Leptin Signaling in Adipose Tissue
Role in Lipid Accumulation and Weight Gain


Rationale: The link between obesity, hyperleptinemia, and development of cardiovascular disease is not completely understood. Increases in leptin have been shown to impair leptin signaling via caveolin-1-dependent mechanisms. However, the role of hyperleptinemia versus impaired leptin signaling in adipose tissue is not known.

Objective: To determine the presence and significance of leptin-dependent increases in adipose tissue caveolin-1 expression in humans.

Methods and Results: We designed a longitudinal study to investigate the effects of increases in leptin on adipose tissue caveolin-1 expression during weight gain in humans. Ten volunteers underwent 8 weeks of overfeeding, during which they gained an average weight of 4.1±1.4 kg, with leptin increases from 7±3.8 to 12±5.7 ng/mL. Weight gain also resulted in changes in adipose tissue caveolin-1 expression, which correlated with increases in leptin (ρ=0.79, P=0.01). In cultured human white preadipocytes, leptin increased caveolin-1 expression, which in turn impaired leptin cellular signaling. Functionally, leptin decreased lipid accumulation in differentiating human white preadipocytes, which was prevented by caveolin-1 overexpression. Further, leptin decreased perilipin and fatty acid synthase expression, which play an important role in lipid storage and biogenesis.

Conclusions: In healthy humans, increases in leptin, as seen with modest weight gain, may increase caveolin-1 expression in adipose tissue. Increased caveolin-1 expression in turn impairs leptin signaling and attenuates leptin-dependent lowering of intracellular lipid accumulation. Our study suggests a leptin-dependent feedback mechanism that may be essential to facilitate adipocyte lipid storage during weight gain. (Circ Res. 2012;111:599-603.)

Key Words: caveolin-1 ▪ impaired signaling ▪ leptin ▪ lipid accumulation ▪ weight gain

Leptin is an important mediator of pathophysiological outcomes in obesity.1 Centrally, leptin plays an important role in maintaining energy homeostasis; therefore, the presence of high leptin in obesity has been suggested as evidence of resistance to central leptin actions. However, what is not clear is whether it is high leptin or impaired leptin signaling in peripheral tissue that contributes to development of obesity-related disorders.1-4 Because adipose tissue plays a role in development of metabolic and cardiovascular disease in obesity, the mechanistic role of leptin in adipose tissue during weight gain is of importance.5 Further, even though the presence of the leptin receptor on adipocytes is well-known, we have little understanding of the role of leptin in human adipose tissue.6 Several studies have indicated that leptin regulates lipid metabolism via modulation of lipid oxidation, lipid lysis, and lipid biogenesis, but these studies are limited to animals and nonadipose tissues.7 Therefore, to understand the implications of increases in leptin during weight gain in human adipose tissue, we investigated the role of leptin in regulation of lipid metabolism in cultured differentiating human preadipocytes. We tested the hypothesis that increases in leptin occurring with weight gain in humans would cause increased caveolin-1 expression in adipose tissue and, in turn, impair leptin signaling.

Methods

Detailed Methods are provided in the Online Supplement. Briefly, we recruited 10 healthy volunteers (7 men and 3 women) aged 23 to 36 years. The volunteers were overfed to increase weight gradually by 5% during an 8-week period. Measurements and adipose tissue biopsies were performed at baseline and after weight gain. The protocol was approved by the Institutional Review Board and informed consent was obtained. In vitro experiments were performed using human white preadipocytes (HWP; PromoCell).
Non-standard Abbreviations and Acronyms

Ad-Cav-1 adenovirus encoding caveolin-1
HWP human white preadipocyte

Results

Effects of Overfeeding on Study Participants
The characteristics of the study participants at baseline and after weight gain are presented in Table. On average, the participants gained 4.1±1.4 kg during the 8-week period of overfeeding. The weight gain was a result of increased fat mass; lean mass did not change. Among the variables measured, only leptin increased significantly with weight gain.

The changes in adipose tissue caveolin-1 expression during weight gain were measured by Western blot analysis. Subjects with the highest leptin increases showed the greatest change in adipocyte caveolin-1 expression and subjects with relatively small increase in leptin showed decreases in caveolin-1 expression (Figure 1A). Also, subjects with smaller increases in leptin with weight gain had higher leptin and adipose tissue caveolin-1 expression at baseline. To test the predictors of adipose tissue caveolin-1 expression, we determined changes in caveolin-1 expression and its relationship with changes in other variables during weight gain (Online Table I). Changes in leptin significantly predicted the changes in caveolin-1 expression (\( \rho = 0.79; P = 0.01 \); Figure 1B), suggesting that leptin regulates adipose tissue caveolin-1 expression in vivo. Because there was a very wide range of changes in leptin (8%–157% increase) with weight gain, there was a similarly variable response in caveolin-1 changes, and the group data from overall changes in adipose tissue caveolin-1 expression did not reach significance (Figure 1C). Additionally, caveolin-1 localization in adipocytes was determined by immunohistochemistry (Figure 1D).

Effects of Leptin and Caveolin-1 Expression
We examined the direct role of leptin on caveolin-1 expression using cultured HWPs and differentiated HWPs. Leptin increased caveolin-1 expression in a dose-dependent manner in both HWPs (\( P = 0.05 \)) and differentiated HWPs (\( P = 0.01 \); Figure 2A and 2B).

Further, we sought to investigate the effect of increased caveolin-1 expression on leptin-dependent activation of cellular signaling pathways. To increase caveolin-1 expression, HWPs were infected with caveolin-1 encoding adenovirus (\( P < 0.0001 \); Figure 2C) and treated with leptin (100 ng/mL). Notably, the caveolin-1 overexpressing cells (caveolin-1 encoding adenovirus–infected) showed increased basal activation of cellular signaling pathways along with impaired leptin-dependent activation of ERK1/2 (\( P < 0.0001 \); Figure 2D and 2E). Additionally, leptin receptor and caveolin-1 interaction was demonstrated using confocal imaging and immune precipitation (Online Figure I).

Effect of Leptin on Lipid Metabolism
To determine the implications of impaired adipose tissue leptin signaling, we first identified the effect of leptin in adipose tissue lipid metabolism. Differentiating HWPs in presence of leptin caused decreased lipid accumulation (\( P = 0.006 \); Figure 3A and 3B). To examine the mechanisms through which leptin may decrease lipid content in differentiated HWPs, we investigated its role in regulation of key proteins involved in lipid metabolism. Perilipin is a protein present on the surface of the lipid droplet that serves as a protective coating, thereby facilitating lipid storage. Leptin decreased the transcription (\( P = 0.02 \)) and translation of perilipin in a concentration-dependent manner (\( P = 0.01 \); Figure 3C and 3E). We also investigated the role of leptin in regulation of fatty acid synthase, which is an important enzyme involved in lipid biogenesis. There was a leptin concentration-dependent decrease in the expression of fatty acid synthase mRNA (\( P = 0.03 \)) and protein (\( P = 0.016 \); Figure 3D and 3F). Additionally, increased caveolin-1 expression, via caveolin-1 encoding adenovirus infection, prevented leptin-dependent attenuation of lipid accumulation (\( P < 0.0001 \); Figure 3G), and also prevented leptin-dependent decreases in perilipin and fatty acid synthase mRNA (Figure 3H and 3I).

Discussion
The main finding of our study relates to the direct role of leptin in adipose tissue lipid metabolism and its implications in weight gain. Using a human weight gain model, we found that changes in leptin correlate with changes in adipose tissue caveolin-1 expression. To our knowledge, this is the first longitudinal study to examine and compare the changes in adipose tissue caveolin-1 expression with changes in leptin during weight gain in humans. Of note, increased caveolin-1 expression in obesity has been observed in a cross-sectional study in humans.8

Modest weight gain in our study subjects resulted in increases in serum leptin that ranged from 8% to 157% despite similar increases in weight. The increases in leptin with weight gain were negatively correlated with baseline body fat percentage (\( \rho = -0.94; P < 0.001 \)) as well as baseline leptin levels (\( \rho = -0.69; P = 0.03 \)). The subjects with smaller increases in leptin during weight gain did not show increases in adipose tissue caveolin-1 expression but had an elevated leptin and adipose tissue caveolin-1 expression at baseline, along with higher body fat percentages compared with those subjects in whom leptin and caveolin-1 expression increased with weight gain.

Table. Effects of Overfeeding on Study Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>Weight Gain</th>
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<tr>
<td>Total body weight (kg)</td>
<td>71.6±14.5</td>
<td>75.7±15.2*</td>
</tr>
<tr>
<td>Total body lean mass (kg)</td>
<td>22±7.9</td>
<td>26.2±7.9*</td>
</tr>
<tr>
<td>Total body fat mass (kg)</td>
<td>46.6±8.9</td>
<td>46.5±8.8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.3±3.7</td>
<td>24.6±3.9*</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>169±20</td>
<td>168±37</td>
</tr>
<tr>
<td>High-density lipoprotein (mg/dL)</td>
<td>46±12</td>
<td>42±9</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>86±27</td>
<td>91±51</td>
</tr>
<tr>
<td>Insulin (μU/mL)</td>
<td>5.2±3.2</td>
<td>6.1±2.6</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>7±3.8</td>
<td>12±5.7*</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>8816±4787</td>
<td>10 081±5539</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>93±5</td>
<td>94±5</td>
</tr>
</tbody>
</table>

\( N = 10 \) (7 males, 3 females). Values are mean±SD. \( *P < 0.05 \) determined by paired Wilcoxon signed-rank test.
gain (body fat of 36±3% vs 28.7±2.4%). The greater level of body fat, leptin, and caveolin-1 at baseline in these “less responsive” subjects suggests that subjects with higher body fat percentages and leptin will have less of a leptin increase with further increases in body fat. Furthermore, there may be a level beyond which leptin is unable to further induce adipose tissue caveolin-1 expression in obese subjects. This “saturating” effect of leptin concentrations on caveolin-1 expression was also observed in our in vitro studies in which the increase in leptin concentrations on caveolin-1 expression was also shown that increased caveolin-1 expression did not reach significance during weight gain in our study, the lack of significance itself highlights the importance of the variability present in the physiological and pathological responses to obesity in the general population. Obesity has multifactorial etiologies, including heritable components such as epigenetic variations that could possibly further account for the variability in leptin and caveolin-1 response to weight gain.

Our study confirms the direct role of leptin in regulation of caveolin-1 expression in HWPs and differentiated HWPs. We also show that increased caveolin-1 expression impairs...
Leptin-dependent activation of STAT3 and ERK1/2 pathways. In these experiments, the adenovirus-mediated increases in caveolin-1 expression were comparable with those seen in adipose tissue during weight gain, indicating that in obesity leptin–cellular signaling may be impaired in adipose tissue. Importantly, caveolin-1 overexpression in HWPs was associated with increased basal activation of these signaling pathways, which itself may contribute to dysfunctional adipose tissue along with prevention of extracellular stimuli from interacting with and regulating adipocyte function. Notably, the role of caveolin-1 in adipose tissue lipid metabolism has been demonstrated in caveolin-1-deficient mice that are resistant to diet-induced obesity despite being hyperphagic and manifest dyslipidemia along with adipocyte abnormalities. In these studies, Razani et al show that caveolin-1 deficiency prevents accumulation of lipids in the white adipose tissue. These findings are consistent with our conclusions that caveolin-1 plays an important role in modulating lipid accumulation during overfeeding.

Our findings suggest not only a role of leptin in adipose tissue but also changing dynamics during weight gain. The leptin-dependent attenuation of lipid accumulation in differentiating HWPs is consistent with previous studies and indicates the role of a caveolin-1–dependent leptin feedback mechanism in preventing antilipogenic effects of leptin.7,10 The development of impaired leptin signaling in adipose tissue during weight gain therefore would allow safe storage of excess energy as lipid in adipose tissue.11 Further, because adipose tissue is the main contributor to systemic leptin levels, we speculate that the cells of adipose tissue would be exposed to higher leptin levels and impaired leptin signaling may develop before other critical nonadipose tissue cells, which may provide benefit by preventing lipotoxicity with short-term modest weight gain. However, additional studies are needed before such conclusions can be drawn.

Leptin acts via the leptin receptor, which is present on cells of liver, kidney, pancreas, muscle, heart, and the vasculature. We previously have shown similar leptin–caveolin-1 interactions in vascular endothelial cells; therefore, our findings do not appear to be confined to adipose tissue.12 If our results also hold true for these other cell types, then the role of leptin in lowering intracellular lipid accumulation via decreasing perilipin and fatty acid synthase expression suggests an antiatherogenic mechanism through which leptin may prevent lipotoxicity in these cells. Several studies in animals have shown that leptin decreases in lipid accumulation in cells of liver, heart, and vasculature,7,13 and leptin resistance is associated with increased lipid accumulation in the liver.14 Alternatively, our findings that peripheral leptin signaling may be impaired in obesity indicates a mechanism through which leptin resistance, but not hyperleptinemia, may be proatherogenic in these tissues, and therapeutics aimed at eliminating leptin resistance may improve pathophysiological outcomes in obesity.

The strength of our study is in the unique longitudinal approach in humans, combined with an in vitro component, which allows investigation into the autocrine role of leptin and weight gain. However, the study was limited to defining the relationship between leptin and adipose tissue caveolin-1 expression. Future studies aimed at investigating the effects of leptin in other peripheral organs, through which leptin may contribute to metabolic and cardiovascular diseases in obe-
sity, are needed. This is important, especially in light of studies aimed at investigating the therapeutic effect of leptin administration in the treatment of diabetes.15,16

In summary, modest weight gain in healthy humans results in proportionate changes in leptin and caveolin-1 expression, consistent with in vitro findings of a cause-and-effect relationship. In adipose tissue, increased caveolin-1 expression, in turn, impairs leptin signaling, which provides an advantage during early stages of weight gain in that the adipocytes can serve as a “reservoir” for increased lipid accumulation in the presence of hyperleptinemia.

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Disclosures
None.

References

Novelty and Significance
What Is Known?
• Increased cardiovascular risk in obesity is mediated, in part, by the expansion of adipose tissue and elevated levels of adipokines, including leptin.
• Although the central role of leptin in energy homeostasis is well-known, its effects on peripheral cells such as adipocytes are unclear.
• In cultured vascular endothelial cells, high levels of leptin increase caveolin-1 expression, which in turn impairs leptin signaling.

What New Information Does This Article Contribute?
• Leptin decreases the accumulation of lipids in adipocytes.
• In humans, increases in leptin seen with modest weight gain could increase adipose tissue caveolin-1 expression.
• Increased caveolin-1 expression in adipose tissue could impair leptin-dependent activation of signaling pathways and allow the storage of lipids in differentiating preadipocytes.

The relative contribution of hyperleptinemia and peripheral tissue leptin resistance to the development of obesity-related disorders remains unclear. We investigated the autocrine role of leptin in adipose tissue and its changing dynamics with weight gain. Our data suggest that increases in leptin, as seen with modest weight gain in humans, increases adipose tissue caveolin-1 expression and impairs leptin-dependent cellular signaling. For the first time to our knowledge, we show that leptin acts directly on differentiating preadipocytes to lower lipid accumulation by decreasing the expression of key proteins involved in lipid biogenesis and storage. Thus, impairment of adipose tissue leptin signal could be beneficial during the early stages of weight gain because this would facilitate safe lipid storage in adipose tissue. However, in established obesity leptin resistance, but not hyperleptinemia, could contribute to lipid accumulation and lipotoxicity in peripheral tissues such as liver, heart, and vasculature. Further studies are needed to investigate the effects of leptin in peripheral tissues. The development of strategies to eliminate leptin resistance in obesity could be of potential clinical benefit in the treatment of obesity and related disorders.
Leptin Signaling in Adipose Tissue: Role in Lipid Accumulation and Weight Gain

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Detailed Methods:

Study Subjects: We recruited 10 healthy volunteers (7 men and 3 women) aged 23-36 years and baseline BMI of 23.3 ± 3.7 kg/m². These volunteers were sedentary, free of any chronic diseases, and did not use any medication with an exception of oral contraceptives. Subjects were excluded if they were pregnant, used tobacco or were employed in shift work. The study was conducted at the Mayo Clinic Center for Translational Science Activities (CTSA) Clinical Research Unit and the protocol was approved by the Institutional Review Board. Informed written consent was obtained from all participants.

Overfeeding protocol: The protocol started with a three day period of dietician supervised meals at the CRU kitchen during which the calories required for weight maintenance were determined and a constant macronutrient composition (40 % carbohydrate, 40 % fat, and 20 % protein) of the diet were ensured. Baseline measures were obtained at the end of this three day period which was followed by an 8 week overfeeding intervention during which the participants were encouraged to either increase the portions of their favorite foods or add 1-3 supplements (400 - 1200 extra kcal) available from the CRU kitchen to boost their caloric intake.1-3 The supplements included choice of ice-cream shake (402 kcal, 40 % fat), a king size chocolate bar (510 kcal), and energy drinks (360 kcal / 8 oz). The protocol aimed to increase weight gradually with the goal to gain approximately 5 % body weight at the end of 8 weeks. To ensure this gradual gain, participants were weighed at least 5 times each week and dietitians were able to monitor and adjust calories accordingly. At the end of 8 weeks of overfeeding, weight gain measures were obtained.

The baseline and weight gain measures included assessment of body composition in duplicate with DXA (DPX-IQ; Lunar Radiation), lipid profile (cholesterol, high density lipoprotein, and triglycerides) with standard turbidimetry methods on Hitachi 912 chemistry analyzer, insulin with 2 site-immunoenzymatic
assay (Beckman Instruments Inc.), glucose was measured using hexokinase reagent using a Hitachi 912 chemistry analyzer (Boehringer Mannheim, Indianapolis, Indiana), adiponectin was measured using an enzyme-linked immunosorbent assay kit (Mediagnost GmbH, Reutlingen, Germany) and leptin with a radioimmunoassay kit (Linco Research, Inc., St. Louis, Missouri). Abdominal subcutaneous adipose tissue biopsies were obtained at the baseline and after weight gain under sterile conditions using a modified needle aspiration technique.4

**Adipose tissue analysis:** Caveolin-1 expression was determined semi-quantitatively in the abdominal adipose tissue samples by Western blot analysis. In each participant, adipose tissue from the two time points were processed and analyzed at the same time. Briefly, adipose tissue was lysed in a buffer containing 50 mM NaCl, 50 mM NaF, 50 mM Na₄O₇P₂, 5 mM EDTA, 5 mM EGTA, 0.1 mM Na₃VO₄, 1 % Triton X-100, 10 mM HEPES, pH 7.4. Equal concentrations of the proteins were loaded, and transferred to PVDF membrane. The membrane was blocked, treated with anti-caveolin-1 antibody (BD Transduction lab, San Diego, CA), and anti-GAPDH antibody (loading control; Abcam Inc, Cambridge, MA), followed by incubation with appropriate HRP-conjugated secondary anti-body and developed with enhanced chemiluminescence (Amersham biosciences, U.K.). The optical density of the band was measured using Scion Image software (Scion Corp.). The protein expression levels were normalized to GAPDH and expressed as relative increase to baseline expression.

**In-vitro experiments:** In-vitro experiments were done using commercially available human white preadipocytes (HWP) (PromoCell, Germany) as detailed in supplementary methods. Cells were grown in Preadipocyte Growth Media containing growth supplements and 5 % fetal calf serum (PromoCell) and all experiments were performed at 3-5 passages with 70-80 % confluence after overnight incubation in serum and growth factor free media. For experiments with differentiated human white preadipocytes (dHWP), HWP were grown to confluence and differentiated in presence of Preadipocyte Differentiation Media (PromoCell) for 72 hours followed by additional growth for 10 days in Adipocyte Nutrition Media.
PromoCell). After the 12-14 days of differentiation, experiments were conducted after overnight incubation in basal adipocyte nutrition media lacking supplements and serum.

To determine the effect of leptin on caveolin-1 protein expression in HWP and dHWP, cells were treated with increasing concentrations of leptin (0 – 150 ng/ml) for 24 hours and the cell lysates were analyzed by Western blot as described above. Adipose tissue is the main contributor to circulating serum leptin; therefore cells of the adipose tissue would be exposed to higher leptin levels compared to cells from other tissues. Hence, the concentrations of leptin used in these in-vitro experiments are physiologically relevant in the adipose tissue milieu of both lean and obese subjects.

Further, the effect of increased caveolin-1 expression on leptin dependent cellular signaling was determined using adenovirus encoding caveolin-1 (Vector Biolabs). HWP were infected with either caveolin-1 containing adenovirus or control null virus at an MOI of 100 for 12 hours, followed by 48 hours of growth prior to leptin treatment. Downstream leptin signaling was determined by Western blot in these cells after 0-30 min of leptin (100 ng/ml) treatment.

To investigate the direct role of leptin in adipocyte lipid metabolism, HWP were differentiated in presence of increasing concentrations of leptin (0 -100 ng/ml) for six days, followed by semi-quantitative determination of accumulated lipid by Oil-Red-O staining. Briefly, after six days of differentiation, the cells were fixed with 4 % paraformaldehyde, stained with 0.3 % Oil-Red-O solution in 60 % isopropanol, and washed with PBS. Intracellular lipid was quantified by treating the stained cells with isopropanol and obtaining absorbance at 490 nm. To determine the effect of increased caveolin-1 expression on leptin-dependent decreases in accumulating lipid, HWP were infected with caveolin-1 encoding adenovirus as described above followed by differentiation in presence of increasing concentrations of leptin. Null-Adenovirus treated HWP were used as control for these experiments.

To examine the molecular mechanisms though which leptin may decrease lipid accumulation in differentiating HWP, we investigated the regulatory role of leptin on expression of key proteins involved
in lipid storage and lipid biogenesis. dHWP were treated with increasing concentrations of leptin (0-150 ng/ml) for 6 hours to determine the effects on mRNA expression and 24 hours to determine the effects on protein expression. Quantitative mRNA analysis was done using commercially available TaqMan probe as per manufacturer’s instruction.6

**Statistical Analysis:** Data from study participants are summarized as mean ± SD. Each measurement at baseline and weight gain was compared using paired Wilcoxon Signed Rank test. The correlation between relative changes in caveolin-1 expression and changes in weight, fat mass, total cholesterol, HDL, triglyceride and leptin was assessed using the Spearman correlation coefficient.

All in-vitro experiments were performed at least four times. Data are presented as mean ± SEM. Statistical significance and pairwise analysis were determined using Wilcoxon rank sum test. A two tailed p values < 0.05 were considered significant. Data were analyzed using JMP 9.0.1 (SAS Institute Inc, Cary, North Carolina).
Online Table I: Predictors of changes in caveolin-1 expression in abdominal adipose tissue during weight gain.

<table>
<thead>
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<th>Variable</th>
<th>Spearman $\rho$</th>
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<tr>
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<td>0.58</td>
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<tr>
<td>Fat mass</td>
<td>0.47</td>
<td>0.17</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-0.02</td>
<td>0.96</td>
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<tr>
<td>HDL</td>
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<td>Triglyceride</td>
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<tr>
<td>Insulin</td>
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<td>0.26</td>
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<tr>
<td>Leptin</td>
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<tr>
<td>Adiponectin</td>
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<td>0.47</td>
</tr>
<tr>
<td>Glucose</td>
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<td>0.62</td>
</tr>
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</table>

N=10, * is p < 0.05 as determined by Spearman’s correlational analysis
References:


**Online Figure I**: Caveolin-1 (Cav-1) and leptin receptor (Ob-R) interactions. Western blot showing the presence of Ob-R in the fraction immuno-precipitated with anti-Cav-1 antibodies in human adipose tissue (A). Confocal images (B) showing co-localization (yellow) of Cav-1 (green) with Ob-R (red) in human adipose tissue sections.