Abstract

Objective: Adipose tissue displays depot-specific metabolic properties and a predominant gene expression of leptin in subcutaneous tissue. The aim of the study was to evaluate leptin mRNA expression in various adipose tissues and to relate it to plasma leptin concentrations. Furthermore, developmental changes in leptin gene expression from childhood to adulthood were examined.

Design and methods: Thoracic subcutaneous and intrathoracic adipose tissue specimens were obtained in 22 adults (51–81 years) and 23 children (0.1–17 years) undergoing cardiac surgery, and abdominal subcutaneous, omental and mesenterial fat specimens were collected from 21 adults (38–79 years) and 22 children (0.2–17 years) before abdominal surgery. Preoperative plasma leptin concentrations were measured by RIA. Leptin mRNA expression was quantified by TaqMan real-time PCR.

Results: In adults, there was no difference between leptin gene expression in subcutaneous and intrathoracic fat, whereas in children leptin mRNA expression was significantly higher in subcutaneous adipose tissue. In omental fat, leptin mRNA levels were significantly lower compared with subcutaneous and mesenterial sites in both children and adults. Adults revealed a significantly higher leptin gene expression in subcutaneous, omental and mesenterial adipose tissues than children. Subcutaneous and omental leptin gene expression are independent factors for plasma leptin concentrations in children and adults.

Conclusion: Leptin is differentially expressed at different adipose tissue sites, a situation which is even more pronounced in children. There is a developmental increase in leptin mRNA expression in adipose tissue during childhood, reaching maximal capacity in adulthood.

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Introduction

Leptin is a peptide hormone controlling appetite and increasing energy expenditure (1, 2). It is mainly produced by differentiated adipocytes (3). Regional differences in leptin secretion (4) and gene expression in adipocytes from subcutaneous compared with omental origin are well established. Human omental adipocytes are known to express less leptin mRNA than subcutaneous adipocytes (5–10). In addition, these two adipocyte subtypes can be distinguished by several other biochemical properties such as an increased lipolytical responsiveness of omental adipocytes to catecholamines (11) and less responsiveness to the anti-lipolytic actions of insulin (12). All these different mechanisms in metabolic regulation may account for the fact that the type of body fat distribution is crucial for predicting metabolic outcomes in obese patients. The visceral quantity of fat, not the total body fat mass per se, is a strong predictor for metabolic adverse consequences of obesity such as diabetes, hyperlipidemia and cardiovascular disease (13).

In children, there are only sparse data on leptin gene expression. An increase in leptin gene expression in subcutaneous adipose tissue with age has been described in obese children (14). In rats, it is known that leptin mRNA expression also increases with age, following characteristic ontogenetic patterns in different adipose tissue depots (15).

To see whether intrathoracic fat also shows different leptin gene expression compared with thoracic subcutaneous adipose tissue and to gain more information about regional differences as well as the influence of age on leptin mRNA expression, we measured the gene expression of leptin in subcutaneous, intrathoracic, omental and mesenterial fat in children and adults undergoing elective surgery. A secondary goal was to evaluate the impact of the different adipose tissues on leptin plasma concentrations in children and adults.
Materials and methods

Patients

Two samples of adipose tissue from subcutaneous and intrathoracic fat were obtained immediately after the beginning and before the end of the operation from patients undergoing cardiac surgery, and from subcutaneous, omental and mesenteric fat from patients with elective abdominal surgery. The patients included 22 adults and 23 children with cardiac surgery and 21 adults and 22 children with abdominal surgery. Patients’ characteristics are listed in Table 1. None of the patients was taking any steroidal medication or insulin. Body height and weight were measured one day before surgery. Body mass index (BMI) was calculated as kg/m^2. Blood samples were drawn after the onset of anesthesia and were kept frozen at −18°C until analysis. The study was approved by the local ethics committee. Informed written consent was obtained on the day before surgery from all patients or parents as appropriate.

RNA extraction and reverse transcription

Tissue samples were kept frozen at −80°C until RNA extraction. Total RNA was extracted from the tissues using guanidine-thiocyanate acid phenol (TRIzol, WAK Chemie, Medical GmbH, Bad Homburg, Germany). The method was modified and optimized using guanidine-thiocyanate acid phenol (TRIzol, for extraction of RNA from adipose tissue. As opposed to the manufacturer’s protocol, we used 1 ml TRIzol/100 mg adipose tissue and added 400 μl CHCl₃.

The RNA concentration was determined spectrophotometrically. One microgram RNA was reversely transcribed in a volume of 20 μl at 39°C for 60 min (all chemicals were obtained from Boehringer Mannheim, Germany).

TaqMan real-time PCR

The method for the measurement of gene expression in various tissues has been described before (16, 17). This approach is based upon the 5’ exonuclease activity of the Taq polymerase. Briefly, within the amplicon defined by a gene-specific oligonucleotide primer pair an oligonucleotide probe labeled with two fluorescent dyes is designed. As long as the probe is intact, the emission of a reporter dye (i.e. 6-carboxy-fluorescein, FAM) at the 5’ end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3’ end. During the extension phase of the PCR, the Taq polymerase cleaves the probe releasing the reporter dye. An automated photometric detector combined with special software (ABI Prism 7700 Sequence Detection System, Perkin-Elmer Applied Biosystems, Foster City, CA, USA) monitors the increasing reporter dye emission. The algorithm normalizes the signal to an internal reference (∆Rn) and calculates the threshold cycle number (C_T), when the ∆Rn reaches 10 times the standard deviation of the baseline. The C_T values of the probes are interpolated to an external reference curve constructed by plotting the relative or absolute amounts of a serial dilution of a known template vs the corresponding C_T values.

Table 1 Characteristics of adults (n = 22) and children (n = 23) undergoing cardiac surgery and of adults (n = 21) and children (n = 22) undergoing abdominal surgery.

<table>
<thead>
<tr>
<th></th>
<th>Adults</th>
<th>Children</th>
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<tbody>
<tr>
<td>Cardiovascular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>68.7 ± 1.7 (range 51–81)</td>
<td>4.1 ± 1.0 (range 0.1–17)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>15/7</td>
<td>13/10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4 ± 0.8 (range 16.2–33.9)</td>
<td>15.9 ± 0.7 (range 12.8–27.2)</td>
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<tr>
<td>Medication</td>
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<tr>
<td>Beta blockers, n = 12</td>
<td></td>
<td>Antihypertensive drugs, n = 1</td>
</tr>
<tr>
<td>Other antihypertensive drugs, n = 20</td>
<td></td>
<td>(no beta blockers)</td>
</tr>
<tr>
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<td>Haemodiluents, n = 4</td>
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<td>Digitalis, n = 10</td>
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<tr>
<td>Molsidomin, n = 9</td>
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<td>Digitalis, n = 3</td>
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<tr>
<td>HMG-CoA reductase inhibitors, n = 12</td>
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<tr>
<td>Abdominal surgery</td>
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<tr>
<td>Age (years)</td>
<td>59.5 ± 2.3 (range 38–79)</td>
<td>7.3 ± 1.4 (range 0.2–17)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>13/8</td>
<td>14/8</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.4 ± 0.79 (range 18.6–33.2)</td>
<td>17 ± 0.8 (range 11.6–25.6)</td>
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<tr>
<td>Medication</td>
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<td>Beta blockers, n = 1</td>
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<td>HMG-CoA reductase inhibitors, n = 2</td>
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</tbody>
</table>

HMG-CoA, hydroxymethylglutaryl coenzyme A.
Commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer) and conditions were employed according to the manufacturer’s protocol. cDNA (2.5 μl) (reverse transcription mixture) and oligonucleotides with a final concentration of 300 nmol/l of primers and 200 nmol/l of TaqMan hybridization probe were added to 25 μl reaction mix. The oligonucleotides of each target of interest were designed by the Primer Express software (Perkin-Elmer) using uniform selection parameters that allowed the application of the same cycle conditions confirmed by primer optimization. All of the primers and probes were purchased from Perkin-Elmer Applied Biosystems. The thermocycler parameters were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A serial dilution of known copy numbers of a PCR product served as reference providing a relative quantification of the unknown samples.

Leptin gene expression was related to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin.

The following primers and TaqMan probes were used. GAPDH: forward 5’-CCATGTCATGCAGGATGGT-3’, reverse 5’-TGTGCTAGTCCCTCCACAGATA-3’, TaqMan probe 5’- (FAM)-CTGCGACCAACTGCTTACGCCACCC-(TAMRA)3’; β-actin: forward 5’-GGGCTGAAGATGACCC AGGATC-3’, reverse 5’-CCATGGTACGGCCAGAGG-3’, TaqMan probe 5’-(FAM)-CCAGGATATGTACCTTATC CAGGC-(TAMRA)3’; leptin: forward 5’-GTGCGGATTCT TGTGCTTCTT-3’, reverse 5’-CAAATTGTAATTCCAACCG GTG-3’, TaqMan probe 5’-(FAM)-CAATGACATTTCACA CACGTCAGTCTCTCCAA- (TAMRA)3’.

**Leptin measurement**

Leptin was measured by a specific radioimmunoassay (18). For the measurement of plasma samples, the standard concentrations ranged from 15.6 to 0.06 μg/l. Samples were diluted 1:2 with assay buffer. Tracer activity was ~ 15 000 c.p.m. per tube, the final dilution of the specific antibody was 1:50 000. One hundred microliters of each tracer, appropriately diluted sample and antibody were mixed and incubated for 24 h at room temperature. Bound and non-bound fractions were separated by goat-anti-rabbit IgG (DSL, Sindheim, Germany) in 4% polyethyleneglycol (PEG) at a final dilution of 1:150. After 2 h at room temperature the tubes were centrifuged (3000 g × 15 min, 4°C) and the radioactivity of the pellets was counted in a gamma counter. Fifty percent binding occurred at 2 μg/l. The sensitivity of undiluted samples was 0.03 μg/l. Inter- and intra-assay coefficients of variation were 8.5% and 3.8% respectively.

**Statistical analysis**

All values are expressed as means±s.e.m. After testing for Gaussian distribution, parametric data of individual groups were compared using Student’s t-test, and non-parametric data were compared using Mann–Whitney–Wilcoxon signed rank test was used for comparison of related interval data. To analyze influence factors on plasma leptin concentrations, simple and multiple regressions were performed after log-transformation of plasma leptin levels. A P-value of ≤ 0.05 was considered significant.

**Results**

**Thoracic tissue**

In adult cardiosurgical patients there was no difference between leptin/GAPDH mRNA expression in subcutaneous and intrathoracic tissue (2.17±0.32 vs 1.65±0.26 relative units (RU), P = 0.22; leptin/β-actin: 0.56±0.07 vs 0.42±0.05 RU, P = 0.11) (Fig. 1A). However, in children with congenital heart disease, we found a significantly higher leptin gene expression in subcutaneous compared with intrathoracic fat (0.89±0.31 vs 0.15±0.06 RU, P = 0.002) (Fig. 1B). This could be confirmed when relating leptin mRNA values to β-actin (2.55±0.82 vs 0.51±0.28, P = 0.001).

**Abdominal tissue**

Leptin gene expression in adult patients was significantly lower in omental compared with subcutaneous adipose tissue (GAPDH: 1.64±0.30 vs 3.67±0.61 RU, P = 0.005; β-actin: 1.64±0.55 vs 4.18±1.40 RU, P = 0.025). A comparison of omental and mesenterial fat revealed a significant difference when related to GAPDH (1.64±0.30 vs 2.72±0.39 RU, P = 0.035) and fell short of being significant when normalised to β-actin (1.64±0.55 vs 2.99±1.15, P = 0.074). There was no significant difference in leptin gene expression between subcutaneous and mesenterial fat in adults (GAPDH: P = 0.196, β-actin: P = 0.521) (Fig. 1C).

In children, the same distribution of leptin mRNA expression in abdominal fat tissue could be observed as in adults. Omental fat revealed lower leptin mRNA values than subcutaneous (GAPDH: 0.05±0.03 vs 0.74±0.31 RU, P = 0.001; β-actin: 0.02±0.01 vs 0.16±0.05 RU, P = 0.001) or mesenterial (GAPDH: 0.05±0.03 vs 0.30±0.14 RU, P = 0.004; β-actin: 0.02±0.01 vs 0.08±0.03 RU, P = 0.01) fat. There was no significant difference between leptin gene expression in subcutaneous and mesenterial fat samples (GAPDH: P = 0.47; β-actin: P = 0.44) (Fig. 1D).

Adults revealed a significantly higher leptin gene expression than children in subcutaneous abdominal (GAPDH: P < 0.0001, β-actin: P < 0.0001), omental (GAPDH: P < 0.0001, β-actin: P < 0.0001), and mesenterial (GAPDH: P < 0.0001, β-actin: P = 0.006) adipose tissue (Fig. 2).
In none of our patient groups was there a significant difference in leptin mRNA expression between males and females.

A correlation between leptin mRNA expression and age could be found for children in subcutaneous thoracic (GAPDH: $r = 0.57$, $P = 0.005$, β-actin: $r = 0.59$, $P = 0.004$), intrathoracic (GAPDH: $r = 0.55$, $P = 0.007$, β-actin: $r = 0.57$, $P = 0.006$) and omental (GAPDH: $r = 0.45$, $P = 0.043$, β-actin: $r = 0.50$, $P = 0.019$), but not in subcutaneous abdominal (GAPDH: $r = 0.35$, $P = 0.109$, β-actin: $r = 0.50$, $P = 0.019$) and mesenterial (GAPDH: $r = 0.35$, $P = 0.129$, β-actin: $r = 0.20$, $P = 0.386$) fat. Leptin gene expression revealed no relation to age in adult patients (subcutaneous thoracic, intrathoracic, subcutaneous abdominal, omental, and mesenterial fat, $P > 0.05$).

In patients with heart disease, a multiple regression analysis revealed that independent factors influencing plasma leptin levels are leptin gene expression in subcutaneous adipose tissue ($P = 0.015$), BMI ($P = 0.008$), gender (plasma leptin levels in women higher than in men, $P = 0.043$) and age (i.e. that in relation to low mRNA expression levels children have

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**Figure 1** Leptin gene expression normalized to GAPDH in subcutaneous thoracic (sc th) and intrathoracic (ith) adipose tissue in (A) adult patients and (B) children undergoing cardiac surgery, and in subcutaneous abdominal (sc abd), omental (om), and mesenterial (mes) fat of (C) adult and (D) pediatric patients with abdominal surgery. * $P = 0.03$; ** $P < 0.005$; ns, not significant.

**Figure 2** Comparison of leptin/GAPDH gene expression in subcutaneous abdominal (subcutaneous abd.), omental, and mesenterial adipose tissue in children and adults. ***$P < 0.0001$. 
relatively higher plasma leptin levels, \( P = 0.047 \), but not leptin gene expression in intrathoracic fat. In patients undergoing abdominal surgery, we found BMI \( (P = 0.003) \), age \( (P < 0.0001) \) and leptin gene expression in omental adipose tissue \( (P = 0.027) \) influenced plasma leptin levels independently. Subcutaneous abdominal and mesenterial fat did not correlate to plasma leptin.

**Discussion**

Leptin gene expression shows regional differences and changes from childhood to adulthood. When analyzing leptin gene expression in different adipose tissues, we found two- to tenfold higher mRNA levels in adults than in children. The well known increase in plasma leptin concentrations from childhood to adulthood (19) might, therefore, be caused not only by the age-dependent increasing fat mass but also by a higher capacity of adult fat cells to produce leptin.

In children, we found increasing leptin mRNA levels with age at some sites of adipose tissue (subcutaneous thoracic, intrathoracic, and omental fat). There is one previous study showing an increase in leptin gene expression with age in the subcutaneous adipose tissue of obese children (14). Our results indicate that there is a differentially regulated development of leptin mRNA expression at different adipose sites during childhood.

In a multiple regression analysis, BMI, gender and leptin gene expression in subcutaneous and omental fat could be shown to independently influence plasma leptin levels. The relation between BMI and plasma leptin levels has been recognised for some time (1). In children, we found increasing leptin mRNA levels with age at some sites of adipose tissue (subcutaneous thoracic, intrathoracic, and omental fat). There is one previous study showing an increase in leptin gene expression with age in the subcutaneous adipose tissue of obese children (14). Our results indicate that there is a differentially regulated development of leptin mRNA expression at different adipose sites during childhood.

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References


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