Interleukin-1β and prostaglandin-synthesizing enzymes as modulators of human omental and subcutaneous adipose tissue function

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Abbreviations:
AKR: aldo-keto reductase; **ATP5O:** ATP synthase O subunit; **BMI:** body mass index; **CCL-5:** C-C motif chemokine ligand 5; **C/EBPα:** CCAAT/enhancer binding protein alpha; **COX:** cyclooxygenase; **G3PDH:** glycerol-3-phosphate dehydrogenase; **ICAM-1:** intercellular adhesion molecule 1; **IL:** interleukin; **KRH:** Krebs-Ringer-Henseleit; **NF-κB1:** nuclear factor kappa B subunit 1; **PG:** prostaglandin; **PPARγ:** peroxisome proliferator activated receptor gamma; **TNF-α:** tumor necrosis factor alpha; **VEGFA:** vascular endothelial growth factor A.

ABSTRACT

IL-1β stimulates expression of prostaglandin (PG)-synthesizing enzymes cyclooxygenase (COX)-2 and aldo-keto reductase (AKR)1B1 in human preadipocytes. We aimed to examine the impact of IL-1β, COX-2 and AKR1B1 on markers of human visceral and subcutaneous adipose tissue function, and to assess whether PG synthesis by these enzymes mediates IL-1β effects. Omental and subcutaneous fat samples were obtained from bariatric surgery patients. PG release and expression of inflammatory and adipogenic markers were assessed in explants treated with COX-2 inhibitor NS-398 or AKR1B1 inhibitor Statil, with or without IL-1β. Preadipocyte differentiation experiments were also performed. IL-1β decreased expression of PPARγ in both fat depots compared to control and increased expression of NF-κB1, IL-6, CCL-5, ICAM-1 and VEGFA, especially in visceral fat for IL-6, CCL-5 and VEGFA. Adding Statil or NS-398 to IL-1β blunted $PGF_{2\alpha}$ and PGE_2 release, but did not alter IL-1β effects on adipose tissue function markers. IL-1β down-regulated adipocyte differentiation whereas NS-398 alone increased this process. However, NS-398 did not prevent IL-1β inhibition of adipogenesis. We conclude that IL-1β induces a pro-inflammatory response in human adipose tissues, particularly in visceral fat, and acts independently of concomitant PG release. IL-1β and COX-2 appear to be critical determinants of adipose tissue pathophysiologic remodeling in obesity.

Keywords: Adipogenesis, Cyclooxygenase, Cytokines, Eicosanoids, Inflammation, Obesity

1. INTRODUCTION

In the past decades, obesity and related metabolic disorders have been the focus of intensive research and dysfunctional adipose tissue has emerged as a key factor linking these conditions [1, 2]. Under a positive energy imbalance, adipose tissue expands through adipocyte hypertrophy by increasing the size of existing adipocytes and/or through adipocyte hyperplasia/adipogenesis by increasing cell number through differentiation of adipocyte precursor cells (preadipocytes) [3]. Because hypertrophied adipocytes may promote adipose tissue macrophage infiltration by increasing the release of pro-inflammatory mediators, altered capacity to generate new adipocytes during fat mass expansion is thought to be a critical component of adipose tissue dysfunction [4, 5]. Moreover, as a result of limited adipogenic capacity, altered ability to store excess lipids in subcutaneous adipose tissue is associated with excess intra-abdominal/visceral adiposity, which is known to increase the risk of metabolic alterations such as dyslipidemia and glucose intolerance [6-8]. This emphasizes the notion that adequate adipose tissue remodeling is crucial during fat mass accretion. A better understanding of inflammation and adipogenic modulators in humans is of particular importance.

Many studies have highlighted the relevance of the pro-inflammatory cytokine interleukin (IL)-1β in adipose tissue, particularly in obesity-associated inflammation and insulin resistance [4, 9-12]. Esser et al. [13] reported that IL-1β gene expression in visceral adipose tissue was positively correlated with body mass index (BMI) and waist circumference, whereas no significant association was found in subcutaneous adipose tissue. Interestingly, obese individuals presenting the metabolic syndrome had increased expression and secretion of IL-1β in visceral adipose tissue compared to lean and metabolically healthy obese patients [13]. Furthermore, higher protein release and mRNA levels of IL-1β were found in the visceral compared to the subcutaneous adipose tissue compartment, but this was only observed in the group of obese patients with the metabolic syndrome [13]. Treatment with IL-1β was shown to upregulate expression of pro-inflammatory genes such as nuclear factor kappa B subunit 1 (NF-κB1), C-C motif chemokine ligand 5 (CCL-5), IL-6 and intercellular adhesion molecule 1 (ICAM-1), while decreasing the

pro-adipogenic genes peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα) in human subcutaneous adipocytes, supporting a role for this cytokine in adipose tissue dysfunction [9]. Consistent with these results, we have previously shown that IL-1β stimulates expression and protein level of cyclooxygenase (COX)-2, an enzyme involved in prostaglandin (PG) synthesis, as well as PGF synthase aldo-keto reductase (AKR)1B1 in human primary preadipocytes [14]. Studies in the 3T3-L1 cell line and murine models have demonstrated the implication of series 2 PGs in the regulation of adipogenesis [15]. Taken together, these results underscore the need to study IL-1β in a depot-specific manner and the potential role of PGs in mediating IL-1β effects on adipose tissue function in humans.

PGs are pro-inflammatory lipid mediators exerting a biological action in almost all organs of the body, but unlike IL-1β, they have been much less studied in relation to human adipose tissue dysfunction [16]. Series 2 PGs are derived from arachidonic acid, which is released from membrane phospholipids by a phospholipase A_2 [16, 17]. Arachidonic acid is then converted to PGH_2 through the action of two PGH synthases, the constitutive enzyme COX-1 or the inducible isoform COX-2, which are the rate-limiting enzymes in PG biosynthesis [16, 17]. PGH_2 can be isomerized into biologically active lipid mediators, including $PGF_{2\alpha}$, PGE_2, PGD_2 and PGI_2, each being synthesized by specific PG synthase enzymes [16-18]. As reviewed in [15], PGD_2 and PGI_2 were shown to enhance adipocyte differentiation whereas $PGF_{2\alpha}$ and PGE_2 suppress the early phase of this process. Our previous results also suggest that in human preadipocytes, the recently identified PGF synthase AKR1B1 plays a predominant role in modulating $PGF_{2\alpha}$ synthesis in response to inflammatory cytokines such as IL-1β [14, 19], pointing toward a possible role of AKR1B1 in impairing fat storage and inflammatory pathways in human abdominal obesity. Moreover, the specific role of the rate-limiting, inducible COX-2 enzyme in modulating adipose tissue homeostasis in humans is largely unknown, whereas results from animal studies are conflicting [15].

Based on these previous findings, the aim of the present study was to examine the depot-specific impact of IL-1β and PG-synthesizing enzymes COX-2 and AKR1B1 on human adipose tissue inflammation and fat storage, in order to further assess whether PG synthesis through these enzymes mediates part of IL-1β effects. We tested the hypothesis that COX-2 and AKR1B1 are critical modulators of adipose tissue dysfunction and are partly responsible for the detrimental effects of IL-1β. We also hypothesized that visceral adipose tissue is more responsive to IL-1β compared to subcutaneous adipose tissue.

2. MATERIALS AND METHODS

2.1 Study population and adipose tissue sampling

Fresh omental and subcutaneous adipose tissue samples were obtained from 18 severely obese individuals (15 women and 3 men) undergoing bariatric surgery at the *Institut universitaire de cardiologie et de pneumologie de Québec* (IUCPQ) (age: 20-63 years, BMI: 40.3-62.0 kg/m^2). Omental and subcutaneous adipose samples were collected from the greater omentum and from the site of surgical incision, respectively. Tissue specimens were immediately carried to the laboratory. Fresh tissue was used to perform explant cultures (n=10, 9 women and 1 man) or preadipocyte isolation (n=8, 6 women and 2 men). Tissue specimens were obtained from the Biobank of the IUCPQ according to institutionally-approved management modalities. All participants provided written, informed consent. The protocol was approved by the Research Ethics Committee of our Institute (Protocol CER-IUCPQ 21049). Primary preadipocytes isolated from the subcutaneous adipose tissue of a 46 years old woman (BMI of 32.1) were also obtained commercially (ZenBio, NC, USA).

2.2 Explant cultures

Portions of fresh omental and subcutaneous adipose tissues were cut into 5-10 mg pieces and then placed in 6-well plates (approximately 50 mg per well). Adipose tissue explants were cultured for 24 hours in serum-free Medium 199 as follows: control, addition of COX-2 inhibitor NS-398 (1 μM) or AKR1B1 inhibitor Statil/Ponalrestat (10 μM), alone or in combination with IL-1β (1 ng/ml). Explants were incubated at 37°C under a 5% CO_2 atmosphere. Culture medium was then recovered for PG measurements ($PGF_{2\alpha}$ and PGE_2) and explants were used for mRNA expression analysis. Our previous findings and prior experiments showed that a 24-hour incubation with inflammatory stimulus (following a time-course experiment: 0, 3, 6, 16 and 24 hours) is optimal and that 1 ng/ml of IL-1β is sufficient to generate robust stimulation of PG-synthesizing enzymes and PG release to a higher extent than tumor necrosis factor alpha (TNF-α) [14]. Concentrations of NS-398 and Statil were determined according to

our previous studies in other models or cell types [14, 20]. Recombinant human IL-1β was purchased from PeproTech (Rocky Hill, NJ, USA). AKR1B1 inhibitor Statil was obtained from Tocris Bioscience (Ellisville, MO, USA) and the COX-2 inhibitor NS-398 from Cayman Chemical (Ann Arbor, MI, USA).

2.3 Preadipocyte isolation and primary cultures

According to a modified version of the Rodbell method [21], fresh adipose tissues were digested with 350 units/ml type I collagenase (Worthington Biochemical Corporation, NJ, USA) for 45 minutes at 37°C in Krebs-Ringer-Henseleit (KRH) buffer. Digested tissues were first filtered through nylon mesh and then two consecutive washes were performed with KRH buffer during which mature adipocytes were separated from the stromal-vascular fraction by floatation. With modifications of the Van Harmelen method [22], preadipocytes were thereafter isolated from the KRH buffer containing the stromal-vascular fraction, which was centrifuged for 5 minutes at 454 g. The pellet was washed in DMEM/F12 culture medium supplemented with 10% fetal calf serum, 2.5 μg/ml amphotericin B and 50 μg/ml gentamicin, before filtration through 140 μm nylon mesh to remove endothelial/mesothelial cells. The cell-containing medium was placed in culture plates and cultured at 37°C under a 5% CO_2 atmosphere. The following days, cells were cultured in DMEM/F12 supplemented with 10% fetal calf serum, 1% penicillin/streptomycin solution, 2.5 μg/ml amphotericin B, 33 μM biotin, 17 μM panthotenate and 100 μM ascorbic acid. Medium was changed every 2-3 days.

2.4 Induction and quantification of adipocyte differentiation

Subcutaneous preadipocytes were seeded in 96-well plates to obtain full confluence within 1-3 days. Differentiation was thereafter initiated with ZenBio (NC, USA) standardized adipocyte differentiation medium (DM-2 for subcutaneous preadipocytes) using previously described conditions (control; NS-398, 1 μM; and Statil, 10 μM; with or without IL-1β, 1 ng/ml) (day 0). On day 7, the differentiation medium (containing treatments) was replaced by a maintenance medium (AM-1 medium, ZenBio, NC, USA) for

14 more days in the absence of treatments. On day 21 of differentiation, pictures were taken with a phase contrast microscope at 10x magnification (Zeiss Axio Observer Z1). Glycerol-3-phosphate dehydrogenase (G3PDH) activity was measured to quantify the extent of differentiation according to tested conditions (after 21 days of preadipocyte differentiation) [23]. Briefly, cells were first washed with PBS and then homogenized in a cold solution containing 20 mM Tris (pH 7.3), 1 mM EDTA and 1 mM β-mercaptoethanol. Samples were stored at -80°C until analysis. A buffer containing 100 mM triethanolamine (pH 7.7), 2.5 mM EDTA, 0.1 mM β-mercaptoethanol and 353 μM NADH, combined with a reactive solution (0.4 mM dihydroxyacetone phosphate), was then used to assess G3PDH activity. Optical density at 340 nm was measured during 3 minutes at 37°C with a BioTek plate reader, and generation of a standard curve was performed to calculate G3PDH activity in mU of purified enzyme. G3PDH activity was normalized according to DNA content, which was quantified by a BioDrop DUO UV/Vis spectrophotometer. Activities were expressed as a function of control wells (fold over control).

2.5 $PGF_{2\alpha}$ and PGE_2 measurements

As described in [14, 24, 25], $PGF_{2\alpha}$ and PGE_2 accumulation was quantified in the culture medium of omental and subcutaneous adipose tissue explants by enzyme immunoassay and acetylcholinesterase-linked $PGF_{2\alpha}$ and PGE_2 tracer (Cayman Chemical, MI, USA). $PGF_{2\alpha}$ and PGE_2 secretion was expressed in pg/ml*mg tissue*24h.

2.6 Messenger RNA expression by quantitative real-time RT-PCR

Gene expression levels of NF-κB1, IL-6, CCL-5, ICAM-1 and vascular endothelial growth factor A (VEGFA) (inflammatory genes) as well as PPARγ and C/EBPα (pro-adipogenic genes) were measured in whole tissue explants of a subsample of participants (n=4 for omental and n=5 for subcutaneous adipose tissue). All quantitative real-time PCR measurements were performed by the gene expression platform of the *Centre hospitalier de l'Université Laval* (Québec, Qc, Canada). As recommended by the

manufacturer, tissues were homogenized in Qiazol buffer (Qiagen, Germantown, MD, USA) and then total RNA was isolated with the RNeasy mini kit on-column DNase treatment (Qiagen, Hilden, DE). A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) were used to assess quantity and quality of total RNA, respectively. For the synthesis of complementary DNA, Superscript III Rnase H-RT (Invitrogen Life Technologies, Burlington, ON, CA), oligo-dT_{18} and random hexamers were used. Complementary DNA was purified (Qiagen, Hilden, DE) and amplified in real-time using the LightCycler 480 (Roche Diagnostics, Mannheim, DE, USA) and the SYBRGreen I Master (Roche Diagnostics, Indianapolis, IN, USA). A melting curve was generated to assess non-specific signal. The number of copies of each mRNA was calculated according to Luu-The et al. [26] using second derivative method and a standard curve of Cq versus logarithm of the quantity. ATP synthase O subunit (ATP5O) served as the housekeeping gene to normalize expression levels of all analyzed genes. GeneTools 2.0 software (Biotools Inc, Edmonton, AB, CA) was used to design oligoprimer pairs and their specificity was verified by blast in the GenBank database. Synthesis was performed by IDT (Integrated DNA Technology, Coralville, IA, USA). Primer sequences were the following (5'→3' sense/antisense): NF-κB1 (AGCCTCTCTATGACCTGGATGACT/GCTGTTTCATGTCTCCTTGTGCTAGT), IL-6 (ACAGCCACTCACCTCTTCAGA/AGTGCCTCTTTGCTGCTTTCA), CCL-5 (CCATATTCCTCGGACACCACAC/TTCTTCTCTGGGTTGGCACACAC), ICAM-1 (CTTCCTCGGCCTTCCCATATT/GCTCCTTCCTCTTGGCTTAGTCA), VEGFA (GGCAGAAGGAGGAGGGCAGAAT/GGCACACAGGATGGCTTGAAGATG), PPARγ (GTCGGATCCACAAAAAAAGTAGAA/AGCGGGAAGGACTTTATGTATGA), C/EBPα (GCGAGCCAGGACTAGGAGATT/CCAGCTCAGCCCCAAGAATTC) and ATP5O (ATTGAAGGTCGCTATGCCACAG/AACGACTCCTTGGGTATTGCTTAA).

2.7 Statistical analyses

$PGF_{2\alpha}$ and PGE_2 release as well as gene expression data were analyzed using a three-way mixed model. Two experimental fixed factors, one associated with the comparison between measurements of two different fat depots (omental vs subcutaneous) and one associated with the comparison of six different conditions (control, NS-398 and Statil, with or without IL-1β) with interaction terms between the fixed factors were defined. These factors were analyzed as repeated measures with two levels using an unstructured covariance matrix for depot and a constant correlation within depot for conditions. G3PDH activity data (subcutaneous depot only) used the same statistical approach with one random factor and one fixed factor. The latter was analyzed as a repeated factor using a structure of correlated measurements with different variances and assuming a constant correlation regardless of the condition. The Akaike Information Criterion fitting criteria was used to determine the most suitable covariance structure for the mixed models. All variables were log-transformed to assess the normality and variance assumptions. The residual maximum likelihood was the method performed for the statistical analyses and the Kenward–Roger method was used to estimate denominator degrees of freedom for the test of fixed effect. The normality assumption was verified with the Shapiro-Wilk test on the error distribution from the Cholesky factorization of the statistical model. The Brown and Forsythe variation of Levene's test statistic was used to verify homogeneity of variances. Differences were considered statistically significant when $p \leq 0.05$. All analyses were conducted using the statistical package SAS, version 9.4 (SAS Institute Inc, Cary, NC, USA) or R (R Core Team 2016, Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS

3.1 $PGF_{2\alpha}$ and PGE_2 release by omental and subcutaneous adipose tissue explants treated with PG synthesis inhibitors, with or without IL-1β

Figure 1 shows $PGF_{2\alpha}$ and PGE_2 release by omental and subcutaneous adipose tissue explants after a 24-hour incubation in the conditions tested (control, NS-398, Statil, IL-1β, IL-1β+NS-398 and IL-1β+Statil). COX-2 inhibitor NS-398 and AKR1B1 inhibitor Statil significantly decreased $PGF_{2\alpha}$ and PGE_2 release in both depots as compared to control ($p<0.01$ for both PGs and depots). In contrast, relative to control, IL-1β strongly enhanced their secretion in omental and subcutaneous tissues ($p<0.0001$ for both PGs and depots). Addition of Statil or NS-398 to IL-1β significantly decreased the stimulatory effect of IL-1β on $PGF_{2\alpha}$ ($p<0.0001$ in both depots) and PGE_2 ($p<0.01$ in both depots). $PGF_{2\alpha}$ and PGE_2 secretion showed depot differences. Omental explants released higher levels of $PGF_{2\alpha}$ than those from the subcutaneous depot ($p=0.03$), both in the absence or presence of IL-1β. PGE_2 was significantly increased in the omental depot only without IL-1β ($p=0.0001$). A similar pattern was observed with the presence of IL-1β, although not statistically significant.

3.2 Messenger RNA abundance of genes related to inflammation in omental and subcutaneous adipose tissue explants treated with PG synthesis inhibitors, with or without IL-1β

Considering that the release of the pro-inflammatory lipid mediators $PGF_{2\alpha}$ and PGE_2 in the culture media was significantly modulated in response to COX-2 inhibitor NS-398, AKR1B1 inhibitor Statil and IL-1β (**Figure 1**), we measured mRNA levels of genes related to inflammation (NF-κB1, IL-6, CCL-5, ICAM-1 and VEGFA) in whole tissue explants of a subset of patients (n=4 for omental and n=5 for subcutaneous adipose tissue) (**Figure 2**). IL-1β treatment significantly induced expression of all genes in both depots as compared to control $p<0.0001$ for NF-κB1, IL-6 and ICAM-1 in both depots; $p=0.001$ (omental) and $p=0.01$ (subcutaneous) for CCL-5; $p<0.0001$ (omental) and $p=0.004$ (subcutaneous) for VEGFA. However, PG inhibitors alone did not have any impact on the expression of these genes in comparison to

control, and consequently, neither the COX-2 inhibitor NS-398, nor the AKR1B1 inhibitor Statil prevented IL-1β-induced up-regulation of pro-inflammatory gene expression (no differences between IL-1β, IL-1β+NS-398 and IL-1β+Statil for all genes in both depots).

Depot differences were also observed. For CCL-5 mRNA abundance, no regional differences were observed when IL-1β was not present in the culture medium, whereas the omental depot was more sensitive to IL-1β than subcutaneous adipose tissue ($p=0.05$ for IL-1β alone, $p=0.005$ for IL-1β+NS-398 and $p=0.05$ for IL-1β+Statil). In a similar fashion, VEGFA expression levels were comparable in both fat compartments in the absence of IL-1β, but the cytokine induced depot differences ($p=0.0006$ for IL-1β alone, $p=0.001$ for IL-1β+NS-398 and $p=0.01$ for IL-1β+Statil). Although omental adipose tissue appeared to express IL-6 at higher levels than the subcutaneous fat compartment in the presence of IL-1β for all conditions, results were only significant for IL-1β combined with Statil ($p=0.10$ for IL-1β alone, $p=0.16$ for IL-1β+NS-398 and $p=0.03$ for IL-1β+Statil). In response to NS-398 alone, IL-6 mRNA levels were higher in the subcutaneous fat compartment than in the omental depot ($p=0.02$). Messenger RNA abundance of NF-κB1 and ICAM-1 showed no depot differences.

3.3 Expression levels of adipogenic genes in omental and subcutaneous adipose tissue explants treated with PG synthesis inhibitors, with or without IL-1ß

We also examined pro-adipogenic genes PPARγ and C/EBPα (**Figure 3**). IL-1β significantly decreased mRNA expression of PPARγ in both fat compartments compared to control ($p=0.02$ for both depots), whereas the decrease did not reach significance for C/EBPα. Although AKR1B1 inhibitor Statil and COX-2 inhibitor NS-398 significantly decreased $PGF_{2\alpha}$ and PGE_2 release (**Figure 1**)**,** these two inhibitors did not modulate PPARγ and C/EBPα mRNA abundance in the basal condition (absence of IL-1β) compared to control and therefore failed to reverse IL-1β-induced inhibition of PPARγ expression (no differences between IL-1β, IL-1β+NS-398 and IL-1β+Statil in both depots). Regarding depot differences,

adipose tissue explants from the subcutaneous fat compartment expressed C/EBPα at higher levels compared to those from the omental depot (p=0.004). Variations between omental and subcutaneous adipose tissue explants did not reach significance for PPARγ (p=0.12). However, the subcutaneous depot tended to express higher levels of PPARγ in the basal condition, and interestingly, the presence of IL-1β attenuated this pattern.

3.4 Effect of PG synthesis inhibitors on preadipocyte differentiation, with or without IL-1β

In order to extend our experiments to assess the impact of IL-1β and PG enzyme inhibitors on adipose tissue expandability in obese subjects, we performed primary preadipocyte differentiation experiments (n=9, subcutaneous adipose tissue). As illustrated in **Figure 4**, IL-1β almost completely inhibited preadipocyte differentiation assessed by G3PDH activity measurements (p<0.0001). Interestingly, in the basal state, COX-2 inhibitor NS-398 significantly stimulated adipogenesis compared to control (p=0.02), whereas G3PDH activity was not statistically different between control and treatment with Statil alone. Addition of NS-398 to IL-1β failed to reverse IL-1β-induced inhibition of adipogenesis (p<0.0001 for control vs IL-1β+NS-398 and p=0.76 for IL-1β vs IL-1β+NS-398). Statil did not reverse the effect of IL-1β (p=0.89 for IL-1β vs IL-1β+Statil). Excluding samples obtained in males from the analyses did not change the results.

4. DISCUSSION AND CONCLUSIONS

This is the first study testing the depot-specific impact of IL-1β and PG-synthesizing enzymes, specifically the rate-limiting enzyme COX-2 and PGF synthase AKR1B1, on markers reflecting adipose tissue inflammation and fat storage in humans. We observed that IL-1β is a strong stimulator of $PGF_{2\alpha}$ and PGE_2 release by human omental and subcutaneous adipose tissue explants and that COX-2 or AKR1B1 inhibition effectively decreases PG secretion in both fat depots. Contrary to our hypothesis, blunted PG secretion failed to prevent IL-1β-induced expression of NF-κB1, IL-6, CCL-5, ICAM-1 and VEGFA in omental and subcutaneous whole tissue explants. COX-2 and AKR1B1 inhibitors also had little effect on gene expression in the absence of IL-1β. A similar pattern was observed for PPARγ expression, which was, however, decreased in response to IL-1β. NS-398 and Statil failed to reverse the strong down-regulation of differentiation by IL-1β. Yet, COX-2 appeared to negatively regulate adipogenesis, as COX-2 inhibitor NS-398 significantly enhanced adipocyte differentiation in the absence of IL-1β.

Previous reports have addressed the role of IL-1β in adipose tissue pathophysiology [4, 9-12]. Gao et al. [9] showed that expression of pro-inflammatory mediators NF-κB1, IL-6, CCL-5 and ICAM-1 was powerfully induced in differentiated human subcutaneous adipocytes after a 24-hour IL-1β treatment with 2 ng/ml [9]. Visceral fat was not tested in that publication. In the present study, we observed that a 24-hour IL-1β treatment with 1 ng/ml was sufficient to significantly upregulate expression of all these inflammatory genes as well as of VEGFA in omental and subcutaneous adipose tissue explants. In addition, for IL-6, CCL-5 and VEGFA, the visceral depot was particularly responsive to IL-1β. This original finding is in agreement with the inflammatory profile of this depot [27].

One important finding is the fact that IL-1β decreased expression of adipogenic gene PPARγ in visceral and subcutaneous explants. C/EBPα downregulation was not significant in either fat compartment, which

may be due to our use of a lower IL-1β dose (1 ng/ml versus 2 ng/ml), compared to a previous study in subcutaneous adipocytes [9]. Our experiments in subcutaneous preadipocytes show that 1 ng/ml of IL-1β almost completely supresses the differentiation process, similar to another study in humans [28]. These results point to IL-1β as a potential key factor impairing adipose tissue expansion in humans and provide support to observational studies by Kursawe et al. [29, 30] in obese adolescents.

Regarding depot differences, we found that subcutaneous explants expressed C/EBPα at higher levels than omental explants, in agreement with an earlier observational study from our group [3, 31]. These results are also consistent with other studies in which a greater differentiation capacity of subcutaneous preadipocytes compared to omental adipocyte precursor cells was noted [8, 31-33]. Even if the depot difference for PPARγ in unstimulated condition did not reach statistical significance, we observed that this trending depot difference was abolished in the presence of IL-1β. This supports the notion that inflammatory signals could attenuate known differences in expansion capacity of the two fat depots.

Available data, most often derived from animal or 3T3-L1 cell line experiments, indicate that PGs are involved in the regulation of adipose tissue function and could mediate the effects of inflammatory cytokines [15, 34]. The COX enzymes have a plethora of regulatory functions; they catalyze the first and rate-limiting step of PG biosynthesis and they have been shown to be involved in the regulation of adipogenesis [15, 17]. For example, Yan et al. [34] showed that in 3T3-L1 cells, COX-2 inhibitors NS-398 or celecoxib stimulated adipocyte differentiation, and partly prevented the potent TNF-α-induced inhibition of adipogenesis. However, other non-human studies on COX-2 generated contrasting results (reviewed in [15]). The impact of COX-2 on adipose tissue function and its possible interaction with inflammatory adipokines have been poorly examined in humans. To our knowledge, we are the first to clearly report the effect of COX-2 inhibition on the differentiation of human adult preadipocytes. We found that COX-2 inhibitor NS-398 increased the differentiation of abdominal subcutaneous

preadipocytes obtained in obese individuals. Hence, COX-2 could be a critical factor in adipose tissue dysfunctional remodeling, which is thought to arise at least partly from impaired adipogenesis of the subcutaneous depot. The involvement of COX-2 in human adipogenesis was also hinted in a study reporting that NS-398 (unknown concentration) was able to reverse the blunted lipid accumulation achieved by applying a compressive force before inducing differentiation of preadipocytes derived from the subcutaneous adipose tissue of an infant [35]. Even if our previous results highlighted a better overall capacity of IL-1β versus TNF-α to stimulate PG synthesis in human preadipocytes and tissue explants [14], the combination of IL-1β and NS-398 did not reverse the down-regulating effect of IL-1β on adipogenesis in our study. Accordingly, the observed effects of IL-1β on adipogenic gene expression were also found to be independent of concomitant PG synthesis, in the same way as for inflammatory genes. Hence, effects of IL-1β do not appear to be mediated by the COX pathway. As other groups underlined the anti-adipogenic properties of TNF-α in humans [28, 36], and that IL-1β is able to stimulate TNF-α production [37], IL-1β could possibly exert part of its anti-adipogenic and inflammatory effects through a TNF-α-mediated pathway.

As the blockade of COX-2 causes a decrease in the synthesis of all PGs and not only the specific lipid mediators implicated in the adverse response, we also focused on the PG terminal synthase AKR1B1 [17]. In 2011, Bresson et al. [19] were the first to demonstrate the PGF synthase activity of human aldose reductase AKR1B1, which was established in the endometrium. AKR1B1 was more efficient than the only other known human PGF synthase AKR1C3 in generating $PGF_{2\alpha}$ and, as opposed to AKR1B1, AKR1C3 was not induced by IL-1β [19, 38]. We later extended these findings to human primary preadipocytes, in addition to highlighting the elevated omental adipocyte $PGF_{2\alpha}$ release of abdominally obese women [14]. Interestingly, AKR1B1 was found to also regulate PGE_2 synthesis in various cell lines through a potential positive feedback loop between $PGF_{2\alpha}$ release and PGE_2 production [38]. We thus hypothesized that AKR1B1-mediated PG synthesis could have inflammatory and anti-adipogenic effects

in human fat tissues from obese individuals. We report for the first time that AKR1B1 inhibitor Statil decreases both $PGF_{2\alpha}$ and PGE_2 release by omental and subcutaneous adipose tissue explants. This, however, did not lead to any further changes in expression of adipose tissue function markers (pro-inflammatory and pro-adipogenic genes). In addition, blocking AKR1B1 did not have any impact on the differentiation of subcutaneous preadipocytes. As opposed to our findings, Pastel et al. [39] recently reported that the AKR1B1 inhibitor Statil was able to enhance the adipogenic differentiation of multipotent cells derived from human adipose tissue. Discrepancies could arise from different experimental conditions such as duration of treatment, differentiation time and method used to quantify adipogenesis or, most importantly, from differences in the model. Additional studies are required to elucidate these differences.

Lastly, limitations of the present study should be mentioned. We performed expression measurements to assess the effects of IL-1β and PG inhibitors on inflammatory and adipogenic regulators. Analysis of protein levels may have generated different results. However, in the context of this study, real-time RT-PCR quantification offers a much more sensitive option than protein-based measurements. The small number of subjects used for gene expression measurements may possibly explain why some depot differences did not reach significance, but our study compares advantageously with other reports on this topic. The limitations associated with our antibody-based PG assay are acknowledged and the possibility that COX-2 and AKR1B1 could modulate other markers of adipose tissue function should be kept in mind. Another acknowledged limitation of the study is that explant cultures examine the response of a variety of cell types and not only adipocytes. The percentage of adipocytes relative to other cell types might be different between subcutaneous and visceral adipose tissue and may have contributed depot differences in the response to IL-1β treatment.

In conclusion, altered adipogenesis seems to be a critical determinant of dysfunctional adipose tissue and concomitant metabolic disturbances. The identification of potential contributors to impaired adipose

storage capacity is crucial. In this study, we identified the possible role of COX-2 in mediating these effects in humans and we provided further indications on the role of IL-1β. We found that IL-1β has powerful inflammatory effects in human adipose tissues, with the visceral fat compartment being more responsive compared the subcutaneous depot. Most importantly, these effects of IL-1β appear to be independent of concomitant PG production in the conditions tested. Further studies are needed to better understand the role of PG-synthesizing enzymes and IL-1β on human adipose tissue function.

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DECLARATION OF INTEREST

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AUTHOR CONTRIBUTIONS

AT obtained funding for the study. JL, AM and AT designed the study. JL, AM, MFG and MP performed laboratory experiments. JL, AM and AT performed data analysis and interpretation and JL wrote the manuscript. FJ and LBB managed all clinical aspects of the study. All authors critically revised the manuscript and approved the final version.

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Figures and figure legends

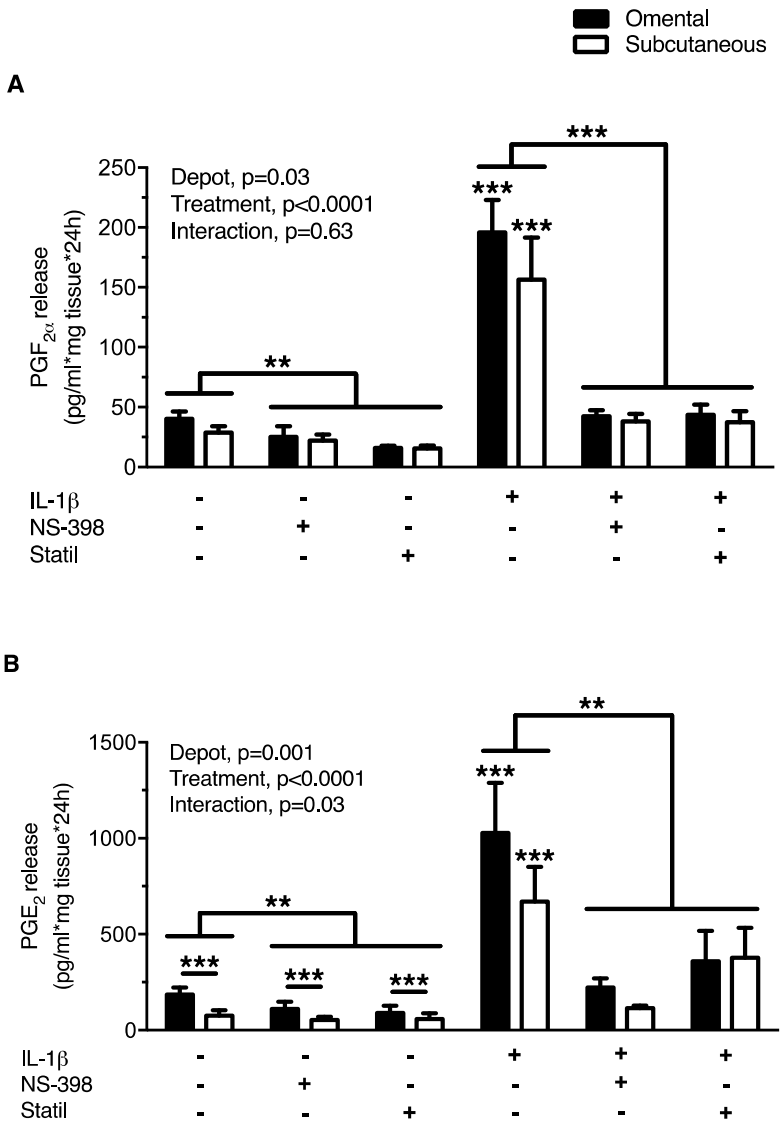


Figure 1: $PGF_{2\alpha}$ (A) and PGE_2 (B) release by omental (black bars) and subcutaneous (white bars) adipose tissue explants in response to COX-2 inhibitor NS-398 (1 μM) and AKR1B1 inhibitor Statil (10 μM), with or without IL-1β (1 ng/ml), after a 24-hour incubation. Omental: n=10 ($PGF_{2\alpha}$ and PGE_2), subcutaneous: n=10 for $PGF_{2\alpha}$ and n=9 for PGE_2. Results are expressed in pg/ml*mg tissue*24h and as means ± standard error of the mean. Interactions reflect treatment condition*depot in the presence of IL-1β. The treatment condition*depot interaction in the absence of IL-1β was non-significant. ** $p \leq 0.01$, *** $p \leq 0.0001$ versus control or as indicated. P-values are based on log-transformed variables.

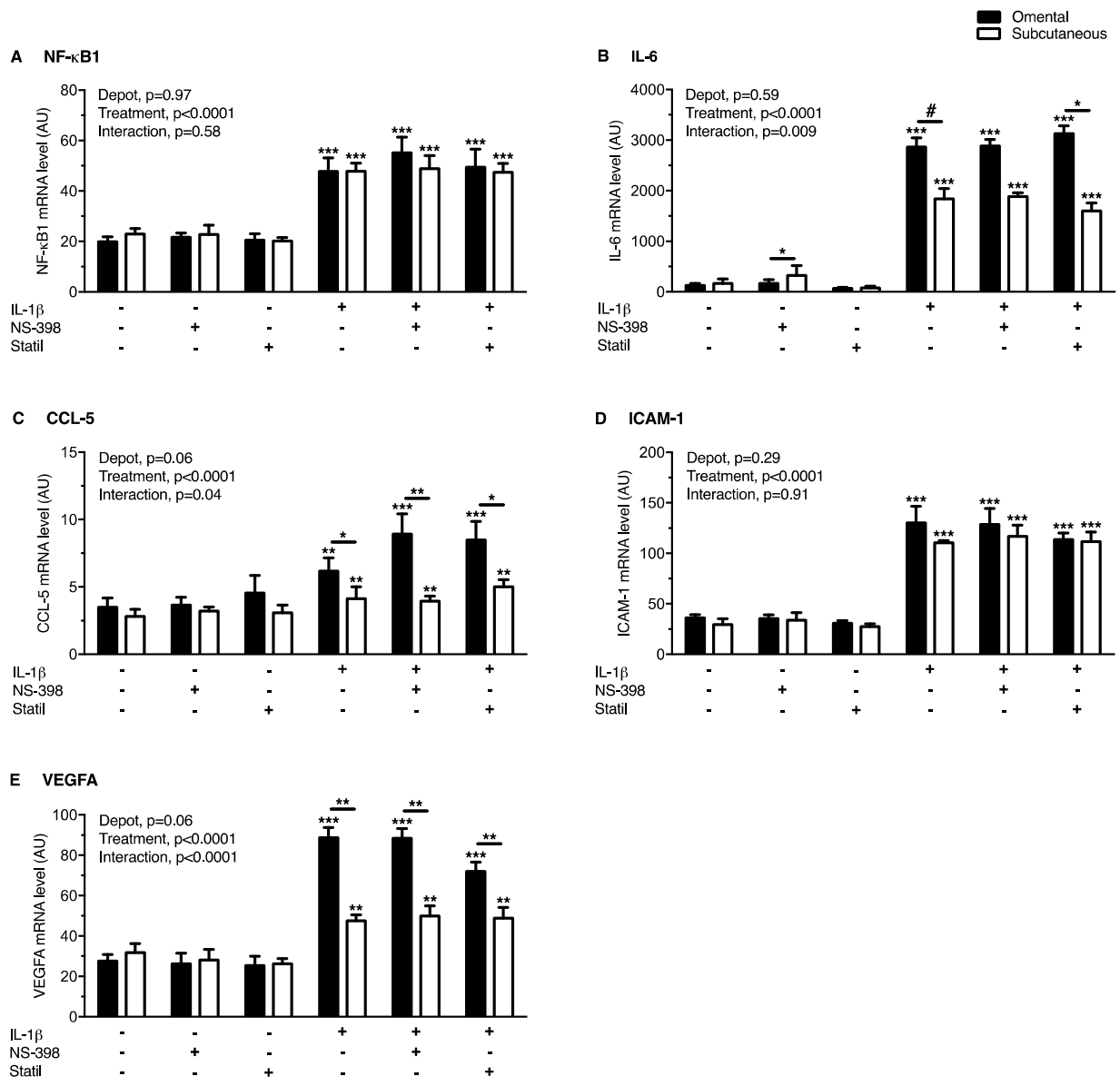


Figure 2: Messenger RNA expression of NF-κB1 (A), IL-6 (B), CCL-5 (C), ICAM-1 (D) and VEGFA (E) in omental (black bars) and subcutaneous (white bars) adipose tissue explants in response to COX-2 inhibitor NS-398 (1 μM) and AKR1B1 inhibitor Statil (10 μM), with or without IL-1β (1 ng/ml), after a 24-hour incubation (n=4 for omental and n=5 for subcutaneous). Expression levels were normalized with ATP5O mRNA expression. Results are expressed as means ± standard error of the mean. AU: arbitrary units. $^{\#}$ p≤0.10, * p≤0.05, ** p≤0.01, *** p≤0.0001 versus control or as indicated. P-values are based on log-transformed variables.

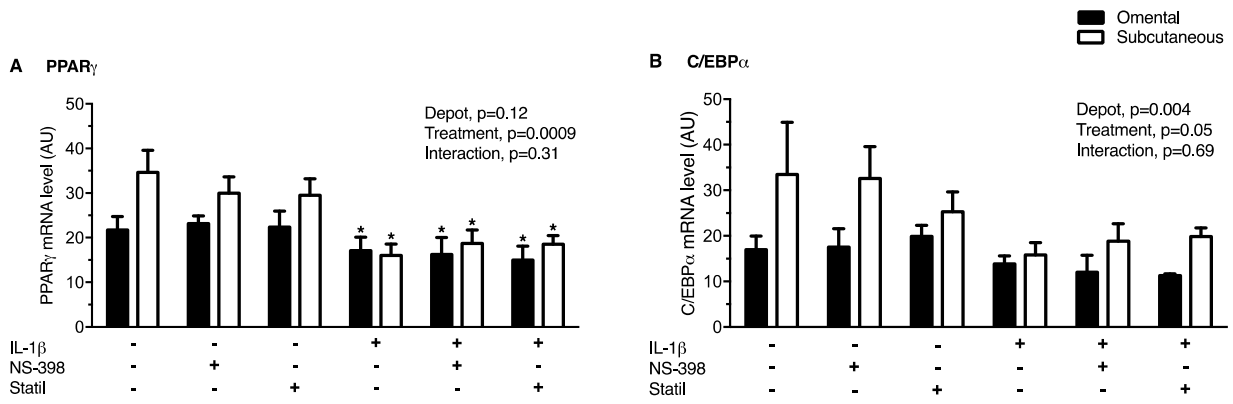
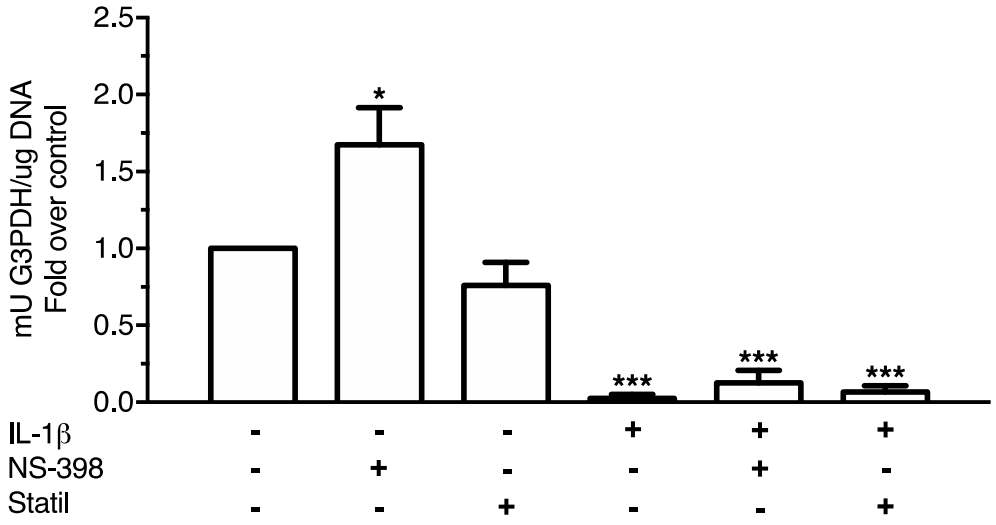


Figure 3: Messenger RNA expression of PPARγ (A) and C/EBPα (B) in omental (black bars) and subcutaneous (white bars) adipose tissue explants in response to COX-2 inhibitor NS-398 (1 μM) and AKR1B1 inhibitor Statil (10 μM), with or without IL-1β (1 ng/ml), after a 24-hour incubation (n=4 for omental and n=5 for subcutaneous). Expression levels were normalized with ATP5O mRNA expression. Results are expressed as means ± standard error of the mean. AU: arbitrary units. * $p \leq 0.05$ versus control. P-values are based on log-transformed variables.

A



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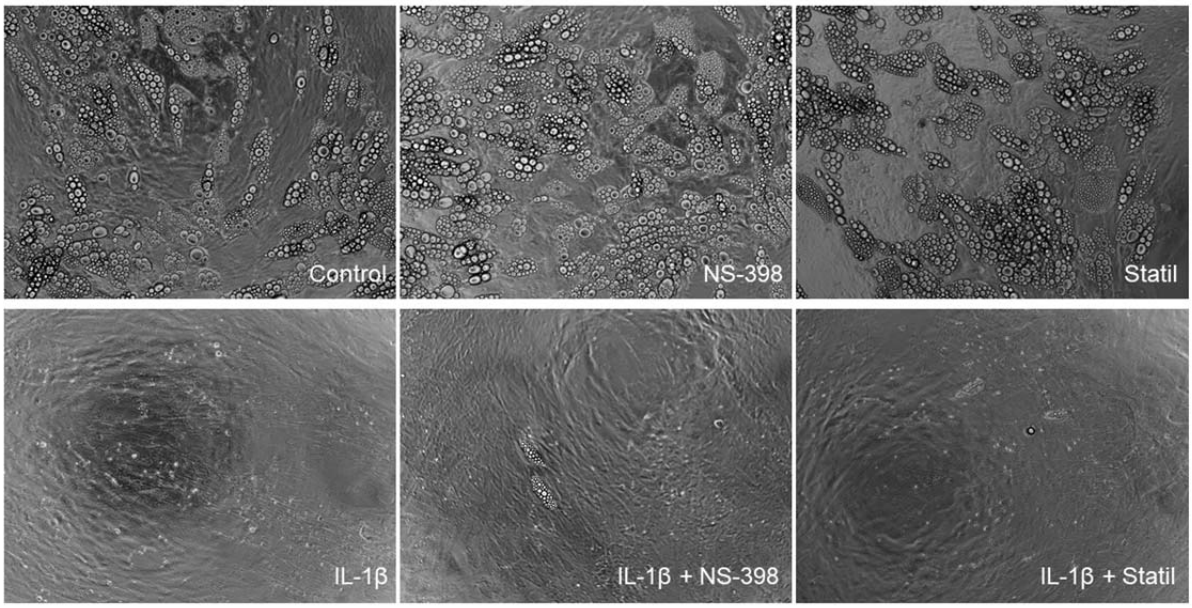

Figure 4: Differentiation of preadipocytes isolated from the subcutaneous adipose tissue of 9 obese patients, after treatment with COX-2 inhibitor NS-398 (1 μM) or AKR1B1 inhibitor Statil (10 μM), alone or in combination with IL-1β (1ng/ml) (day 21 of differentiation). A) The extent of differentiation was assessed by measuring G3PDH activity (mU). Results are expressed as means ± standard error of the mean. * $p \leq 0.05$, *** $p \leq 0.0001$ versus control. P-values are based on log-transformed variables. B) Pictures of differentiated cells from a representative culture (10x magnification).