CLINICAL STUDY

Interleukin-18 in plasma and adipose tissue: effects of obesity, insulin resistance, and weight loss

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Abstract

Objective: Interleukin (IL)-18 is associated with obesity, insulin resistance, and cardiovascular disease. The present study compared 1) IL-18 in adipocytes versus stromal vascular (SV) cells, 2) IL-18 in plasma and adipose tissue (AT) in obese versus lean subjects, and 3) IL-18 in plasma, AT, and skeletal muscle (SM) in obese subjects after weight loss.

Subjects and methods: At baseline, plasma and AT IL-18 in 23 obese subjects were compared with that in 12 lean subjects. The obese subjects were submitted to a 15-week lifestyle intervention (hypocaloric diet and daily exercise) after which plasma samples, AT, and SM biopsies were obtained. Analyses were performed by ELISA and RT-PCR respectively.

Results: IL-18 expression in isolated adipocytes was ~2% of that in SV cells. Plasma IL-18 was higher in obese subjects (P<0.001) and associated with insulin resistance (HOMA; P<0.001). AT expression of IL-18, CD14, and CD68 was higher in obese (P<0.01). The intervention reduced body weight (P<0.001), plasma IL-18 (P<0.001), and increased insulin sensitivity (HOMA; P<0.05). AT and SM expression of IL-18 remained unchanged after the intervention. Changes in plasma IL-18 were associated with changes in insulin sensitivity (P<0.05) but not with BMI or AT expression of IL-18.

Conclusion: Plasma IL-18 is associated with changes in insulin resistance and reduced after weight loss. AT expression of IL-18 is increased in obesity but not affected by weight loss, indicating that changes in plasma IL-18 are related to insulin resistance rather than changes in obesity per se.

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Introduction

Visceral obesity is a chronic low-grade inflammatory state associated with the development of insulin resistance, type 2 diabetes, and cardiovascular disease (1). The background for the obesity-related low-grade inflammation remains unknown but human adipose tissue (AT) is characterized by the ability to produce and release a variety of inflammatory proteins, collectively known as adipokines: e.g. adiponectin (2), tumor necrosis factor (TNF)-α (3), interleukin (IL)-6 (4), IL-8 (5), and monocyte chemoattractant protein (MCP)-1 (6). With increasing adiposity, human AT is infiltrated by monocytes and macrophages in both s.c. AT (7) and visceral AT (8). Only adiponectin and leptin are adipocyte specific, and inflammatory cells (e.g. monocytes and macrophages) within the stromal vascular (SV) fraction primarily account for the production of several of the other known adipokines (8, 9).

IL-18 is found to be produced and released from human AT and isolated adipocytes in vitro (10–12) as well as from skeletal muscle (SM) (13). As for the majority of the other known adipokines, IL-18 production primarily originates from the non-fat cells within the AT (14, 15). IL-18 is a 18.3 kDa pro-inflammatory cytokine belonging to the IL-1 family (16), displaying chemoattractant properties by inducing mononuclear cell recruitment and thereby inflammation in vitro and in vivo (17). Low-grade inflammation seems to be a central feature in several metabolic diseases, and plasma IL-18 is found to be elevated in obesity (18), in women with polycystic ovary syndrome (19), and in patients with type 2 diabetes (20, 21). In addition, IL-18 is expressed in the atherosclerotic plaque and involved in atherogenesis (22–24) as well as associated with plaque instability and cardiovascular death (23, 25, 26). Weight loss induced by diet (18) or bariatric surgery (27) induces a significant reduction in plasma IL-18, but so far only few investigations have evaluated whether the reduction in plasma IL-18 is paralleled by a decrease in IL-18 mRNA expression in human AT.

The present study is an extension of a previously published study (28) with focus on IL-18 in order to (1) compare AT IL-18 mRNA expression in the adipocyte fraction with that in the SV fraction; (2) investigate the...
possible differences in IL-18 in plasma and AT in lean and obese subjects; and (3) investigate the effects of weight loss induced by a 15-week life-style intervention on IL-18 in plasma, AT, and SM as well as the relationship with adiposity (body mass index (BMI), kg/m²), homeostasis model of insulin resistance (HOMA), macrophage/monocyte specific markers (CD68 and CD14), and various adipokines (adiponectin, IL-6, IL-8, TNF-α, and MCP-1).

Subjects and methods

Subjects

The present study is divided into three sub-studies as follows:

Study 1. An in vitro comparison between isolated adipocytes and SV cells obtained from s.c. abdominal AT from six normal to overweight women (mean age: 31.0 ± 5.4 years, mean BMI: 24.0 ± 1.8 kg/m²) undergoing liposuction for cosmetic reasons.

Study 2. A cross-sectional baseline investigation comparing plasma levels of IL-18 in 12 lean subjects (four females and eight males; mean age: 32.0 ± 3.0 years, mean BMI: 22.8 ± 0.5 kg/m²) with that in 23 obese subjects (12 females and 11 males; mean age: 32.2 ± 1.9 years, mean BMI: 45.8 ± 1.9 kg/m²) and AT-mRNA levels of IL-18, CD14, and CD68 in four lean subjects (two females and two males; mean age: 30.3 ± 4.5 years, mean BMI: 23.3 ± 0.9 kg/m²) to that of 19 obese subjects (ten females and nine males; mean age: 33.5 ± 2.1 years, mean BMI: 47.4 ± 2.0 kg/m²).

Study 3. A weight loss study described in detail in (28), investigating the effect of the 15-week life-style intervention (hypocaloric diet and moderate daily physical activity) on plasma levels of IL-18 in 23 obese subjects (12 females and 11 males; mean age: 32.2 ± 1.9 years, mean BMI: 45.8 ± 1.9 kg/m²), AT-mRNA levels of IL-18 in 19 obese subjects (ten females and nine males; mean age: 33.5 ± 2.1 years, mean BMI: 47.4 ± 2.0 kg/m²), and SM-mRNA levels of IL-18 in 14 obese subjects (seven females and seven males; mean age: 32.3 ± 2.2 years, mean BMI: 48.0 ± 2.1 kg/m²). The association between IL-18 and various anthropometrical and inflammatory markers was also investigated, elaborating on previous findings reported in (28).

All subjects included in the three sub-studies were Caucasian. None of the subjects in the in vitro study (study 1) and none of the lean subjects (study 2) received any medication. In the intervention study (study 3), five of the obese subjects were treated for hypertension (with calcium channel blockers, angiotensin-I-converting enzyme inhibitors, or angiotensin-II receptor blockers), four were treated for asthma (inhalation only), and five received oral contraceptives, but all received the same dose and type of medication throughout the study.

Preparation of isolated adipocytes and SV cells

As described in (5), AT was transported to the laboratory and washed several times in isotonic saline. Adipocytes were isolated by collagenase digestion (0.15 mg/g AT) of AT fragments in 10 mmol/l HEPES buffer for 45–60 min at 37 °C. The isolated adipocytes were washed three times in buffer containing 5% albumin and resuspended in medium 199 containing 1% BSA and 25 mM HEPES. Finally, 200 µl cell suspension containing 10% adipose cells (~100 000 adipocytes) were aliquoted in separate tubes and snap-frozen in liquid nitrogen and kept at −80 °C until RNA extraction. After the initial collagenase digestion, the remaining SV fraction was centrifuged for 15 min at 6300 g, resuspended in 9 ml buffer, and filtered through a nylon mesh. This procedure was repeated three times after which the supernatant was removed, and the SV fraction was snap-frozen in liquid nitrogen and kept at −80 °C until RNA extraction.

AT and SM biopsies

AT samples from 4 lean and 19 obese subjects were obtained from the s.c., abdominal AT depot at the level of the umbilicus as previously described (5). In brief, the skin was anesthetized with lidocaine (10 mg/ml) before a small incision was made and ~200 mg AT were removed under sterile conditions. Immediately after removal, the AT sample was washed in isotonic saline and snap-frozen in liquid nitrogen and kept at −80 °C until RNA extraction. SM samples were obtained from the vastus lateralis muscle. Skin and muscle fascia were anesthetized with lidocaine (5 mg/ml) and under sterile conditions, a 1 cm incision was made and ~100 mg muscle tissue were removed using the Bergström technique. Immediately after removal, the muscle biopsy was dissected free of visible fat and snap-frozen in liquid nitrogen and kept at −80 °C until RNA extraction.

Determination of inflammatory markers in plasma

Plasma samples were measured using specific high-sensitive human ELISA. The IL-18 assay (Quantikine, R&D Systems Europe Ltd, Abingdon, UK) had an intra-assay coefficient of variation (CV) of 7.3% (n = 5). The adiponectin assay (B-Bridge International Inc., San Francisco, CA, USA) had an intra-assay CV of 8.1% (n = 12). The IL-6 assay (Quantikine, R&D Systems Europe Ltd) had an intra-assay CV of 5.0% (n = 12). The IL-8 assay (Quantiglo, R&D Systems Europe Ltd) had an intra-assay CV of 5.5% (n = 12). The TNF-α assay (Quantikine HSTA00C, R&D Systems Europe Ltd) had an intra-assay CV of 5.0% (n = 12). The MCP-1 assay (R&D Systems Europe Ltd) had an intra-assay CV of 3.5% (n = 12).
**Determination of mRNA levels**

RNA was isolated using Trizol reagent (Gibco BRL, Life Technologies) and cDNA was made with random hexamer primers using the GeneAmp PCR kit (Applied Biosystems, Foster City, CA, USA). The following oligonucleotide primer pairs were used: IL-18: 5’-GCTTG-GAATCTAAATATTATCGT-3’, 5’-GAAGATTCAATGCTGACTCTG-3’; CD14: 5’-TAAAGGACTGCGCAAGC-3’, 5’-AGCCAAGCCAAGCTTAC-3’; CD68: 5’-GCTACATGGCCGGTAACTCAGT-3’, 5’-ATGGATGGAGGGGCATA-3’; TNF-α: 5’-CGAGTTGACAAGCCTGTC-3’, 5’-GTTGAGGGTGAGGACT-3’; IL-6: 5’-AAATGCGAGCTGCTGACGAAG-3’, 5’-AACAACAAACTCGAGCGCCCATGCTAC-3’; IL-8: 5’-TTGGCAAGCTTCCGTGACGA-3’, 5’-AATTCTTCACTGACCCGACACCTCT-3’; MCP-1: 5’-CGACATCTCTGAACTGACTACT-3’, 5’-CAGTCTGCCGCTCTGGTCAC-3’; adiponectin: 5’-CATGACCAGGAAACACGACCT-3’, 5’-TGAAATGCTGAGCGCAT-3’; β-actin: 5’-ACGGGTCATCCACACTGTCG-3’, 5’-CTAGAAGCCATTGCGTTGGAGCTG-3’. The mRNA levels of the target genes were expressed relative to the housekeeping gene (β-actin). The expression of β-actin was unchanged before and after the intervention (threshold cycles (Ct)): 25.3 ± 0.3 vs 24.9 ± 0.3; P = 0.29). Quantification was performed with a SYBR-Green real-time PCR assay using an iCycler PCR machine (Bio-Rad Laboratories Inc). In brief, PCR amplification was performed with PCR Mastermix containing target primers, HotStar Taq DNA polymerase, SYBR-Green, and PCR buffer. All samples were determined as duplicates. Samples were incubated for an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles each consisting of 95 °C for 30 s, 57 °C for 30 s, and 74 °C for 60 s. Relative gene expression of target gene to β-actin was calculated as described in User Bulletin no. 2, 1997 from Perkin–Elmer (Perkin–Elmer Cetus, Norwalk, CT, USA).

**Statistical analysis**

The SigmaStat 3.1 statistical packet (Systat Software Inc., Point Richmond, CA, USA) was used for the calculations. Normality of distribution was tested using the Kolmogorov–Smirnov test. All anthropometrical and metabolic parameters were found to be normally distributed. AT IL-18 was found not to be normally distributed when a Mann–Whitney rank sum test was applied for comparing mRNA levels in the adipocyte fraction with that in the SV fraction. IL-18 in plasma and AT was found not to be normally distributed when a Mann–Whitney rank sum test was applied for examining the relationship at baseline between lean and obese subjects. IL-18 in plasma, AT, and SM was found not to be normally distributed when a Mann–Whitney rank sum test was applied for comparing circulating levels of IL-18, and IL-18 mRNA levels in AT- and SM biopsies before and after the intervention. A bivariate correlation analysis with a Pearson correlation coefficient (r) was used to investigate the possible relationship between metabolic and inflammatory markers at baseline as well as changes in these parameters after the intervention. Values are presented as mean ± S.E.M. Threshold for significance was set at P < 0.05.

**Ethics**

Informed, written consent was obtained from all subjects, and experiments were performed in accordance with the Helsinki II Declaration. The study was approved by the Ethical Committees of Aarhus and Copenhagen.

**Results**

**Isolated adipocytes versus SV cells**

In general, a low expression of IL-18 mRNA was found in the AT biopsies. After separation (by collagenase digestion) of the adipocyte and SV fraction, it was found that IL-18 mRNA expression in the adipocyte fraction was only ~2% of that in the SV fraction (P < 0.05).

**IL-18 in lean and obese subjects**

Circulating levels of IL-18 was higher in obese subjects compared with lean (243.3 ± 20.3 vs 141.9 ± 19.7 pg/ml; P < 0.001, Fig. 1). IL-18 mRNA expression was significantly higher in AT biopsies from obese compared with lean (P < 0.01, Fig. 2). As a marker for macrophage infiltration in the AT, mRNA expression of CD14 and CD68 was found to be higher in obese compared with lean (6.3 ± 1.4 vs 0.2 ± 0.1 arbitrary units, P < 0.01, and 0.43 ± 0.08 vs 0.02 ± 0.01 arbitrary units, P < 0.01). No gender difference in IL-18 was found in neither plasma (P = 0.58, data not shown) nor in AT (P = 0.96, data not shown).

**Effects of weight loss**

As described in detail in (28), the 15-week intervention reduced body weight by ~18 kg (138.3 ± 5.9 vs 120.7 ± 5.4 kg; P < 0.001), BMI (45.8 ± 1.9 vs 40.0 ± 1.8 kg/m²; P < 0.001), waist circumference (142.6 ± 4.0 vs 132.2 ± 4.2 cm; P < 0.001), and total body fat mass (46.0 ± 2.5 vs 41.4 ± 2.3 kg; P < 0.001). In parallel, insulin sensitivity was improved according to an OGTT (P < 0.001) and HOMA (HOMA=fasting insulin × fasting glucose/22.5) (29) (P < 0.05). No significant difference was observed in the blood pressure (systolic BP: 131.1 ± 2.7 vs 128.6 ± 2.7 mmHg; diastolic BP: 83.8 ± 2.3 vs 79.2 ± 3.0 mmHg).

The intervention reduced circulating IL-18 by 22% (243.3 ± 20.3 vs 190.9 ± 10.1 pg/ml; P < 0.001, Fig. 1)
but was without effect on IL-18 mRNA expression in AT (Fig. 2) and SM (Fig. 3).

**Correlations between IL-18 and metabolic and inflammatory parameters**

At baseline, plasma IL-18 in obese subjects correlated significantly with BMI ($P<0.01$) and HOMA ($P<0.001$) but not with AT IL-18, CD14, or CD68 mRNA expression (Table 1). Plasma IL-18 did not correlate with waist circumference or various inflammatory parameters (e.g. TNF-$
\alpha$, IL-6, adiponectin, MCP-1, and IL-8; data not shown). HOMA correlated significantly with BMI ($r_p=0.71$; $P<0.001$, data not shown). At baseline, AT IL-18 mRNA expression did not correlate with HOMA, CD14 or CD68 (Table 1) in addition to various inflammatory proteins known to be expressed in human AT (e.g. TNF-$
\alpha$, IL-6, adiponectin, MCP-1, and IL-8; data not shown). After the 15-week intervention, changes in plasma IL-18 ($\Delta$plasma IL-18) correlated significantly with $\Delta$HOMA ($P<0.05$) but not with $\Delta$BMI ($P=0.27$) or $\Delta$AT IL-18 mRNA ($P=0.33$). $\Delta$HOMA correlated with $\Delta$BMI ($r_p=0.54$; $P<0.05$, data not shown). Interestingly, when using a multiple stepwise regression analysis, $\Delta$HOMA was found to be the main (and significant) predictor ($P<0.05$) and $\Delta$BMI to be a non-significant ($P=0.08$) predictor of $\Delta$IL-18. Neither $\Delta$plasma IL-18 nor $\Delta$AT IL-18 mRNA correlated with $\Delta$CD14, $\Delta$CD68, ATNF-$
\alpha$, ATIL-6, Adiponectin, $\Delta$MCP-1, or $\Delta$IL-8 mRNA (data not shown; Table 2).

**Discussion**

The present study is an extension of a previously published study (28) with novel findings on IL-18 in plasma and AT. Plasma IL-18 and AT IL-18 expressions were found to be increased in obese when compared with lean subjects. Plasma IL-18 at baseline and after the 15-week life-style intervention correlated significantly with insulin resistance (HOMA) but not with BMI or AT IL-18 expression, in accordance with the findings in a multiple stepwise regression analysis where changes in HOMA were found to be the main (and significant) predictor of changes in plasma IL-18. Finally, the 15-week life-style intervention significantly reduced plasma IL-18 but was without effect on IL-18 mRNA expression in AT and SM from the same obese subjects.

IL-18 is reported to be expressed in human AT (10–12) and SM (13). In accordance, we found IL-18 to be expressed in AT and SM of severely obese subjects but did not observe any effect of weight loss. In our human AT samples, mRNA expression of IL-18 was primarily found in the SV fraction with only $\approx 2\%$ in the...
The underlying cause of the association between plasma IL-18 and the development of cardiovascular disease seems to be through an IL-18-induced inflammatory cell recruitment (17) and subsequent induction of macrophage-derived interferon-γ, TNF-α, IL-6, IL-8, and IL-1β within the atherosclerotic plaque (22, 31).

In the present paper, a 15-week life-style intervention consisting of hypocaloric diet and supervised daily moderate physical activity significantly reduced plasma levels of IL-18. This finding is essentially in line with other studies, in which a weight loss induced reduction in plasma IL-18 was obtained through hypocaloric diet (1 300 kcal/day) and behavioral counseling in 40 obese women (18) or bariatric surgery (27). Interestingly, plasma IL-18 is reported to be associated with traits of the metabolic syndrome (BMI, waist circumference, triglyceride, high-density lipoprotein (inversely), fasting glucose, and insulin) in a dose-dependent manner but independent of obesity (36). This report is in line with two recent papers, in which increased plasma IL-18 was associated with the development of type 2 diabetes independent of fat mass (21, 37). We found plasma IL-18 to be correlated with both HOMA and BMI at baseline but interestingly the changes in plasma IL-18 (∆plasma IL-18) after the 15-week intervention was only correlated with ∆HOMA and not with ∆BMI. These findings may suggest that plasma IL-18 is more related to the degree of insulin sensitivity/insulin resistance than to obesity per se. Recently, clamp-induced hyperglycemia (15 mmol/l) was reported to increase plasma IL-18 (38), indicating that weight loss as reported in the present study and other studies may decrease plasma IL-18 through an improvement in insulin sensitivity rather than a reduction in obesity. In addition to hyperglycemia and insulin resistance, obesity is associated with a low-grade inflammation suggested to involve an increased infiltration of macrophages/monocytes in the AT (7, 8). In accordance, we found a significant increase in macrophage/monocyte infiltration in the AT obtained from our population of severely obese subjects (BMI = 47.4) as compared with our population of lean subjects (BMI = 23.3). The significant weight loss obtained after the 15-week intervention has previously been demonstrated to decrease mRNA expression of CD14 and CD68 (28), but this attenuation of AT inflammation was found to be without an effect on AT IL-18 mRNA expression in the present study. This is in contrast to a recent study by Leick et al. in which an 8-week exercise program tended to (P = 0.06) reduce AT IL-18 expression (33). However, in contrast to the present study Leick et al. did not find any change in plasma IL-18, body weight, or insulin sensitivity as assessed by HOMA after the intervention.

In conclusion, plasma IL-18 and AT-mRNA expressions of IL-18, CD14, and CD68 are increased in obese compared with lean subjects. A 15-week combination of hypocaloric diet and regular moderate

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<th>Baseline correlations between interleukin (IL)-18 in plasma and adipose tissue (AT) and metabolic and inflammatory parameters.</th>
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<td>Plasma IL-18</td>
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<td>AT CD14 mRNA</td>
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Adipocyte fraction confirming findings by Clement et al. (14) and Fain et al. (15). Even though IL-18 mRNA was higher in obese compared with the lean subjects, a relatively low AT-mRNA expression was found in the present study in accordance with previous reports (10, 11). Although Wood et al. were unable to detect IL-18 protein in the medium from isolated human adipocytes incubated up to 24 h (11), two recent studies have reported release of IL-18 protein from isolated human mammary adipocytes (12) and, whole human AT cultures (15). However, in both studies, IL-18 protein release was ~1000 times lower than the protein release of IL-8 and IL-6 (5, 30). This indicates that AT-derived IL-18 is of minor or no importance to plasma IL-18 levels. However, IL-18 may exert autocrine or paracrine pro-inflammatory effects within the AT via release of IL-8 and IL-1β through a TNF-α dependent mechanism in monocytes (31). At the same time, lipopolysaccharide can induce IL-18 expression in monocytes (32) and TNF-α can induce IL-18 expression in isolated adipocytes in vitro (11) but not in AT in vivo (13). These interactions between IL-18 and other pro-inflammatory adipokines within the AT may create a vicious circle leading to a final net release of pro-inflammatory proteins to the circulation.

Esposito et al. were the first to link high plasma levels of IL-18 to obesity (18). We found plasma IL-18 to be significantly correlated with BMI, thus confirming this finding. In addition, we elaborated on the findings by Esposito et al. by parallel AT biopsies and found a significant increase in IL-18 mRNA expression in the AT from obese compared with the lean subjects confirming recent findings by Leick et al. (33). IL-18 is attracting increasing interest since plasma IL-18 is found to be associated with metabolic and cardiovascular diseases such as obesity (18), polycystic ovary syndrome (19), type 2 diabetes (20, 21), lipodystrophy (34), atherogenesis (22–24), and plaque instability (23, 26, 35).
physical activity induced an increase in insulin sensitivity paralleled by a decrease in plasma IL-18 but was without effect on AT expression of IL-18. This is of specific interest since IL-18 in whole human AT samples as well as in isolated adipocytes, and the adjacent SV fraction only displayed very low expression levels. The findings in the present paper suggest that the increase in plasma IL-18 in obesity is associated with the degree of insulin sensitivity rather than obesity per se, and that AT-derived IL-18 may be of only minor importance in obesity-related low-grade inflammation.

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