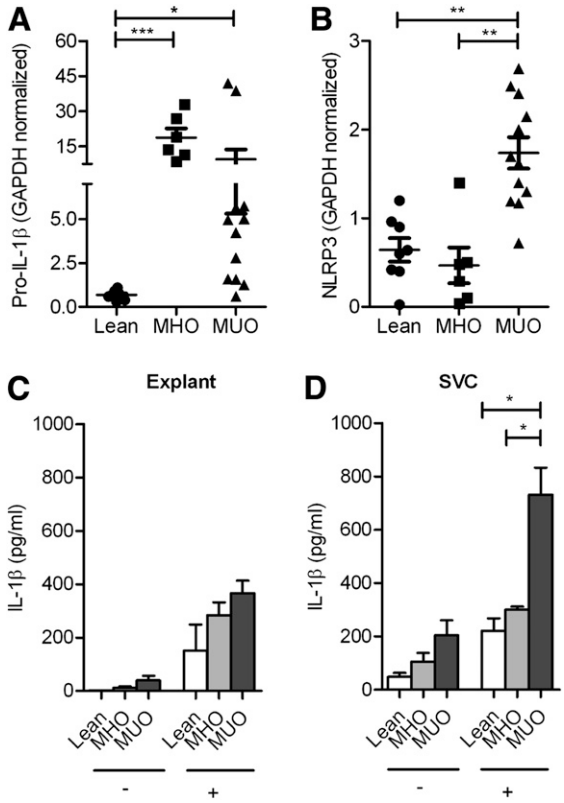

Figure 4. Higher levels of IL-1β and NLRP3 in MUO SVC. (A and B) Basal expression of pro-IL-1β (A) and NLRP3 (B) mRNA in lean donor ($n = 8$) and MHO ($n = 6$) and MUO ($n = 12$) SVCs was quantified by qRT-PCR. (C and D) Explants (200 mg fat tissue; C) or SVCs (5×10^5 SVCs; D) from lean donors and MHO and MUO patients ($n = 6$, from each group) were cultured in the presence of LPS (overnight) plus ATP (last 5 h) or medium alone for 18 h, and the levels of IL-1β were determined by ELISA. The data represent the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

In contrast, the addition of adenosine did not increase IL-1β levels in the SVC culture (284 pg/ml ± 62; Fig. 5C). Adenosine alone did not increase the levels of IL-1β in the explants or SVCs.

These findings could be explained by the endogenous production of adenosine when ATP was added to the culture, mediated by CD39 in SVCs but not in explants as a result of the lack of expression of this ectoenzyme in adipocytes (the main cell subset present in explants). As illustrated in Fig. 5D, we confirmed by immunohistological examination the absence of CD39 expression in adipocytes and its presence in the pancreatic epithelial cells that were used as the positive control.

A2AR is coupled to a stimulatory adenylate cyclase and up-regulates cAMP production. The contribution of adenosine to IL-1β production was tested by adding the adenylate cyclase activator FSK, which increased IL-1β production by SVC (376 pg/ml ± 33 vs. 537 pg/ml ± 51, $P < 0.05$, for LPS plus ATP and LPS plus ATP plus FSK, respectively; Fig. 5E). These results demonstrated that adenosine contributes to the obesity proinflammatory state of VAT. Of note, FSK alone did not modulate IL-1β secretion by SVC.

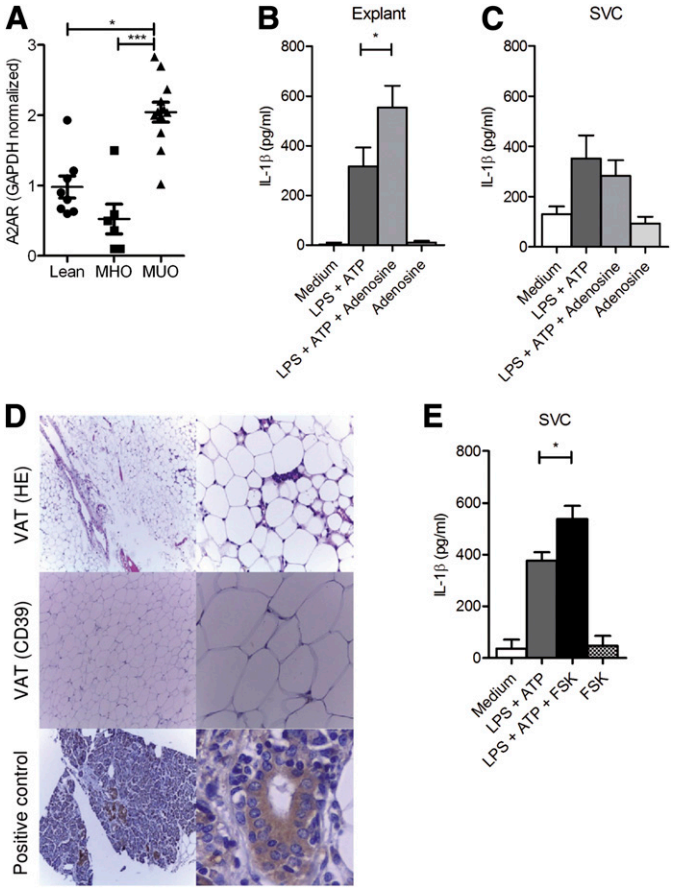

Figure 5. Adenosine modulates IL-1β production in VAT. (A) Basal expression of A2AR mRNA in lean donor ($n = 8$), MHO ($n = 6$), and MUO ($n = 12$) SVCs was quantified by qRT-PCR. (B and C) Explants (200 mg fat tissue; B) or SVCs (5×10^5 SVC; C) from lean donors were cultured in the presence of LPS (overnight) plus ATP (last 5 h), LPS plus ATP plus adenosine (the last hour), or medium alone for 18 h, and the levels of IL-1β were determined by ELISA ($n = 6$). (D) Immunohistochemistry of VAT. (Top) H&E staining; (middle) CD39 staining in VAT; (bottom) CD39 staining in pancreatic tissue, used as the positive control ($n = 3$). The images are shown at ×100 (left) and ×400 (right). (E) SVCs from lean donors were cultured in the presence of LPS (overnight) plus ATP (last 5 h) plus FSK (last hour), FSK alone, or medium alone for 18 h. The levels of IL-1β were determined by ELISA ($n = 6$). The data represent the mean ± SEM. $^*P < 0.05$; $^{***}P < 0.001$.

Clinical relevance of the purinergic signaling pathway in VAT

BMI is widely used as a surrogate measure for body fat, and this parameter has been shown to correlate with other measures of adiposity [35]. In addition to body-fat composition, other factors might be involved in metabolic changes, as some obese individuals remain metabolically healthy. In this sense, it is still unclear how obesity promotes insulin resistance, yet results from clinical, epidemiologic, and molecular studies have converged to highlight the role of inflammation [5]. In an attempt to clarify how fat inflammation promotes metabolic changes only in some individuals, we evaluated the potential association between activation of the purinergic signaling pathway in VAT with BMI or the degree of MS of donors.

As shown in **Fig. 6A**, there was a positive correlation between the capacity of ATP release and P2X7R, A2AR, CD39, and CD73 frequency within SVC and BMI ($r = 0.56$, $P < 0.05$; $r = 0.5$, $P < 0.01$; $r = 0.63$, $P < 0.001$; $r = 0.6$, $P < 0.001$; and $r = 0.58$, $P < 0.001$, respectively).

The data also showed a strong, positive association between MS and purinergic signaling pathway components ($r = 0.61$, $P < 0.01$; $r = 0.65$, $P < 0.001$; $r = 0.64$, $P < 0.001$; $r = 0.72$, $P < 0.001$; and $r = 0.68$, $P < 0.001$ for ATP release capacity and P2X7R, A2AR, CD39, and CD73 frequency within SVCs, respectively; Fig. 6B). These findings suggest that obesity and metabolic disease are accompanied by the increased activity of this pathway at the tissue level. Of note, the exclusion of MHO not only showed an improved correlation between this pathway and BMI (data not shown), but MS also appeared to be more tightly correlated to purinergic signaling in VAT.

DISCUSSION

It is established that VAT releases proinflammatory mediators, the production of which can be dysregulated in obesity, contributing in an important manner to insulin resistance. In the present study, we identified purinergic signaling as a novel mechanism that is involved in the maintenance of obesity-related inflammation in the VAT of obese patients with metabolic disorders.

ATP and adenosine are increased in the extracellular space in response to insults and function both as sensory and efferent signals to modulate immune responses [29, 36–38]. We show here that the VAT of obese patients was capable of releasing higher levels of ATP in response to an injury compared with lean donors. This is functionally relevant, as VAT inflammation could result in higher levels of this nucleotide in the extracellular space. Within this context, ATP acting through P2X7R could chronically perpetuate inflammatory responses in VAT. According to this finding, the mRNA levels of P2X7R were increased in MUO SVCs compared with those of lean donors and MHO individuals.

CD39 and CD73 ectoenzymes calibrate the duration and magnitude of the purinergic signals delivered to immune and nonimmune cells through the conversion of ATP to adenosine [39]. Although CD73 is widely expressed among epithelial and immune cells [40], our data show that CD39 expression was restricted to CD45PAN$^+$ SVCs, suggesting that this population is responsible for diminishing the ATP concentration once it is released into the extracellular space. It has been described that T cells resident in inflamed sites express high levels of the CD39 ectoenzyme [23]. In this sense, the higher expression of CD39 and CD73 in MUO CD45PAN$^+$ SVCs could be explained by the chronic inflammatory process in fat deposits. Moreover, these findings were not associated with increased ATPase activity in this tissue.

Inflammasome activation and IL-1β secretion in adipose tissue have been implicated in obesity-associated insulin resistance [12, 41]. Our data show that MHO and MUO individuals had increased levels of pro-IL-1β compared with lean donors, confirming the VAT proinflammatory state of obese patients.

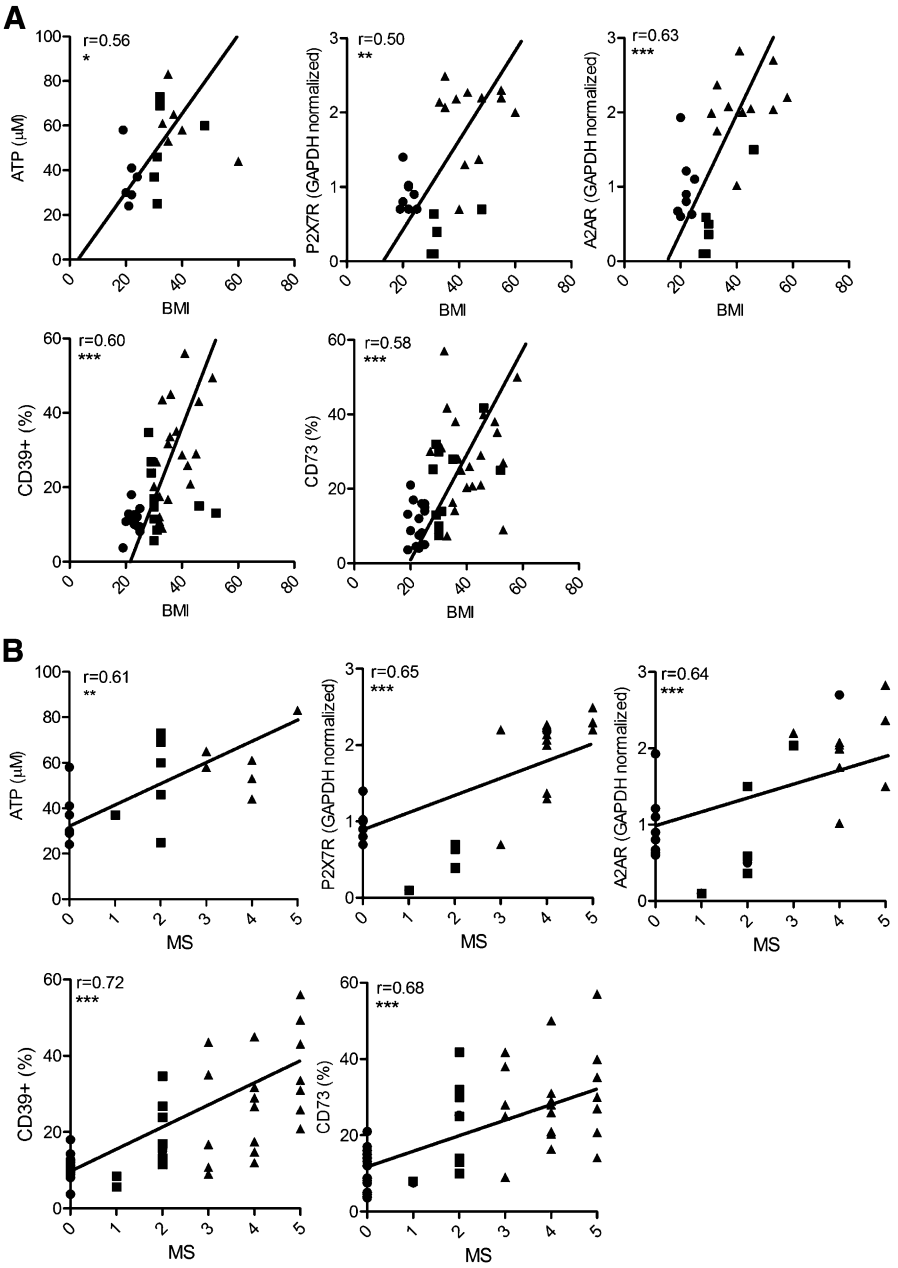

Figure 6. The expression of purinergic signaling components in VAT correlates positively with the degree of obesity and metabolic disease. Graphs showing correlations between the ATP release capacity ($n = 18$) and P2X7R ($n = 26$), A2AR ($n = 26$), CD39 ($n = 45$), and CD73 ($n = 45$) expression and lean donor (circles), MHO (squares), and MUO (triangles) BMI (A) or MS (B). Correlations were evaluated by use of a Spearman rank correlation coefficient test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Interestingly, higher NLRP3 mRNA expression and IL-1β production were only found in the MUO group, suggesting that metabolic changes in obesity can increase chronic inflammasome activity in VAT.

Although 2 distinct pathways are required for acute inflammasome activation [42, 43], an outstanding question in obesity is regarding which stimuli are involved in chronically sustaining IL-1β production in adipose tissue. P2X7R-deficient mice on a high-fat diet exhibit no changes in metabolic phenotype or inflammasome activation, suggesting the existence of other endogenous signals that could contribute to this phenomenon [44].

We show that SVCs are capable of producing adenosine in the presence of ATP and hypothesized that adenosine could also be increased as a result of the high levels of extracellular ATP in the VAT of obese patients. The fact that A2AR mRNA levels were increased in MUO SVCs but not in MHO and lean donors supports the notion that this mediator could sustain inflammasome activation in the VAT of these patients. Although there is some evidence that adenosine, acting through A2AR, mediates proinflammatory effects in mice by modulating IL-1β production [31, 45], no studies in humans have demonstrated similar effects. To our knowledge, this is the first report to demonstrate that SVCs from human VAT, after receiving signals 1 and 2, are dependent on adenosine for enhanced inflammasome activity and IL-1β production. Our results show that the addition of adenosine to human explant cultures increases IL-1β production. Interestingly, no differences in IL-1β levels were observed between the LPS/ATP or LPS/ATP/adenosine SVC culture

condition. One possible explanation is that compared with explants, SVCs express higher levels of CD39, hydrolyze ATP, and produce endogenous adenosine. In accordance with this, immunohistological examination demonstrated no expression of CD39 in adipocytes, which represent the main cell type in explants. Finally, the contribution of adenosine to IL-1β production by SVCs was evident when FSK, an adenylate cyclase activator, was added to the culture.

In spite of the fact that waist-circumference measurement allows a better characterization of body-fat distribution than BMI, this parameter might be less useful for the identification of MUO individuals, who are frequently under lifestyle intervention [3, 46]. However, the use of BMI as a diagnostic tool may have its limitations, as it underestimates the percentage of body fat, especially in the overweight category [4]. In this sense, it is important to mention that our study did not include overweight donors. Interestingly, we found a significant association between BMI and the purinergic signaling pathway in VAT. However, the fact that MS had the best correlation with some of the purinergic components suggests that the local activation of this signaling pathway is related to the severity of metabolic disease. Concerning the biologic implications of the study, these findings open a new avenue of research, not only regarding the biologic mechanisms underlying MUO but also the biologic basis of MHO. In the future, additional studies should investigate the transitory nature of MHO and predictors of this phenotype. Finally, this concept might be used in future clinical practice to design intervention strategies tailored toward the metabolic profile of an individual.

In conclusion, the purinergic signaling pathway in the VAT of MUO individuals can sustain chronic local inflammation through adenosine production. Although tissue inflammation was found in all obese patients, the higher expression of P2X7R/A2AR associated with a higher level of NLRP3, pro-IL-1β, and IL-1β in the MUO group suggests that this pathway is involved in the development of metabolic disorders related to obesity. The characterization of the proinflammatory role of adenosine in obese patients may be relevant for understanding other chronic inflammatory diseases and the discovery of new therapeutic targets.

AUTHORSHIP

J.P. and L.A. provided the conception and design. J.P., A.F., M.L., J.R.S., A.D., L.F., and L.A. did the analysis and interpretation. J.P., L.F., and L.A. drafted the manuscript for important intellectual content.

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DISCLOSURES

The authors declare no conflict of interest.

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