IGF-1 receptor deletion in adipocytes

Autocrine IGF-1 action in adipocytes controls systemic IGF-1 concentrations and growth

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Objective: Insulin-like growth factor-1 (IGF-1) and the IGF-1 receptor (IGF-1R) have been implicated in the regulation of adipocyte differentiation and lipid accumulation \textit{in vitro}.

Research Design and Methods: To investigate the role of IGF-1 receptor \textit{in vivo}, we have inactivated the IGF-1 receptor gene in adipose tissue (IGF-1R\textsuperscript{aP2Cre} mice) using conditional gene targeting strategies.

Results: Conditional IGF-1R inactivation resulted in increased adipose tissue mass with a predominantly increased lipid accumulation in epigonadal fat pads. However, insulin-stimulated glucose uptake into adipocytes was unaffected by the deletion of the IGF-1R. Surprisingly, IGF-1R\textsuperscript{aP2Cre} mice exhibited markedly increased somatic growth in the presence of elevated IGF-1 serum concentrations and IGF-1 mRNA expression was significantly increased in liver and adipose tissue. IGF-1 stimulation of wild type adipocytes significantly decreased IGF-1 mRNA expression, while the opposite effect was observed in IGF-1R deficient adipocytes.

Conclusions: IGF-1 receptor signaling in adipocytes does not appear to be crucial for the development and differentiation of adipose tissue \textit{in vivo}, but we identified a negative IGF-1 receptor-mediated feedback mechanism of IGF-1 on its own gene expression in adipocytes, indicating an unexpected role for adipose tissue IGF-1 signaling in the regulation of IGF-1 serum concentrations in control of somatic growth.
Insulin and the structurally related insulin-like growth factors (IGFs) provide essential signals for the control of embryonic and postnatal development (1) through binding of their respective tyrosine kinase receptors. Conventional gene targeting strategies used to abrogate insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) signaling reveal severe phenotypes of both IR- and IGF1R-deficient mice. While inactivating the IR results in embryonic lethality (2), mice with a whole body inactivation of the Igf1r gene die shortly after birth (3), whereas heterozygous Igf1r knockout mice have increased longevity, most likely due to greater resistance to oxidative stress (4). These data point to a role of IGF-1 receptor signaling in the regulation of lifespan. To discern the relevance of IGF-1 receptor signaling in individual tissues, mice with tissue-specific inactivation of the IGF-1 receptor have been created. Tissue-specific knockout of the Igf1r gene in osteoblasts displayed significant defects in bone formation with low levels of osteoblastogenesis (5), revealing a function of the IGF system in formation and maintenance of bone tissue. Mice with simultaneous functional inactivation of both insulin and IGF-1 receptors in skeletal muscle have a low muscle mass and display muscle hypoplasia (6), suggesting that IGF-1 is a vital regulator of muscle development. Conversely, muscle-specific overexpression of IGF-1 receptor resulted in local myofiber hyperplasia (7). β-cell specific deletion of the IGF-1 receptor did not alter islet development, but led to hyperinsulinemia and glucose intolerance in βIGF1RKO mice (8). This model suggests that tissue-specific alterations in the IGF-1 signaling might have secondary effects on the organism.

Until now, the role of IGF-1 receptor signaling in white adipose tissue remains unclear. In 3T3-L1 adipocytes, IGF-1 is an essential regulator of differentiation (9) and it was recently shown that IGF-1 stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes in vitro (10). However, the physiologic role of IGF-1 receptor signaling in adipose tissue in vivo has not been systematically studied. To investigate the role of the IGF-1 receptor in the development and metabolism of adipose tissue, we generated mice lacking the IGF-1R in adipose tissue (IGF-1R<sup>ap2Cre</sup>) using a conditional gene targeting approach based on the Cre recombinase. Subsequently, we characterized the consequences of IGF-1 receptor deletion in adipose tissue on morphologic and metabolic parameters of IGF-1R<sup>ap2Cre</sup> mice up to an age of thirty-two weeks.

**RESEARCH DESIGN AND METHODS**

**Generation of IGF-1R<sup>ap2Cre</sup> mice.** IGF-1 receptor flox/wt mice were created by inserting loxP (locus of crossover (x) in bacteriophage P1) sites flanking exon 3 of the Igf1r gene using conditional gene targeting strategies and maintained on a pure C57BL/6 genetic background. A targeting vector was constructed encompassing 4.5 kb of intron 2 of the murine IGF-1R gene, a loxP site, an FRT-flanked neomycin resistance gene, 600 bp fragment containing exon 3, a second loxP site, and 1.3 kb of intron 3. Cre-mediated recombination and subsequent excision of exon 3 of the IGF-1R results in a frame shift after 213 codons, with an appended sequence of 27 amino acids followed by a stop codon in exon 4. Gene targeting was performed in Bruce4 ES cells. For transfection, 1x10<sup>7</sup> ES cells were transfected with 40 µg DNA. Approximately 9 days after transfection, G418/Ganciclovir double-resistant colonies were picked and expanded on 96-well tissue culture dishes. Genomic DNA was extracted...
from each clone and analysed by Southern blot and PCR analysis. Recovery, microinjection and transfer of 3.5 day p.c. embryos was performed according to standard procedures. Chimeric animals (80-90% chimerism based on coat color) were bred with FlpE deleter on a C57BL/6 background. Genotyping was performed by PCR on DNA extracted from tail biopsies using customized primers: 5’- TCC CTC AGG CTT CAT CCG CAA -3’ (sense) and 5’- CTT CAG CTT TGC AGG TGC ACG -3’ (antisense). The targeting strategy is shown in Figure 1A. Mice on a mixed (C57BL/6 x 129/Sv) genetic background carrying the aP2 Cre transgene were made by cloning a 1.4kb acI/SalI complementary DNA fragment encoding the Cre recombinase, modified by inclusion of a Nuclear localization sequence (NLS) and a consensus polyadenylation signal, immediately downstream of the 5.4 kb promoter/enhancer of fatty-acid-binding protein aP2 (11).

IGF-1R<sup>AP2Cre</sup> were derived by crossing IGF-1R<sup>flox/wt</sup> mice with IGF-1R<sup>flox/wt</sup> mice expressing the Cre recombinase under the control of the aP2 promoter/enhancer (IGF-1Rhet<sup>AP2Cre</sup>). All mice were housed in pathogen-free facilities in groups of 3-5 at 22 ± 2°C on a 12-hour light/dark cycle. Animals were fed a standard chow diet (Altromin, Lage, Germany). In a subgroup of 6-8 female IGF-1R<sup>AP2Cre</sup> and 6 wildtype (WT) mice, a high fat diet (HFD) study was performed with a special HFD containing 55.2% of calories from fat (C1057, Altromin, Lage, Germany). Animals had ad libitum access to water at all times, and food was only withdrawn if required for an experiment. All experiments were performed in accordance with the rules for animal care of the local government authorities (Bezirksregierung Köln, Cologne, Germany) of the German Government and were approved by the Institution's animal care and use committee.

**Molecular characterization and genotyping of the IGF-1R<sup>AP2Cre</sup> mice.** Genotyping was performed by PCR using genomic DNA isolated from the tail tip. In brief, genomic DNA was prepared by using the DNasey kit (Qiagen, Hilden, Germany). The following two primer pairs were used to genotype Igf-1r loxP sites: 5’-TCC CTC AGG CTT CAT CCG CAA-3’ (forward) and 5’-CTT CAG CTT TGC AGG TGC ACG-3’ (reverse) as well as the aP2 Cre recombinase: 5’-CGC CGC ATA ACC AGT GAA AC-3’ (forward) and 5’-ATG TCC AAT TTA CTG ACC G-3’ (reverse). PCR was performed for 25 cycles (loxP sites) or 30 cycles (aP2 Cre) of 95°C, 59°C (loxP sites) or 58°C (aP2 Cre), and 72°C (30s each) using the Qiagen Taq Polymerase and a Peltier Thermal Cycler, PTC-200 (BioRad, Hercules, California, USA). DNA from WT mice produced a 300 bp band, and a 380 bp band was detected in IGF-1R lox mice (Figure 1B). For western blot analysis, tissues were removed and homogenized in homogenization buffer with an ultra-Turrax homogenizer (IKA Werke, Stauffen, Germany), proteins were isolated using standard techniques and western blot analysis was performed with antibodies raised against IGF-1 receptor β subunit (Santa Cruz Biotechnology Inc., C20) and β actin (Abcam, Cambridge, UK) as loading control.

**Phenotypic characterization.** Ten mice of each genotype (IGF-1R<sup>AP2Cre</sup>, IGF-1Rhet<sup>AP2Cre</sup>, WT, IGF-1R<sup>flox/flox</sup>, aP2 Cre) of both sexes were studied from an age of 4 weeks up to 32 weeks of life. Body weight was recorded weekly up to an age of 14 weeks, and thereafter in 2-weeks intervals, body length (naso-anal length) was measured once at week 32. At an age of 16 weeks, both sexes of a subgroup of twenty (10 IGF-1R<sup>AP2Cre</sup> and 10 controls) underwent a food intake measurement over a time period of one week. The daily food intake was calculated as the average intake of chow within the time stated.
Intraperitoneal (i. p.) glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed at the age of 12 and 24 weeks. GTT was performed after an overnight fast for 16 hours by injecting 2g/kg body weight of glucose and measuring the blood glucose levels after tail vein incision at 0 (baseline), 10, 30, 60 and 120 min after injection. ITT was performed in random fed animals by injecting 0.75 U/kg body weight of human regular insulin (40U Actrapid, Novo Nordisk, Copenhagen, Denmark). Glucose levels were determined in blood collected from the tail tip immediately before and 15, 30, and 60 minutes after the i. p. injection.

Mice were sacrificed at the age of 32 weeks by an overdose of anesthetic (Sevofluran, Abbott, Germany). Liver, heart, brain, lung, spleen, subcutaneous and epididymal adipose tissue were immediately removed. The organs were weighed and related to the whole body mass to obtain relative organ weights. Serum was collected at 32 weeks and concentrations of insulin, leptin, adiponectin and IGF-1 were measured.

**Analytical procedures.** Blood glucose values were determined from whole venous blood samples using an automated glucose monitor (GlucoMen®, Menarini Diagnostics). Insulin, leptin, growth hormone and adiponectin serum concentrations were measured by ELISA using mouse standards according to the manufacturer’s guidelines (Mouse/Rat Insulin ELISA, Linco, St. Charles, USA; Mouse Leptin ELISA; Mouse Adiponectin/Acrp30 ELISA, both R&D Systems, Minneapolis, USA). IGF-1 serum concentrations were measured by a RIA (Mediagnost, Reutlingen, Germany).

**Adipocyte isolation, size distribution and primary culture.** Animals were sacrificed and subcutaneous and epididymal fat pads were removed. Adipocytes were isolated by collagenase (1 mg/ml) digestion. To determine cell size distribution and adipocyte number, 200 µl aliquots of adipocytes were fixed with osmic acid, incubated for 48 hours at 37°C and counted in a Coulter counter (Multisizer III, Beckman Coulter GmbH, Krefeld, Germany). Isolated adipocytes were resuspended in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 Ham’s medium (Sigma, St. Louis, MO) and centrifuged at 350 g for 10 min to separate mature adipocytes from the stromal vascular cell pellet, which was discarded. Floating fat-filled adipocytes were then incubated for 6 hours with different concentrations (0, 0.1, 1, 10, 100 ng/ml of recombinant IGF-1 (US Biological, Swampscott, MA).

**Histology.** Tissue was fixed in 4% buffered formaldehyde, trimmed into small cubes, rinsed in phosphate-buffered saline and dehydrated in a graded series of 50-100% ethanol followed by propylene oxide. Tissue was infiltrated with propylene: Epon 812 mixtures by increasing the resin concentration gradually. Sections at 0.5 µm were cut with a Reichert ultramicrotome, mounted on polylysine-covered object slides and stained with azur-II-methylene blue (Richardson-stain). Digitized pictures were taken with the light microscope Axioplan 2 (Zeiss, Jena, Germany) using the measurement facilities of "Imagic Access" (Imagic, Glattburg, Switzerland). Multiple sections (separated by 70-80 µm each) were obtained from gonadal and subcutaneous fat pads and analyzed systematically with respect to adipocyte size and number. For each genotype and gender at least 10 fields (representing approximately 100 adipocytes) per slide were analyzed.

**Glucose transport.** For the determination of glucose transport, isolated adipocytes from the different fat depots were stimulated with 100 nM insulin for 30 min, then incubated for 30 min with 3 µM U-14C-glucose. Immediately after incubation, adipocytes were
fixed with osmic acid, incubated for 48 hours at 37°C and radioactivity was quantified after the cells had been decolorized (12).

**Tissue-specific IGF-1 mRNA expression.**
IGF-1 mRNA expression was measured by quantitative Realtime PCR using the standard curve method in a fluorescent temperature cycler using the TaqMan assay. Fluorescence was detected on an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). Total RNA was extracted from epigonalad and subcutaneous WAT, primary adipocytes culture after incubation with or without IGF-1, liver, brain, skeletal muscle, and BAT using TRIzol (Life Technologies, Grand Island, NY) and 1 µg RNA was reversed transcribed with standard reagents (Life Technologies, Grand Island, NY). From each RT-PCR, 2 µl were amplified in a 26 µl PCR reaction using the Brilliant SYBR Green QPCR Core Reagent Kit from Stratagene (La Jolla, CA) according to manufacturer’s instructions. The following primer pairs were used: muIGF-1 5’ GCTGCTGAAGCCATTCATTT 3’ (sense) and 5’ TTGCTCTTAAGGAGGCCAAA 3’ (antisense); mu36B4 5’aacatgctcaacatctcccc 3’ (sense) and 5’cgcactcctccgactcttc 3’ (antisense). Expression of IGF-1 and 36B4 mRNA were quantified by using the second derivative maximum method of the TaqMan software (Applied Biosystems, Darmstadt, Germany) determining the crossing points of individual samples by an algorithm which identifies the first turning point of the fluorescence curve. Amplification of specific transcripts was confirmed by melting curve profiles (cooking the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR.

**Data analysis and statistics.** Data are given as mean ± SD. Data sets were analyzed for statistical significance using a 2-tailed unpaired Student’s t test or differences were assessed by one-way analysis of variance corrected by Bonferroni-Holm using the Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA), version 14.0. P values less than 0.05 were considered significant.

**RESULTS**

**Generation of IGF-1R<sub>aP2Cre</sub> mice.**
Mice lacking the IGF-1R in adipose tissue (IGF-1R<sub>aP2Cre</sub>) were generated by crossing mice carrying the loxP-flanked Igf-1r allele with transgenic mice expressing the Cre recombinase under control of the adipose-specific fatty acid binding protein (aP2) promoter. The targeting strategy is shown in Figure 1A. IGF-1R<sub>aP2Cre</sub> mice were obtained with the expected Mendelian frequency. Cre expression was expected to be restricted to white adipose tissue (WAT) and brown adipose tissue (BAT). PCR analysis of the genomic DNA showed that the wildtype Igf-1r allele was absent in IGF-1R<sub>aP2Cre</sub>, whereas IGF-1R<sub>het<sub>aP2Cre</sub> mice contained both the wildtype and loxP-flanked Igf-1r allele (Fig. 1B). Western blot analysis of adipose tissue lysates clearly indicated that Igf-1r protein was reduced by ~90% in WAT of IGF-1R<sub>aP2Cre</sub> mice (Fig. 1C) and completely lost in BAT (data not shown). A ~ 40% reduction of IGF-1R protein expression was found in the brain, whereas no changes in the overall expression of the IGF-1R protein were detected in liver, skeletal muscle (Fig. 1C), kidney, and bone (data not shown). IGF-1 receptor expression was unaffected in all tissues of WT, IGF-1R<sub>flox/flox</sub> and aP2 Cre mice, indicating that neither the loxP modification of the IGF-1R locus nor expression of the aP2 transgene alone affects IGF-1R expression. These genotypes (WT, IGF-1R<sub>flox/lox</sub>, aP2 Cre) had similar physiologic and metabolic characteristics and were therefore considered controls in all subsequent analyses.
Phenotype of IGF-1R<sup>aP2Cre</sup> mice.
Both sexes of IGF-1R<sup>aP2Cre</sup> mice exhibited normal growth until the age of 10 weeks. However, by 10 weeks of age male and female IGF-1R<sup>aP2Cre</sup> mice had gained more weight than control group littermates (Fig. 2A, B). Moreover, we found that IGF-1R<sup>aP2Cre</sup> mice had significantly increased naso-anal body length when compared to littermate controls (females p<0.01 and males p<0.001) at an age of 24 and 32 weeks (Fig. 2C). Heterozygous deficiency of the Igf-1r gene had no significant influence on body growth and weight (data not shown). Daily food intake was indistinguishable between IGF-1R<sup>aP2Cre</sup> mice and all control groups (data not shown). Because IGF-1R<sup>aP2Cre</sup> mice exhibit ~15% increased body weight, organ and fat pad weights were calculated as percentage of the whole body weight at an age of 24 weeks. We found a disproportional increase of fat pad (Fig. 2D), liver (Fig. 2E) and heart weights in IGF-1R<sup>aP2Cre</sup> mice. In contrast, relative brain weight was significantly reduced in IGF-1R<sup>aP2Cre</sup> compared to controls (Fig. 2F). Relative organ weights of BAT (Fig. 2G), skeletal muscle, kidney, spleen and bone were not different between IGF-1R<sup>aP2Cre</sup> mice and control mice (data not shown).

Metabolic parameters.
To determine the physiological consequences of reduced adipose tissue IGF-1 receptor expression, we monitored blood glucose, insulin, total cholesterol and triglyceride concentrations and performed serial glucose and insulin tolerance tests over an age range from 12 to 32 weeks (Table 1). Fasted blood glucose was significantly higher in IGF-1R<sup>aP2Cre</sup> mice at an age of 12 weeks, whereas no such differences were found at 24 weeks of age (Table 1). In parallel, fed blood glucose was significantly higher at an age of 12 weeks in males (Table 1). There were no differences in serum insulin and leptin concentrations between IGF-1R<sup>aP2Cre</sup> and control mice at 12 and 24 weeks (Table 1). Cholesterol and triglyceride serum concentrations were also not significantly different between IGF-1R<sup>aP2Cre</sup> and control mice (Table 1). Adiponectin serum concentrations were higher in control compared to IGF-1R<sup>aP2Cre</sup> mice, however, these differences were only significant at 12 weeks (Table 1). Independent of age, intraperitoneal glucose tolerance tests demonstrated normal glucose tolerance in male (Fig. 3A, B) and female (data not shown) IGF-1R<sup>aP2Cre</sup> and control mice. Intraperitoneal insulin tolerance tests at 12 and 24 weeks of age in male (Fig. 3C, D) and female mice (data not shown) were indistinguishable between IGF-1R<sup>aP2Cre</sup> and control mice. Taken together, reducing IGF-1R expression in adipose tissue results in increased somatic growth in the absence of major metabolic alterations.

IGF-1 serum concentration is markedly increased in IGF-1R<sup>aP2Cre</sup> mice
Since IGF-1R<sup>aP2Cre</sup> mice exhibited an increased postnatal somatic growth, we next determined circulating plasma IGF-1 concentrations as the major determinant of somatic growth in mammals. IGF-1 serum concentration is significantly increased in IGF-1R<sup>aP2Cre</sup> mice starting at an age of 12 weeks up to 24 weeks of age (Fig. 4A). In parallel to higher IGF-1 serum concentrations, we found significantly higher serum concentrations of the insulin-like growth factor binding protein (IGFBP-) 3 (Fig. 4B). On the other hand, serum concentrations of IGFBP-1 and -2 were not significantly different between IGF-1R<sup>aP2Cre</sup> and control mice (data not shown). Growth hormone (GH) and IGF-2 serum concentrations were indistinguishable between IGF-1R<sup>aP2Cre</sup> and control mice (Table 1), indicating that dysregulation of IGF-1 concentrations is not a result of altered pituitary regulation and thus
appears to be primarily regulated in peripheral organs of IGF-1R<sub>Apa2Cre</sub> mice. Therefore, we determined IGF-1 and IGFBP3 mRNA expression in adipose tissue and liver of IGF-1R<sub>Apa2Cre</sub> and control mice to assess whether increased IGF-1 and IGFBP3 serum concentrations correlate with increased expression in those organs important for the synthesis of these molecules. Indeed, we found significantly higher IGF-1 mRNA expression both in adipose tissue (Fig. 4C) and liver (Fig. 4D) of IGF-1R<sub>Apa2Cre</sub> mice. In parallel, there was a trend for increased IGFBP3 mRNA expression in liver and adipose tissue of IGF-1R<sub>Apa2Cre</sub> compared to control mice (data not shown).

To determine, whether increased adipose tissue IGF-1 expression in IGF-1R<sub>Apa2Cre</sub> mice was indeed the result of an altered adipocyte autonomous autocrine regulatory loop, we isolated adipocytes from wildtype and IGF-1R<sub>Apa2Cre</sub> mice. Treatment of control adipocytes with recombinant IGF-1 (100 ng/ml) for 6 hours <i>ex vivo</i> caused a significant reduction of its own mRNA expression (Fig. 4E). Conversely, in adipocytes from IGF-1R<sub>Apa2Cre</sub> mice, stimulation with IGF-1 did not inhibit <i>IGF-1</i> mRNA expression, but rather resulted in a paradoxical stimulation of <i>IGF-1</i> mRNA expression.

**Consequences of IGF-1R KO on adipose tissue morphology and metabolism.**

Adipose tissue mass is increased in IGF-1R<sub>Apa2Cre</sub> mice compared to controls (Fig. 2D). The increased adipose tissue mass was related to both increased number and mean volume of adipocytes in IGF-1R<sub>Apa2Cre</sub> mice (data not shown). Mean adipocyte size (diameter) is significantly higher in IGF-1R<sub>Apa2Cre</sub> (105.4 ± 12 µm) when compared to control (87.3 ± 9.3 µm) mice (p<0.05). These differences are more pronounced in epigonal than in subcutaneous fat (Fig. 5A, B). We found a 1.4fold increase in insulin receptor mRNA expression in adipocytes from IGF-1R<sub>Apa2Cre</sub> mice (data not shown). To determine the consequences of IGF-1 receptor knockout on adipocyte glucose transport, basal and insulin-stimulated glucose uptake in isolated adipocytes was studied. In adipocytes from IGF-1R<sub>Apa2Cre</sub> mice, basal glucose uptake is unchanged compared to WT mice (Fig. 5C). Insulin stimulated glucose uptake 2.8 fold in IGF-1R<sub>Apa2Cre</sub> and 2.1 fold in WT adipocytes (p<0.05). Despite larger adipocyte size in IGF-1R<sub>Apa2Cre</sub> mice, we found significantly increased insulin sensitivity in IGF-1R<sub>Apa2Cre</sub> compared to WT adipocytes.

**DISCUSSION**

Insulin-like growth factors (IGF-1 and 2) lead to many different biological effects in target cells such as hepatocytes, myocytes and adipocytes (13). IGF-1 action is propagated by the IGF-1 receptor. IGF-1 receptor deficient mice are severely growth retarded, exhibit multiple growth- and differentiation-dependent abnormalities and die shortly after birth (3). Disruption of IGF-1 receptor in specific tissues or cell types such osteoblasts (5), skeletal muscle (6) and β-cells (8) further revealed an essential role of IGF-1R signaling in differentiation and growth.

IGF-1 was shown to be an essential regulator of differentiation in 3T3L1 adipocytes (9). Moreover, IGF-1 stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes <i>in vitro</i> (10). However, the physiologic role of the IGF-1 receptor signaling in adipose tissue <i>in vivo</i> has not been systematically studied. We therefore created mice lacking the IGF-1R in adipose tissue (IGF-1R<sub>Apa2Cre</sub> mice). To specifically target adipose tissue, we used transgenic mice that express the Cre recombinase cDNA from the adipose-specific fatty acid binding protein (aP2) promoter/enhancer (14). aP2 Cre mice have been used for adipose-selective inactivation of the GLUT4 and insulin...
receptor genes (11, 15, 16). Unexpectedly, deletion of IGF-1R was not restricted to white and brown adipose tissue. In most IGF-1R<sup>aP2Cre</sup> mice, we found a reduction of IGF-1R protein also in brain, suggesting transgene expression in neuronal tissue in addition to fat. Although aP2 promoter provides adipocyte-restricted expression postnatally, it was recently shown that aP2 has a wider embryonic expression pattern than previously appreciated (17). Throughout embryonic development, the aP2 Cre transgene was consistently found in different cell types of the nervous system (17). Expression of the aP2 Cre transgene during embryonic development might explain the reduced relative brain mass in IGF-1R<sup>aP2Cre</sup> mice. We excluded aP2 Cre transgene expression in other tissues including liver and skeletal muscle by protein expression analyses. Whole body expression of the transgene would cause growth retardation as demonstrated in IGF-1<sup>-/-</sup> mice (4, 18). In contrast, IGF-1R<sup>aP2Cre</sup> mice have increased somatic growth, further suggesting that tissues other than fat and brain are not affected by the ectopic aP2-Cre transgene expression. However, we have to discuss our data with the caution that reduced IGF-1R expression in brain may contribute to the phenotype of IGF-1R<sup>aP2Cre</sup> mice. IGF-1R<sup>aP2Cre</sup> mice are viable, fertile and do not exhibit growth retardation.

We demonstrate that IGF-1R<sup>aP2Cre</sup> mice have increased somatic growth starting at an age of 10 weeks. IGF-1R<sup>aP2Cre</sup> mice are both longer and heavier than their wild type littermates. The increased growth is not the result of increased food intake in IGF-1R<sup>aP2Cre</sup> mice. Interestingly, adipose tissue, liver and heart exhibit higher relative organ weights than other organs including kidney, skeletal muscle and spleen, which are increased in size proportionally to the higher body weight of IGF-1R<sup>aP2Cre</sup> mice. Increased growth in IGF-1R<sup>aP2Cre</sup> mice is most likely the result of ~ 20% increased IGF-1 serum concentrations. In patients with acromegaly, elevated circulating IGF-1 levels cause increased adult growth and may lead at least in some patients to enlarged organs including hepatomegaly (19) and acromegalic cardiomyopathy (20). In analogy to the phenotype of IGF-1R<sup>aP2Cre</sup> mice, transgenic expression of human IGF-1 in mice causes a similar phenotype with 1.3fold increased weight as a result of selective organomegaly without an apparent increase in skeletal growth (21). Moreover, liver-specific IGF-1 overexpression causes 50% increased serum IGF-1 levels and enhanced somatic growth (22).

Since circulating GH and IGF-2 levels are normal, increased IGF-1 serum levels in IGF-1R<sup>aP2Cre</sup> mice are most likely the result of increased IGF-1 expression both in adipose tissue and liver and not of changes caused by an IGF-1R deficiency in the central nervous system. It is difficult to dissect whether adipose or liver-derived IGF-1 primarily increases circulating IGF-1 levels. Interestingly, we identified a negative feedback mechanism for IGF-1 mRNA expression in adipose tissue. In WT adipocytes, IGF-1 suppressed its own mRNA expression<sup>ex vivo</sup>, whereas this negative feedback was blunted in adipocytes isolated from IGF-1R<sup>aP2Cre</sup> mice. However, increased IGF-1 expression in liver is more difficult to explain.

It is difficult to dissect whether adipose or liver-derived IGF-1 primarily increases circulating IGF-1 levels. Our experimental design does not allow for the distinction between hepatic or adipose tissue derived IGF-1. It has been shown that IGF-1 and IGF-1R mRNA levels are differentially regulated in different tissues (23). Interestingly, presumably adipose tissue derived IGF-1 does not inhibit hepatic IGF-1 expression as it was shown in the liver of GH-deficient dwarf rats after IGF-1 treatment (24). Another potential explanation for the absence of a negative feedback of IGF-1 on its own expression in
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liver might be that hepatocytes only express the IGF-1R in very little amounts (25). Alternatively, circulating IGF-1 could be modulated indirectly by affecting its binding proteins. Increased IGFBP-3 mRNA expression in liver in response to increased circulating IGF-1 from adipose tissue could be a potential mediator. Increase in IGFBP-3 was the best and independent predictor of increased IGF-1 serum concentrations in our IGF-1R<sup>aP2Cre</sup> mice.

Moreover, deletion of the IGF-1 gene in liver demonstrated that liver-derived IGF-1 is not crucial for somatic growth (26), suggesting that increased somatic growth in IGF-1R<sup>aP2Cre</sup> mice is primarily due to increased adipose tissue expression. However, previous data suggest that systemic IGF-1 can feedback on hepatic IGF-1 mRNA expression in the GH-deficient state. Our IGF-1R<sup>aP2Cre</sup> mice have normal GH serum concentrations (Table 1), suggesting that under normal growth hormone levels, hepatic IGF-1 expression does not depend on the same feedback mechanism. Moreover, we can not explain, why increased IGF-1 levels did not cause decreased GH serum concentrations. Therefore, we can not exclude that reduction of IGF-1R receptor signalling in individual tissues causes a disruption of physiologic feed-back mechanisms in other systems or tissues. Adipose tissue derived signals other than circulating IGF-1, GH, and/or IGF-2 might cause increased circulating IGF-1 levels and increased IGF-1 expression in liver and adipose tissue. Further studies are necessary to characterize these signals.

Circulating IGF-1 was shown to reduce IGF-1 mRNA expression in liver, whereas GH increases IGF-1 gene expression (23). In our model with normal GH, but increased IGF-1 serum concentrations, one would expect decreased rather than the observed increased hepatic IGF-1 mRNA expression. One hypothesis is that lack of IGF-1R signaling in adipose tissue generates signals, which indirectly increase both adipose and hepatic IGF-1 expression. However, growth hormone and IGF-2 receptors expression were indistinguishable between IGF-1R<sup>aP2Cre</sup> and control mice in both liver and adipose tissue, suggesting that no liver specific compensatory mechanism accounts for the unsuppressed IGF-1 mRNA expression in liver. In parallel to increased IGF-1 serum concentrations, circulating IGFBP3, but not IGFBP1 and -2, was significantly increased in IGF-1R<sup>aP2Cre</sup> mice. Overexpression of human IGFBP3 in transgenic mice results in selective organomegaly including liver and heart (24), suggesting that increased IGFBP3 levels contribute to organomegaly in IGF-1R<sup>aP2Cre</sup> mice.

Increased circulating IGF-1 by 50% was shown to improve glucose tolerance in mice with a liver-specific overexpression of the Igf-1 gene (22). Interestingly, we did not observe any improvement of glucose tolerance and insulin sensitivity in IGF-1R<sup>aP2Cre</sup> mice, suggesting that 20% increased IGF-1 levels are not sufficient to enhance whole body insulin sensitivity. IGF-1R<sup>aP2Cre</sup> mice do not exhibit significant metabolic alterations. Interestingly, at the age of 12 weeks, when growth curves diverge, fasting blood glucose concentrations are significantly higher in IGF-1R<sup>aP2Cre</sup> than in controls. Since glucose tolerance is normal at this age, it is difficult to establish causality for elevated glucose concentrations. Higher adipose tissue mass could contribute to these metabolic alterations. We also found lower adiponectin serum concentrations in IGF-1R<sup>aP2Cre</sup> mice of both genders. Lower circulating adiponectin levels further suggest that metabolic alterations in IGF-1R<sup>aP2Cre</sup> mice at 12 weeks of age are a consequence of increased fat mass. Serum adiponectin negatively correlates with fat mass in mice (16) and men (28). Insulin and IGF-1 are capable of binding to each other’s receptor although with different affinities (29). Mice with a targeted disruption
of the insulin receptor in adipose tissue (FIRKO mice) are protected from obesity and its metabolic alterations (16). Moreover, these mice exhibit specific morphologic changes in adipose tissue, which could be due to a defect in adipose tissue development. We therefore expected alterations in adipose tissue of IGF-1R<sup>aP2Cre</sup>, since IGF-1R was shown to be important in the growth and differentiation of adipocytes in vitro (9). Despite similarities in the structure and intracellular signaling between insulin and IGF-1 receptors, we did not find a severe metabolic phenotype in mice lacking the IGF-1 receptor in adipose tissue. However, we found significantly increased insulin stimulated glucose uptake into isolated adipocytes from IGF-1R<sup>aP2Cre</sup> mice, suggesting increased glucose uptake and subsequent increase in lipogenesis as primary causes leading to increased lipid load per adipocyte and adipose tissue hypertrophy in IGF-1R<sup>aP2Cre</sup> mice.

This further suggests that IR signaling is more important for fuel metabolism, whereas IGF-1R mediates growth and that one receptor can not functionally compensate for the other receptor’s absence (13).

Our data further show that IGF-1 receptor signaling in adipocytes is not crucial for the growth, development and differentiation of adipose tissue in vivo. Unexpectedly, both the number and the size of adipocytes is increased in adipose tissue of IGF-1R<sup>aP2Cre</sup> mice, either suggesting that intact IGF-1R is not necessary for adipose tissue development or that other mechanisms exist in vivo, which can compensate for the disruption of IGF-1R. In accordance with our results, it was recently shown that most IGF receptor deficient adipocyte cell lines were capable of normal differentiation (13). Moreover, important markers of adipogenesis including PPARγ and GLUT4 were expressed despite the lack of IGF-1R (13). One potential mechanism could be that insulin receptor mediates the IGF-1 effects on growth and differentiation in vivo. This hypothesis is supported by increased insulin receptor gene expression in adipose tissue of IGF-1R<sup>aP2Cre</sup> mice. However, in vitro there was no compensation of IR when the IGF-1R was absent. Therefore, it appears that IGF-1R is not crucial for adipose tissue development. Interestingly, adipocytes from IGF-1R<sup>aP2Cre</sup> mice were more sensitive to insulin-stimulated glucose uptake than adipocytes from control mice. Our findings are in accordance with previously reported increased insulin sensitivity in IGF-1 receptor-deficient brown adipocytes (30). Moreover, downregulation of IGF-1R in breast cancer cells was shown to increase insulin sensitivity (31). Diminished IGF-1R/IR hybrid receptor formation resulting in enhanced holo-IR formation could contribute to increased insulin sensitivity in IGF-1R deficient adipocytes. In addition, increased IR number might enhance insulin effects on IGF-1R<sup>aP2Cre</sup> adipocytes.

In conclusion, IGF-1 receptor signaling in adipocytes is not crucial for the development and differentiation of adipose tissue in vivo, but seems to participate in the regulation of IGF-1 serum concentration.

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Figure 1. Targeting Strategy, Assessment of IGF-1 receptor Recombination and IGF-1 receptor expression.
(A) Schematic representation of the loxP-flanked IGF-1R allele before and after recombination (Cre-expression). The knockout allele is shown below the floxed allele, indicating the deletion of exon 3 in the event of recombination of the *igf-1r* gene. BamHI, restriction sites; triangle, loxP site; ellipse, FRT site (B) Results from PCR analysis of DNA prepared from isolated adipocytes. DNA from isolated adipocytes of WT mice produced a 300 bp band (lane 1), whereas a single 380 bp band was detected for IGF-1R^{aP2Cre} mice (lane 3). Heterozygous expression of the transgene was detected by both a 300 bp and a 380 bp band (lane 2). (C) Western blot analysis for IGF-1 receptor β subunit and β actin as loading control of white adipose tissue (WAT), liver, brain, and skeletal muscle of two representative wildtype (WT) and IGF-1R^{aP2Cre} mice.
Figure 2

A  

Males

B  

Females

C  

Body length (cm)

D  

Relative gonadal fat pad weight (%)

E  

Relative liver weight (%)

F  

Relative brain weight (%)

IGF-1 receptor deletion in adipocytes
Figure 2. Growth phenotype of IGF-1R\textsuperscript{ap2Cre} mice and controls.
IGF-1R\textsuperscript{ap2Cre} mice show identical growth until an age of 10 weeks. Thereafter, body weight of IGF-1R\textsuperscript{ap2Cre} mice is significantly increased both in males (A) and females (B) until an age of 32 weeks. (*, p<0.05, **, p<0.01, ***, p<0.001). (C) Body length (naso-anal length) is markedly increased in IGF-1R\textsuperscript{ap2Cre} mice at an age of 24 weeks (n=10 per genotype and gender). (D-F) Organ weights relative to body weight in 24 weeks old IGF-1R\textsuperscript{ap2Cre} and control mice (n=10 per genotype and gender). IGF-1R\textsuperscript{ap2Cre} mice exhibit increased relative epigonadal fat pad (D) and liver weight (E). Relative brain weight (F) was significantly reduced in IGF-1R\textsuperscript{ap2Cre} mice, possibly as a consequence of heterozygous transgene (aP2) expression in brain, whereas relative brown adipose tissue (BAT) weight (G) was indistinguishable between IGF-1R\textsuperscript{ap2Cre} and control mice.
Figure 3. IGF-1R<sup>αP2Cre</sup> mice have normal glucose tolerance and insulin sensitivity. (A, B) Glucose tolerance tests performed on 12-hour fasted 12-week old (A) and 24-week old (B) male wildtype (control) and IGF-1R<sup>αP2Cre</sup> mice. Results are expressed as mean ± SEM from 10 animals per genotype. (C, D) Insulin tolerance tests on random fed 12-week old (C) and 24-week old (D) male wildtype (control) and IGF-1R<sup>αP2Cre</sup> mice. Results are expressed as mean ± SEM from 10 animals per genotype.
Figure 4

A  Serum IGF-1 (ng/mL)

B  Serum IGF-1 (mg/L)

C  Adipose tissue

D  Liver

E  Adipose tissue

F  Liver

IGF-1 receptor deletion in adipocytes
Figure 4. IGF-1, IGFBP3 serum concentrations and IGF-1 mRNA expression in adipose tissue and liver of IGF-1R<sup>ap2Cre</sup> and WT (control) mice.

(A) IGF-1 serum concentrations are significantly higher in IGF-1R<sup>ap2Cre</sup> compared to WT mice. (**, p<0.01, n=10 per genotype and gender).

(B) Higher IGFBP3 serum concentrations in IGF-1R<sup>ap2Cre</sup> compared to WT mice. (*, p<0.05, n=5 per genotype and gender).

(C, D) IGF-1 mRNA expression in adipose tissue (C) and liver (D). (*, p<0.05, **, p<0.01; n=10 per genotype and gender).

(E, F) IGF-2R mRNA expression in adipose tissue (E) and liver (F).

(G, H) Growth hormone receptor (GHR) mRNA expression in adipose tissue (G) and liver (H).

(I) IGF-1 mRNA expression in isolated adipocytes from 24-week old male (n=5 per genotype) after 6 hours incubation with or without 100 ng/ml recombinant murine IGF-1. (*, p<0.05) Values are means +/- SEM. AU, arbitrary units. Igf-1 mRNA expression was calculated relative to the mRNA expression of 36B4.
Figure 5

A

Epigonal fat

Subcutaneous fat

Brown adipose tissue

IGF-1 receptor deletion in adipocytes
Figure 5. Hypertrophy of adipocytes in white adipose tissue and normal insulin stimulated glucose uptake into isolated adipocytes of IGF-1R<sup>aP2Cre</sup> mice.

(A) Hematoxylin and eosin staining of white (epigonadal and subcutaneous) and brown adipose tissue sections from random fed, 24-week old male IGF-1R<sup>aP2Cre</sup> and WT (control) mice. Initial magnification, 10X. (B) Distribution curves of epigonadal and subcutaneous (SC) isolated adipocytes from IGF-1R<sup>aP2Cre</sup> and WT mice (pooled from five mice per genotype). SC size distribution was indistinguishable between IGF-1R<sup>aP2Cre</sup> and control mice, whereas both mean and maximum epigonadal adipocyte size is significantly increased in IGF-1R<sup>aP2Cre</sup> mice. (C) Relative glucose uptake into isolated adipocytes from 24-week old male IGF-1R<sup>aP2Cre</sup> and WT (control) mice. Insulin stimulation was performed for 30 min at 100 nM. Data represent mean ± SEM of five independent experiments. (*, p<0.05)
Table 1. Metabolic parameters in 12- and 24-week old male and female IGF-1R<sup>aP2Cre</sup> and control mice (n = 10 per group and gender)

|                | Age | Males |        | Females |        |
|----------------|-----|-------|--------|---------|--------|
|                | weeks | Control | IGF-1R<sup>aP2Cre</sup> | Control | IGF-1R<sup>aP2Cre</sup> |
| Blood glucose, fasted |       |        |        |         |        |
| (mg/dl)        | 12   | 82 ± 18* | 111 ± 35 | 74 ± 17** | 99 ± 20 |
|                | 24   | 91 ± 22 | 106 ± 33 | 88 ± 18 | 91 ± 20 |
| Blood glucose, fed |       |        |        |         |        |
| (mg/dl)        | 12   | 149 ± 16** | 169 ± 15 | 141 ± 18 | 147 ± 16 |
|                | 24   | 144 ± 17 | 153 ± 12 | 137 ± 15 | 140 ± 15 |
| Serum insulin (ng/ml) |       |        |        |         |        |
| fasted         | 24   | 1.8 ± 1.5 | 1.5 ± 0.5 | 0.4 ± 0.2 | 0.6 ± 0.4 |
| Serum leptin (pg/ml) |       |        |        |         |        |
| fed            | 24   | 10.1 ± 4.8 | 8.2 ± 4.8 | 10.6 ± 5.4 | 12.1 ± 5.2 |
| Growth hormone (ng/ml), fed |       |        |        |         |        |
| 24             | 11.4 ± 5.2 | 10.8 ± 6.1 | 9.5 ± 4.7 | 10.2 ± 5.8 |
| IGF-2 (µg/ml), fed |       |        |        |         |        |
| 24             | 1.13 ± 0.5 | 1.08 ± 0.4 | 0.97 ± 0.6 | 1.11 ± 0.6 |
| Serum adiponectin |       |        |        |         |        |
| (ng/ml), fed   | 12   | 7.5 ± 1.6*** | 4.7 ± 0.8 | 16.1 ± 1.2*** | 8.9 ± 2.2 |
|                | 24   | 9.5 ± 4.1 | 6.4 ± 1.9 | 16.5 ± 6.8 | 12.5 ± 7.7 |
| Serum cholesterol, fed |       |        |        |         |        |
| (mmol/l)       | 24   | 2.5 ± 0.5 | 2.4 ± 0.5 | 1.9 ± 0.3 | 2.1 ± 0.2 |
| Serum triglycerides, fed |       |        |        |         |        |
| (mmol/l)       | 24   | 1.7 ± 0.7 | 1.5 ± 0.9 | 1.1 ± 0.1 | 1.8 ± 1.2 |

* Significant differences from control: (*, p< 0.05, **, p<0.01, ***, p<0.001)