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Olov Åstrand, Martin Carlsson, Ingela Nilsson, Torbjörn Lindström, Magnus Borga and Fredrik Nyström

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Weight gain by hyper-alimentation elevates CRP levels but does not affect circulating levels of adiponectin or resistin in healthy subjects

Running title: Adiponectin and resistin unchanged by weight-gain

Olov Åstrand MSc[1], Martin Carlsson MD PhD[2], Ingela Nilsson BSc[2], Torbjörn Lindström MD PhD[1], Magnus Borga professor [3,4], and Fredrik H. Nystrom MD PhD professor[1,5], for the Fast Food Study Group. [1]Department of Medical and Health Sciences, Faculty of Health Sciences. [2]Department of Clinical Chemistry, Kalmar County Hospital, [3]Center for Medical Image Science and Visualization (CMIV), [4]Department of Biomedical Engineering, [5]Diabetes Research Centre. Linköping University, Sweden.

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Address correspondence to:
Fredrik H. Nystrom MD PhD professor
Department of Medical and Health Sciences
Linköping University
SE 581 85 Linköping, Sweden
Telephone; +46 101 037749
Fax; +46 13 145949
E-mail; fredrik.nystrom@lio.se

Abstract

Objective: Increase of resistin and/or reduction of adiponectin have been implicated in the development of insulin resistance following weight gain. We aimed to study this prospectively in humans.

Design: Prospective and interventional with parallel control group.

Methods: Twelve healthy men and six healthy women (age 26±6.6 years) and an age-matched control group were recruited. Subjects in the intervention group aimed for a body-weight increase of 5-15% by doubling the baseline caloric intake by eating at least two fast food-based meals a day in combination with adoption of a sedentary lifestyle for four weeks.

Results: Body-weight increased from 67.6±9.1 kg to 74.0±11 kg, p<0.001, by the intervention. Insulin levels increased (before: 27.4 ±12 pmol/L after: 53.0±22 pmol/L, p= 0.004) while plasma levels of adiponectin (before: 5038±3736 ng/mL, after: 6739±7949ng/mL, p= 0.18) and resistin (before: 21.8±19 ng/mL, after: 14.4±6.8 ng/mL, p= 0.074) remained unchanged by the weight gain and were similar as in controls. On the other hand, leptin levels increased about threefold following the intervention (before: 5.7±7.4, after: 16±20 ng/mL, p= 0.008), and also the inflammatory marker CRP increased from 0.34±0.44 mg/L to 0.71±0.87 mg/L, p= 0.03, when two outliers >10 mg/L were disregarded.

Conclusions: Hyper-alimentation reduces insulin sensitivity when weight gain of 9% is combined with reduction of exercise. However, levels of resistin and adiponectin were unaffected by the intervention, while CRP levels increased within this short time period suggesting that low grade inflammation can occur early in the process of developing a metabolic syndrome.

Introduction

The worldwide increase in the prevalence of obesity is expected to relate to a marked rise in the number of patients with type 2 diabetes. However, the exact mechanisms behind development of the insulin resistance that is a consequence of obesity is not known in humans. A lot of attention has recently been paid to the secretory properties of adipocytes and of the fat tissue. Secreted hormones from fat tissue are collectively denominated adipokines and according to experimental studies several key candidates among these proteins are capable of affecting both glucose metabolism and energy balance (1). Leptin is secreted by fat cells and acts on the central nervous system where specific receptors are expressed in hypothalamic neurons that can regulate appetite. Circulating leptin levels in humans are proportional to adipose tissue mass and are also associated with presence of cardiovascular disease (2; 3). Indeed, several atherogenic properties of leptin have been described *in vitro* (3). The adipokine adiponectin is also secreted by adipocytes and is found in the circulation in several isoforms. Adiponectin might protect against the development of type 2 diabetes (4) and thus seems to counteract insulin resistance and in many studies adiponectin levels are elevated by physical exercise in humans (5). However, it is mainly the heavy-molecular weight (HMW) isoform that has been linked with insulin-sensitizing effects in humans (6; 7). While leptin and adiponectin are produced by the fat cells, the 12-kDa peptide resistin is derived from macrophages in the fat tissue (8) and levels of circulating resistin are associated with prevalence of insulin resistance in humans in several (9; 10), but not all (11), cross-sectional studies. It has been proposed that resistin acts as a mediator of the chronic inflammatory process that has been the focus of much research in obesity and insulin resistance (12; 13). C-reactive protein (CRP) is an acute-phase reactant and is considered a classic marker of inflammation. CRP levels within the range detected with high-sensitivity assays, levels <1, 1

to 3 and >3mg/L, correspond to low-, moderate-, and high-risk groups for future cardiovascular events while higher levels are seen during acute inflammation such as in infectious disease (14).

 The clinical correlates of levels of adipokines is corroborated by the finding that circulating levels of resistin in humans is lowered by treatment with the anti-diabetic insulin-sensitizing PPAR gamma agonist drugs rosiglitazone and pioglitazone while such treatment concomitantly increases levels of adiponectin (15-17).

The great majority of prospective interventional studies of the development of insulin resistance are based on in vitro experiments, or studies in animals. In contrast, little is known about the early phases in the development of insulin resistance in humans. This is likely a consequence of that it is cumbersome, and even unpleasant, to participate in trials in which the participants are expected to gain weight to an extent large enough to affect clinical markers of insulin sensitivity. Consequently most studies on insulin resistance in humans are observational, or the effects are supposed to be possible to derive from trials in which already obese subjects are subjected to weight reduction by different procedures, or are given pharmacological substances. In contrast, we performed a prospective study of fast food based hyper-alimentation in healthy subjects who were asked to double the regular caloric intake and also to abandon physical exercise during four weeks. The aims of this analysis were to prospectively investigate development of reduced insulin sensitivity, and markers of the metabolic syndrome, in relation to changes in circulating levels of leptin, resistin, and HMW adiponectin and also to study this in relation to the presumed increase in intra abdominal obesity as determined by magnetic resonance imaging.

Methods

Intervention group

We recruited 12 males and 6 females as volunteers for the intervention arm of the study. Age and gender matched subjects for the control group, were recruited in parallel. The design was thus prospective and interventional with a parallel control group. The participants of the intervention arm had to accept an increase in body weight of 5-15% and were subsequently asked to eat at least two fast food-based meals a day, preferably at well known fast food restaurants such as McDonald's and Burger King. The results of the intervention on liver enzymes and on gender differences in changes of body composition has been published earlier (18; 19). The food expenses were reimbursed consecutively and information based on the food receipts were also used for estimation of the actual food composition and caloric intake. Physical activity was not to exceed 5000 steps per day. The maximal weight gain was set to 15% and subjects were asked to terminate the study as soon as possible after re-performing the same study investigations as were done at baseline if this level of body weight increase was reached within the four week period. All participants were free from significant diseases as judged by medical check-up and history at recruitment.

Subjects in the intervention group were contacted and given advice by professional dieticians, by weekly meetings or by phone, during the study. The aims of these advices were to affect the caloric intake to correspond to a doubling of the regular caloric requirement they had before entering the trial period. If the subject was not able to ingest the hamburger-based diet, it was changed to whatever food the participant could presently accept with the main aim to achieve the calculated caloric intake and also to accomplish a food composition rich in animal protein and

fat. The exact composition of the diet, for example data on unsaturated or saturated fat, saccharides and complex carbohydrates, was calculated from reports done three days before the study and another two three-day periods: one at the end of the first, and one during the third study week (or a week earlier in the one subject that ended the trial after just two weeks).

Blood for routine laboratory tests was drawn in the fasting state at baseline, i.e. before starting on the extra caloric intake, after two weeks on the fast food based diet, and at the end of the study, i.e. either at the end of fourth week or earlier if prematurely terminated. Since very few studies that deliberately aimed to reduce insulin sensitivity have been performed earlier, blood was also drawn in the non-fasting state at the end of the first and the third study weeks, as a precaution, to monitor changes in serum liver enzyme levels and non-fasting lipid levels.

HMW Adiponectin, resistin and leptin were measured using ELISA-kits (Linco Research, Missouri, USA) in duplicates. The intra-assay-coefficient of variation for leptin was 2.4% for low and 3.5% for high controls and the corresponding total-assay-coefficient of variations were 5.9% and 4.1% respectively. The methodological error for leptin was 10.3% (coefficient of variation). The intra-assay-coefficient of variation for HMW adiponectin was 5.9% for low and 11.4% for high controls and total-assay-coefficients of variation were 9.0% and 12.0 %, respectively. The methodological error for HMW adiponectin was 7.9%. The intra-assay-coefficient of variation for resistin was 6.1% for low and 10% for high controls and the total-assay-coefficients of variation were 24% and 11 % respectively. The methodological error for determination of resistin levels was 9.3%. Serum-insulin was assayed using immunoassay methods (AutoDelfia, Perkin Elmer, Linköping, Sweden). Total-cholesterol, HDL-cholesterol and triglycerides were determined by colorimetric analyses (Siemens, Liederbach, Germany) and LDL-cholesterol was

calculated according to Friedewald (total-cholesterol - HDL-cholesterol - 0.456 x total triglyceride concentration). Glucose was determined by the hexokinase method (Siemens, Liederbach, Germany) and high sensitivity (hs) CRP by spectrophotometry (Wide range CRP, Siemens, Liederbach, Germany). Homeostasis model assessment (HOMA) index of insulin resistance was calculated as: glucose concentration x insulin concentration/22.5 (20), while the Quantitative insulin sensitivity check index (QUICKI) was calculated as: 1/[log (insulin concentration) + log (glucose concentration)] (21).

The subjects were subjected to Dual Energy X-ray Absorbimetry (DEXA: Hologic 4500, Hologic, Waltham, MA, USA), for analysis of body composition. The technique for measurement of basal metabolic rate has been described earlier (22) and was based on analysis of CO_2 production and O_2 consumption with Delta Trac equipment (SensorMedics, Yorba Linda, California, USA). The measurement of intra abdominal and subcutaneous fat volumes by magnetic resonance imaging has also been described in detail earlier (19).

All anthropometric measurements were made by two research nurses. The control group performed the laboratory investigations, measurement of basal metabolism, and anthropometric measurements at baseline and after 4 weeks.

Statistics

Statistical calculations were done with PASW 18.0 software (SPSS Inc. Chicago, IL, USA). Linear correlations were calculated as stated in the text. Comparisons within and between groups were done with Student's paired and unpaired 2-tailed t-test or as stated in the results section. Mean values and standard deviations are given, unless otherwise stated. Statistical significance

was considered at the 5% level (p≤ 0.05). Data on HMW adiponectin was missing in two controls at baseline and in one subject each of the two groups at the end of the study due to technical reasons, and data on hs-CRP was missing in two subjects of the intervention group at study end. Since the detection limit of hs-CRP was 0.30 mg/L, levels below the detection limit were set at 0.15 mg/L in the statistical analyses and in the figure.

Ethics

The study was approved by the Regional Ethics Committee of Linköping and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participating subjects.

Results

All subjects in the intervention group except one were students. Seventeen of the 18 participants met the goal to increase 5-15% body weight by the intervention while one participant increased 3.3% in body weight. Four men and one woman reached the maximal 15% increase in body weight. Mean daily caloric intake during the total intervention period increased +70 ± 35 % (men +68 ± 31 % women +74 ± 45 %). There was no statistically significant change in the food intake of macronutrients when comparing the registrations from the first and third weeks, nor did we find any gender differences regarding macronutrient composition of the hyper-alimentation. The subject with the steepest weight increase started at 79.8 kg and reached 91.9 kg already after two weeks (+15%), and thus terminated the study early. One male participant developed an ALT level of 447 U/l (7.6 μkat/l) during the third week (18), and was asked to reduce his caloric intake for reasons of medical safety at this time point.

Table 1 shows baseline anthropometric and laboratory data of all the participants, and the effects of the intervention. Data were similar in control and subjects of the intervention group at baseline and were unchanged in controls during the 4 week observation period (Table 1). Subjects of the intervention group displayed a pronounced increase in body weight and a concomitant increase in fasting insulin levels (Table 1). When analyzed according to gender there was almost a twofold increase in insulin levels in men (before: 27.4 ±12 pmol/L after: 53.0±22 pmol/L, p= 0.004) with no statistically significant changes in the women (before: 35.0±16 pmol/L after: 42.5±20 pmol/L, p= 0.17) (19). HOMA index of insulin resistance (20) increased and QUICKI index of insulin sensitivity (21) was lowered in the intervention group, but unchanged in controls (Table 1). Mean circulating plasma resistin and HMW adiponectin were similar in controls and in subjects of the intervention group before and at the end of the study and remained statistically unchanged in controls (controls: HMW adiponectin before: 3244±3155 ng/mL, after: 2709±2583 ng/mL, p= 0.28, resistin before: 18.5±17 ng/mL, after: 13.6±3.4 ng/mL, p= 0.24, intervention group: HMW adiponectin before: 5038±3736 ng/mL, after: 6739±7949 ng/mL, p= 0.18, resistin before: 21.8±19 ng/mL, after: 14.4±6.8 ng/mL, p= 0.074, no statistical significant differences compared with control group at any time point). Figures 1 a (HMW adiponectin) and b (resistin) show individual changes in the subjects of the intervention group during the weight gain. There were also no statistically significant changes in the levels of resistin or HMW adiponectin when data were analyzed in men and women separately (not shown). Leptin, on the other hand, increased on average about threefold in subjects of the intervention group (before: 5.7±7.4 ng/mL, after: 16±20 ng/mL, p= 0.008, Figure 1 c), while being similar in both groups at baseline (control group baseline: 7.0±9.6, after 4 weeks: 6.3±7.5 ng/mL, p= 0.6 for change and p=0.6 for comparison of baseline levels with intervention group). The relative increase in leptin was of a similar
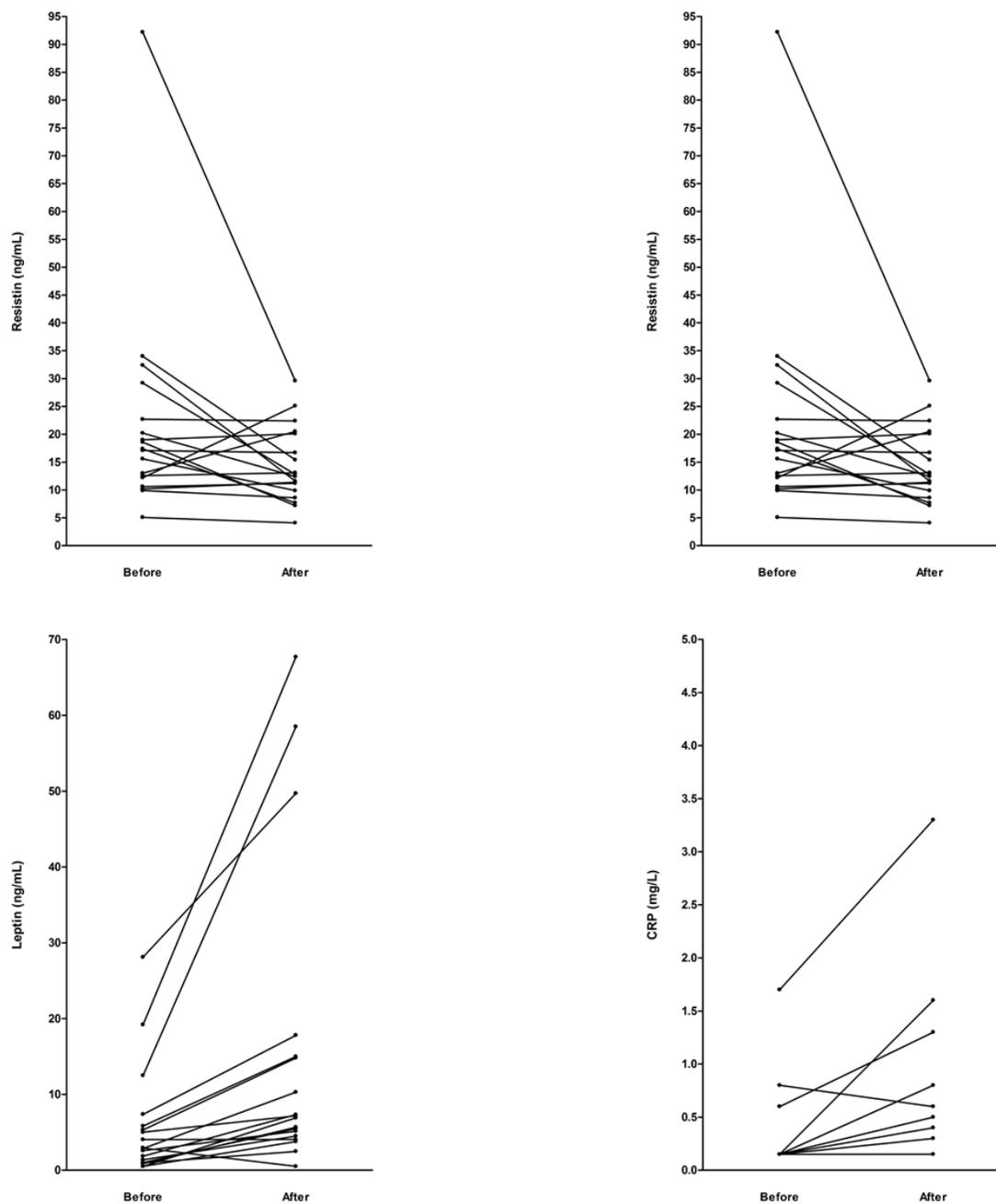
Figure 1. Circulating fasting levels of adiponectin (a), resistin (b) leptin (c) and hs-CRP (d) before and after aiming to double the calculated regular caloric intake and combining this with adoption of a sedentary behavior for four weeks. The change in leptin levels was statistically significant, before: 5.7±7.4 ng/mL, after: 16±20 ng/mL, p= 0.008 in paired t-test. Observe that in figure d five subjects had an hs-CRP level of 0.15 mg/l both before and after the intervention, and that these subjects thus can not be individually visualized. Also note that two subjects with an hs-CRP above 10 mg/l were excluded from the graph. Levels of hs-CRP were 0.34±0.44 mg/L before and 0.71±0.87 mg/L after the intervention in these subjects, p= 0.03 in paired t-test.

magnitude, about three-fold, in both men (2.3±1.7 after: 5.9±3.7 ng/mL, p= 0.003) and women (before: 12.5±10, after: 36.0±27 ng/mL, p= 0.03). Unpaired comparisons between the intervention group and control group resulted in a p-value bordering on statistical significance when comparing leptin levels after 4 weeks between the groups (p= 0.07).

At baseline HOMA (index of insulin resistance) and QUICKI (index of insulin sensitivity) both related to leptin levels (HOMA: r= 0.55, p= 0.001, QUICKI: r= -0.35, p= 0.04) in the total material but only QUICKI related to levels of HMW adiponectin (HOMA: r= -0.27, p= 0.14, QUICKI: r= 0.45, p= 0.01). There were no statistically significant correlations between HOMA and QUICKI to levels of resistin (all p > 0.3) at baseline. There were also no statistically significant correlations between the concentrations of the three adipokines (all p > 0.14) at baseline.

In this group of healthy non-obese subjects only circulating leptin of the three adipokines studied related to total amount of adipose tissue by DEXA (r= 0.79, p< 0.001) and amount of adipose tissue by MRI of abdominal region (r= 0.47, p= 0.049). When analyzing changes in adipokines in relation to changes of the adipose tissue measures, again, only changes of circulating leptin were statistically significant related to measures of fat mass (ratio of leptin after/before correlated to the corresponding ratio of increase in intra abdominal fat mass: r= 0.75, p< 0.0001, and to amount of increase in total fat tissue-ratio by DEXA: r= 0.76, p< 0.0001).

Two subjects in the intervention group had CRP above 10 mg/L, one at baseline and another participant at the study end. When analyzing the data in the remaining group, after removing these two outliers, a statistically significant increase in CRP levels following the weight gain was

seen (hs-CRP before: 0.34±0.44 mg/L, after: 0.71±0.87 mg/L, p= 0.03, see also Figure 1 d). In the controls there was no change in levels of hs-CRP during the observation period (before: 1.2±2.4 mg/L, after: 0.57±0.81 mg/L, p= 0.24. Resistin levels were 20.6±20 ng/mL before and 14.5±7.2 ng/mL after the intervention (p= 0.17) when these two outliers with respect to hs-CRP were excluded. Corresponding values for HMW adiponectin were 5085±3782 ng/mL before and 6817±8326 ng/mL, after, p= 0.23 while the levels of leptin were: 5.8±7.8 ng/mL before and 17±22 ng/mL after, p= 0.013 after exclusion of the two outliers.

Discussion

Despite successfully reducing insulin sensitivity by combining hyper-alimentation with a sedentary behavior, we found no changes in circulating levels of resistin or HMW adiponectin, even though the weight gain was 9%. On the other hand, levels of leptin were increased almost three-fold by the intervention. The increase in fasting levels of insulin was particularly pronounced in the men, but also when analyzed according to gender, the levels of resistin and HMW adiponectin were statistically unchanged following the weight gain.

Although our study was not numerically large, we know of no other larger studies with participants of both genders that induced weight gain and concomitant increase in fasting insulin of a similar magnitude as in our study. We found it unlikely that low statistical power was the reason for the lack of increase in resistin or decreases of HMW adiponectin since there were no trends for such changes to occur in our study, although we acknowledge the small size of the study as a limitation. Also, the graphical appearance in Figures 1 a and b clearly display that individual participants exhibited increases or decreases of resistin and HMW adiponectin during

the intervention in a manner seeming to be arbitrary, which suggests that other factors than

weight gain determined the changes of the levels of these hormones. Also when analyzing the

changes of intra abdominal fat tissue by MRI-based quantification of adipose tissue volume, we

found no significant relationships with either circulating levels of HMW adiponectin or resistin.

However, it should be noted that the lack of correlation in the cross-sectional analysis within the

group at baseline was hampered by the small variation in weight in this group of healthy non-

obese subjects.

The results of our study thus suggests that increases in resistin and/or decreases of HMW

adiponectin are not primary phenomena that are necessary for the earliest steps in the

development of reduced insulin sensitivity caused by weight gain and low levels of physical

activity. According to our study, such changes are instead likely to be secondary phenomena that

still might be important for the long term changes and manifestations of insulin resistance.

Indeed, although re-feeding of patients with anorexia nervosa might not be a model for

development of insulin resistance, it has earlier been demonstrated that during the early stage of

weight increase, HMW adiponectin levels increase as do markers of reduced insulin sensitivity

(23). Also suggestive of a sufficient weight gain for relevant changes of hormone levels to occur

in our study was the recent finding that a weight loss of 5-10%, but not 5%, was sufficient to

induce changes of adipokines in severely obese women (24). We have earlier shown that

saturated and in particular mono-unsaturated fatty acids can stimulate the transcription factor

PPAR gamma in primary human fat cells (25), and PPAR gamma stimulation promotes release of

HMW adiponectin (26). Thus, during hyper-alimentation it is possible that increase in fatty acids

could induce release of HMW adiponectin despite weight gain and reduced insulin sensitivity,

again, however, these effects might only be present during the short hyper-alimentation phase,

and not during more permanent obesity. Our study adds to the idea that there is no proof yet that adiponectin actually modulates insulin sensitivity in humans and that low adiponectin levels may be a consequence of the hyperinsulinemia in insulin resistance as described in a recent review on adiponectin in human metabolic syndrome by Cook et al. (27).

Intriguingly, we did find a small but statistically significant increase in hs-CRP levels following the intervention. This should be interpreted with caution, however, since the data relied on comparisons of 14 samples and hs-CRP is a rather unspecific marker of inflammation that can react in response to numerous stimuli, making it difficult to interpret the results on an individual level or even in small groups, such as in our study. The finding of increased levels of hs-CRP following weight gain is however corroborated by an observational study in which spontaneous weight-gain during 9 years was associated with an increase in hs-CRP (28), and with cross-sectional findings of elevated levels of hs-CRP in obese and overweight subjects (29). Tam et al. also recently demonstrated a significant increase in hs-CRP by overfeeding for 28 days that caused an average weight gain of 2.7 kg (30). The finding of a small but statistically significant increase in inflammation, hs-CRP, but lack of a corresponding increase in levels of resistin suggests that resistin is not an orchestrator of inflammation in the early stage of insulin resistance, as has been suggested by others (12; 13). Indeed, some quite recent studies even showed an inverse relation between resistin and markers of insulin resistance (11; 31).

In contrast to HMW adiponectin and resistin, levels of leptin increased quite dramatically in both men and women of the intervention group. Since the increase in fasting insulin and other markers of reduction of insulin sensitivity only occurred to a significant degree in the males, leptin is probably not a key player to be causative in terms of mediating insulin resistance. Indeed, leptin

might even have a protective role against obesity and its' consequences since administration of leptin can increase the basal metabolic rate when administered after weight-reduction (32) and subcutaneous injections of leptin reduces glucose levels in leptin deficiency (33). On the other hand, whether leptin causes inflammation in humans when administered as subcutaneous injections (34), or not (35), is presently controversial. The effect of leptin resistance in obesity, which has been demonstrated in humans (36), could also not be determined in our study but could have affected the levels of the studied hormones.

In summary we found no increases in HMW adiponectin or resistin despite reduced insulin sensitivity in 18 subjects that combined weight gain of 9% by hyper-alimentation with reduction of physical activity. However, levels of hs-CRP did increase following the intervention suggesting that low grade inflammation can indeed occur early in the process of development of the metabolic syndrome. According to our intervention study the adipokines resistin and HMW adiponectin are unlikely to be the mediators of such primary events in the development of a metabolic syndrome following weight gain. Since the study was of limited size, future studies are called for to re-assess the findings.

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