Insulin-like Growth Factor II (IGF-II) in Adipocyte Regulation: Depot-Specific Actions Suggest a Potential Role Limiting Excess Visceral Adiposity

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Running head: Fat-depot regulatory role of IGF-II in children

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Abstract

The IGF system has an important role in growth and development. IGF-II is a recognized fetal growth promoter. However, its physiological post-natal role remains uncertain although it is maintained in the circulation at a substantially high level throughout life. IGF-II has been strongly linked to obesity in genetic studies and more recent evidence suggests a metabolic role. We examined fat depot differences in IGF-II’s action on differentiation and metabolism. We speculate a specific effect on visceral adipocytes in relation to the differential distribution of insulin receptors between visceral and subcutaneous fat depots. Using a previously established adipocyte, cell culture system of matched pairs of visceral and subcutaneous fat biopsies from 20 normal weight children undergoing routine surgery for non-malignant, non-septic conditions. Preadipocytes were differentiated for 14 days in the presence or absence of IGF-II. Oil Red O staining, western blotting and reverse transcription polymerase chain reaction techniques were employed to assess levels of adipogenesis markers and levels of the insulin receptor and insulin receptor isoforms. Our data indicate that IGF-II promotes preadipocyte differentiation in subcutaneous preadipocytes but showed a protective, opposing effect restricting visceral preadipocyte differentiation, confirmed by reductions in the differentiation markers PPARγ and adiponectin and in triglyceride staining. Additionally, IGF-II reduced mRNA expression of the insulin receptor in adipocytes, and downregulated IR-A and GLUT4 abundance and corresponding glucose uptake in visceral adipocytes. In conclusion, IGF-II is a regulator of preadipocyte differentiation and metabolism by acting as a differential modulator of fat accumulation favoring less visceral fat deposition in children.

Keywords: IGFs, childhood, adipocytes, visceral fat, subcutaneous fat

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1. INTRODUCTION

Childhood obesity is a global health issue with more than 42 million overweight children worldwide (WHO Health Report 2015). It is of particular concern because 25% of obese adults tend to be overweight as children, and early overweight onset is associated with greater negative adult health consequences (12). In addition, overweight children are at increased risk of developing obesity-related systemic and psychological problems (29).

Humans have evolved for an active lifestyle with significantly more intermittent feeding compared to lower mammals, whose activity is closely tied to their feeding frequency. Adipocytes are the only cells specifically designed to store sufficient energy to sustain such a lifestyle. Concurrent with this, visceral and subcutaneous adipocytes in humans have evolved with clear functional distinctions and different pathogenic significance; in contrast, such depot-specific distinctions in adipocyte function are much less marked in rodents (1). Although the majority of body fat is subcutaneous, visceral fat has attracted more attention due to its association with metabolic syndrome, type II diabetes and cardiovascular risk (7). Subcutaneous fat is less of a metabolic risk and is considered to be protective against metabolic abnormalities (42). There are multiple intrinsic characteristic differences between fat depots in humans (1, 22). The distribution of insulin receptors (IR) in mature adipocytes differs between the fat depots with a higher abundance of insulin receptors in visceral compared with subcutaneous fat. The majority of this increase is due to insulin receptor isoform A (IR-A); the insulin receptor comes in two isoforms—IR-A and IR-B—according to the presence or absence of exon 11, and a major functional difference is the high affinity of IR-A for insulin-like growth factor II (IGF-II) (6). These site-specific differences in adipose tissue structure and metabolism are important to consider in relation to the pathogenesis of obesity.

The IGF system has an established role in adipose tissue growth and metabolism. IGF-I is a potent promoter of preadipocyte growth and adipogenesis (15, 46) and fat depot differences in IGF-I responses have been reported (16). IGF-II is recognized for being an embryonic and placental growth factor, but its physiological role postnatally is still to be determined. The reason for this might be because IGF-II is not expressed postnatally in mouse models, whereas humans maintain extremely high levels of IGF-II, averaging around 700 ng/ml (19, 21, 23), with concentrations considerably higher than those of IGF-I (4). Most of the circulating IGF-II is present in a ternary complex with IGF
binding protein-3 that has limited access to the tissues and therefore tissue IGF-II concentrations, to which adipocytes are exposed, are much lower (1-10% of circulating levels) but still considerably higher than those of insulin (reference).

IGF-II expression has been strongly related to weight and adiposity; the level of IGF-II gene methylation is associated with birth weight (3) and the expression of IGF-II in utero promotes adipogenesis and fat storage during pregnancy (44). Additionally, methylation status of the IGF-II gene at birth has been linked to early childhood weight (26), and the level of IGF-II in the circulation during childhood has been closely related to fat mass (34). In adults, polymorphic genetic differences in IGF-II expression are correlated with weight gain: homozygous individuals with Apal AA have a significant increase in IGF-II levels and this has been associated with less body weight and lower risk of pathological body mass index (BMI) in comparison to those with Apal GG, who had lower levels of IGF-II (14, 32). Furthermore, IGF-II level has been proposed as a prognostic marker to predict future weight gain because lower baseline circulating levels of IGF-II are associated with a higher risk of obesity and future weight gain (39). With a limited understanding of IGF-II’s role postnatally and the lack of data on children, we aimed to study the physiological role of IGF-II by conducting a series of experiments on primary cultures of matched pairs of subcutaneous and visceral adipocytes from children, to test the hypothesis that IGF-II is an important regulator of adipocyte physiology with specific effects on visceral adipocytes. We hypothesized that the differential distribution of insulin receptor isoforms between visceral and subcutaneous adipocytes may enable IGF-II to exert distinct effects on fat cells in different fat depots.
2. MATERIALS AND METHODS

Subjects

Samples were obtained from young children admitted to a regional children’s hospital for elective surgery. The study was approved by the NRES Committee South West – Exeter (REC reference: 14/SW/0109). An invitation to the study and a written information sheet were sent by post to all potential subjects along with the surgery admission letter. Parents/legal guardians were approached with a verbal explanation of the study on the morning of admission for surgery, and consent was obtained from the families of 20 participants. Children recruited were of normal weight and were admitted to the Bristol Royal Hospital for Children for routine renal surgery (non-malignant, non-septic operations). Fat tissue samples were collected by specialist pediatric surgeons during the operation. Subcutaneous and intra-abdominal peri-nephric (visceral) fat biopsies (0.2–0.5g) were collected and transferred immediately to the laboratory. Clinical data for the participants are shown in Table 1.

Reagents

All reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless stated otherwise. Recombinant, human IGF-II peptide was purchased from Gropep (Adelaide, South Australia, Australia). Dulbecco’s Modified Eagle Medium/Ham’s F-12 (DMEM/Ham’s F-12), fetal bovine serum (FBS), and Hank’s balanced salt solution (HBSS) were obtained from Gibco (Paisley, UK), penicillin/streptomycin and L-glutamine from Lonza (Berkshire, UK), fungizone from Fisher Scientific (Paisley, UK), and insulin from Novo Nordisk (West Sussex, UK). Tissue culture plastic materials were purchased from Greiner Labortechnik Ltd (Tyne and Wear, UK) and phosphate buffered saline (PBS) was acquired from Oxoid (Basingstoke, UK). Nitrocellulose and enhanced chemiluminescence (ECL) western blotting reagents and 2-deoxy-[^3H]d-glucose (2DG) were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK), and the bicinchoninic acid assay (BCA) protein assay™ and SuperSignal West-Dura chemiluminescence reagents were obtained from Pierce (Rockford, IL, USA).

Isolation, culture and differentiation of adipocyte precursor cells

The techniques of preadipocyte isolation, culture, differentiation, and characterization have been described previously (16). In brief, small sections (0.2–0.5g) of adipose tissue collected during surgery under sterile conditions were transported immediately to the laboratory. The tissue was
washed three times in 10ml Hank's balanced salt solution (HBSS) and then cut into 1mm³ pieces and digested with 10ml of type II collagenase (1mg/ml) in HBSS for 60 min at 37°C in a shaking water bath (150 cycles/min). Adipocytes were separated from the stromal-vascular cells by centrifugation at 80g for three minutes. The pellet of sedimented stromal-vascular cells, including fibroblasts, endothelial cells, preadipocytes etc (but excluding mature adipocytes) was suspended in preadipocyte growth media (DMEM/Ham's F12) medium (v/v 1:1) supplemented with 20% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and seeded into T75 flasks coated with 0.2% gelatin; these were maintained at 37°C in a humidified atmosphere of 5% CO2. The medium was changed every 72 hours until cells became confluent. For differentiation, preadipocytes obtained from the visceral and subcutaneous biopsies were cultured in gelatin-coated 6-well plates at a seeding density of 0.2 X10⁶ and after 16 hours, they were induced to differentiate using a technique established previously (16). In brief, preadipocytes were washed with PBS twice and cultured for 14 days with a chemically defined medium (DMEM/Ham's F12) (1:1, v/v) supplemented with 15 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mmol/l sodium bicarbonate (NaHCO₃), 10 g/ml apotransferrin, 33 mol/l biotin,100 nmol/l insulin (for the first ten days only), 0.2 nmol/l 3,3,5-triiodothyronine, 17 mol/l pantothenate, and, for the initial three days of culture, 10 M rosiglitazone and 25 mol/l 3-isomethyl-l-methylxanthine in the absence or presence of a continuous exposure to 7.5 ng/ml or 62.5 ng/ml IGF-II. To maintain consistency, experiments were only performed using cell passages 3–5.

**Western blotting**

Protein cell lysates (50 μg), as estimated by BCA protein assay (Thermo Fisher Scientific, 23225), were separated using sodium dodecyl-polyacrylamide electrophoresis (SDS-PAGE) 10% gels. Following transfer to nitrocellulose membranes (Amersham, RPN119B), non-specific binding sites were blocked using 5% non-fat dried milk for insulin receptor β subunit (IRβ); glucose transporter 4 (GLUT4), glyceraldehyde-3P-dehydrogenase (GAPDH), and β-actin or 5% bovine serum albumin for peroxisome proliferator-activated receptor gamma (PPARγ); and adiponectin or 3% bovine serum albumin for fatty acid synthase (FASN) in tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7.8, 150 mM NaCl) 0.1% Tween 20 (TBS-T) for one hour at room temperature before overnight probing (4°C) with the following primary antibodies: adiponectin (1:500 abcam ab22554), PPARγ (1:1000 E-8: sc-7273 Santa Cruz Biotechnology), anti-insulin receptor β subunit (1:1000 C-19: sc-711 Santa Cruz
Biotechnology), GLUT4 (1:1000 abcam ab654), FASN (1:5000 Biosciences 610963), GAPDH (1:5000 Millipore MAB 374), and β-actin (1:10,000 Sigma-Aldrich A5441). After washing, membranes were incubated for one hour at room temperature with horseradish-peroxidase conjugated secondary antibodies: anti-mouse for PPARγ (1:2000); anti-rabbit (1:2000) for adiponectin; IRβ, anti-goat antibody, for GLUT4 (1:2000); and anti-mouse antibody (1:2000) for FASN and (1:5000) for β-actin or GAPDH. Proteins were visualized using enhanced chemiluminescence and detected using the ChemiDoc XRS+ System and Image Lab Software (BioRad, 170-8265). Quantification of western immunoblots was performed using Image J 1.46r software.

### Quantitative RT PCR

Cells were seeded (0.2×10⁶) in 6-well plates and at day 14 of differentiation, total RNA was extracted using TRIzol reagent (Invitrogen). Two micrograms of total RNA were used for cDNA synthesis with Green JumpStart SYBR (Sigma, H5041). Real-time PCR was carried out using StepOne plus the Realtime PCR (qPCR) System (Applied Biosystems, 4376600). qPCR reactions were run in duplicate in three independent experiments and to control the variability in expression, data were normalized to the geometric mean of a housekeeping gene (GAPDH) and were analyzed using the 2⁻ΔΔCT method. PCR primers were designed using OligoPerfect online software from Qiagen under consideration of the special design criteria for real-time RT-PCR primers, spanning the junction between exons. Primers were purchased from Thermo Scientific and primer sequences were as follows:

- **PPARγ FOR**: 5’GGTGGCCATCCGCTCT3’ REV: 5’TGCTTTTGGCATACTCTGTGATCT3’
- **Adiponectin FOR**: 5’TCAGCATTCAGTGTGGGATTG3’ REV: 5’GGTAAAGCGAATGGGCATGTG3’
- **IR FOR**: 5’TGACAACGACCAGTGTGGAG3’ REV: 5’GCAGCCGTGTGACTTACAGA3’
- **IR-A FOR**: 5’ TTCGGCCGCGGAATGCTG3’and REV: 5’CCGAGTGGCCTGGGGACGA3’
- **IR-B FOR**: 5’AAAACCTCTTCAGGCACTGG3’ REV: 5’GAGGAAGTGTTGGGGAAAGC3’
- **GAPDH FOR**: 5’GATCATCAGCAATGCCTCCT3’ REV: 5’TGTGGTCAATGCTTCCA3’

### Oil Red O triglyceride staining

To evaluate the level of preadipocyte differentiation, cells were stained using Oil Red O (ORO). Stock was made up by dissolving 0.25 g Oil Red O stain powder (Sigma O0625) in 50ml of isopropanol and a working solution of ORO was made by adding 10ml of ORO stock solution to 6.67ml of distilled water. Fully differentiated cells were washed twice in PBS before being fixed in 10% formalin for 10
minutes and then stained with ORO for 10 minutes. Following stain removal, cells were washed with 60% isopropanol to eliminate any excess stain. The cells were then washed with distilled water and viewed under light microscopy. Following image capture, the level of staining was quantified by leaching the stain with 100% isopropanol (1ml/well) followed by spectrophotometry (FLUOstar OPTIMA, BMG LABTECH) at 490nm.

Glucose uptake assay

Differentiated adipocytes at day 14 were washed with PBS twice, and serum-starved for five hours in serum-free media before incubation with 900µl glucose-free Krebs-Ringer phosphate (KRP)/well at 37°C for 15 minutes. Following this incubation, cells were transferred to a water bath, also at 37°C, and stimulated with 60 ng/ml insulin or IGF-II per well for 15 minutes. After stimulation, 100µl radiolabeled glucose solution (2DG) in KRP buffer was added to each well for ten minutes. Glucose transport was terminated by transferring the dishes to ice, removing the KRP buffer, and washing the cells gently three times with ice cold PBS before disruption with 1% Triton X-100 PBS. The cell-Triton X solution was then added to 10ml of ultima gold scintillation fluid (Perkin Elmer, Bucks, UK); the cell-associated radioactivity was counted with a liquid scintillation counter. Basal glucose uptake was measured in the absence of insulin or IGF-II.

Statistical analysis

SPSS 12.0.1 for Windows using one-way ANOVA was used to analyze data, followed by least significant difference (LSD) post-hoc test, with a significant statistical difference at p<0.05.
3. RESULTS

Characterization of subcutaneous and visceral preadipocyte differentiation

Paired biopsies from subcutaneous and visceral fat were prepared as described in the Methods section (16). Figure 1A shows representative phase contrast micrographs of subcutaneous and visceral preadipocytes on day 0 that display a fibroblastic morphology and differentiated adipocytes on day 14 stained with Oil Red O stain for triglycerides. Figure 1B represents the corresponding spectrophotometric absorbance analysis of Oil Red O stain that shows a significant increase in fat deposition with differentiation; the staining was undetectable for subcutaneous and visceral preadipocytes but there was an 18.5-fold increase for subcutaneous adipocytes (p<0.001) and a 16.5-fold increase for visceral adipocytes (p<0.001) in Oil Red O staining absorbance vs. corresponding preadipocytes. To further confirm successful differentiation, protein levels of two differentiation markers (PPARγ and adiponectin), which are known to be increased during adipogenesis (13), were analyzed using western blotting (Figure 1C). A significant increase in protein abundance of both differentiation markers, PPARγ (p<0.01) and adiponectin (p<0.001), was seen in adipocytes from both cell types vs. corresponding preadipocytes indicating differentiation (Figure 1D). A similar increase was seen for mRNA expression of these markers using qPCR; PPARγ expression significantly increased for subcutaneous and visceral adipocytes in comparison to preadipocytes (p<0.001); the adiponectin marker also showed a significant increase in both adipocytes compared with preadipocytes (p<0.001) (Figure 1E). A difference in adiponectin and PPARγ mRNA expression in visceral adipocytes in comparison to subcutaneous adipocytes was seen at day 14 of differentiation.

Of the stromal-vascular cell fraction isolated approximately 20-30% of the cells differentiated into mature adipocytes (presumably reflecting the proportion of preadipocytes in the crude mixed-cell fraction) and there were no differences in this between subcutaneous and visceral cultures as assessed by fat deposition or protein markers of differentiation (figure 1).

IGF-II promoted differentiation of subcutaneous but not visceral preadipocytes

After characterization of preadipocyte differentiation, we studied the role of IGF-II in preadipocyte differentiation and adipogenesis. We examined the effect of IGF-II treatment at a low (7.5 ng/ml) and
high (62.5 ng/ml) dose following a preliminary dose-response assessing IGF-II induced preadipocyte proliferation (data not shown).

Visceral and subcutaneous preadipocytes were differentiated for 14 days with normal glucose (5mM/l) differentiation media, or differentiation media supplemented with the IGF-II concentrations as described above.

IGF-II (62.5 ng/ml) treatment enhanced fat deposition in subcutaneous fat in comparison to the control; this was observed under the microscope (Figure 2A) and by Oil Red O staining, as the absorbance analysis showed a significant increase in comparison to the control (p<0.01) (Figure 2B). In addition, the protein abundance of the differentiation markers PPARγ and adiponectin showed a significant increase in relative fold change in comparison to the control (1.86 vs. 1.0 for adiponectin; p<0.05) and (3.8 vs. 1.0 for PPARγ; p<0.01). This IGF-II induced increase in PPARγ in subcutaneous adipocytes was mainly due to an increase in PPARγ2 protein (Figure 2C–2E). Interestingly, in terms of visceral preadipocyte differentiation, IGF-II reduced the amount of preadipocyte differentiation as seen under the microscope (Figure 2A). Oil Red O absorbance analysis showed a reduction in triglycerides with 62.5 ng/ml IGF-II treatment (p<0.05) (Figure 2B). A decrease in the relative fold change of protein abundance of the differentiation markers in comparison to the control was also observed for adiponectin (0.4 vs. 0.9; p<0.05) and PPARγ (0.3 vs. 1.2; p<0.05) (Figure 2C–2E).

Differentiation with IGF-II decreased fat deposition in visceral preadipocytes: effect on the abundance of the insulin receptor, GLUT4, and fatty acid synthase (FASN)

To further confirm that IGF-II reduced differentiation and fat deposition in visceral preadipocytes whilst enhancing it in subcutaneous preadipocytes, we examined the protein abundance of the insulin receptor, GLUT4, and FASN using western blotting after 14 days of differentiation of paired subcutaneous and visceral preadipocytes with IGF-II in two doses (7.5 ng/ml and 62.5 ng/ml). With visceral adipocytes, there was a significant IGF-II-induced reduction in insulin receptor abundance (Figure 3A, 3B), in comparison to the control; the relative fold change reduction was significant for both doses, 7.5 ng/ml (1.0 vs. 1.4; p<0.05) and 62.5 ng/ml (0.9 vs.1.4; p<0.01). A consistent decrease in GLUT4 protein abundance (Figure 3A, 3C) was also seen in visceral adipocytes when differentiated with IGF-II at 7.5 ng/ml (0.8 vs.1.4; p<0.01) and at 62.5 ng/ml (0.7 vs. 1.4; p<0.01). FASN was also
reduced in visceral adipocytes (Figure 3A, 3D) for 7.5 ng/ml IGF-II (0.9 vs. 1.8; p<0.001) and for 62.5 ng/ml IGF-II (0.5 vs. 1.8; p<0.001). This was reflected in a 16% reduction in insulin-stimulated radioactive 2DG glucose uptake into visceral adipocytes in comparison to the control (p<0.01) (Figure 3E) following IGF-II (62.5ng/ml) treatment. In contrast, for the subcutaneous differentiated adipocytes, IGF-II enhanced insulin receptor abundance and the relative fold change in comparison to the control was 2.2 vs. 1.0 (p<0.001) for 7.5ng/ml IGF-II and 3.1 vs. 1.0 (p<0.001) for 62.5 ng/ml IGF-II. When contrasting fat depots, there was a significant difference in insulin receptor protein abundance, with IGF-II (62.5 ng/ml) increasing insulin receptor abundance 2.2-fold in subcutaneous adipocytes in comparison to visceral adipocytes (p<0.001) (Figure 3B). GLUT4 relative protein abundance increased in subcutaneous adipocytes with IGF-II (62.5 ng/ml) (1.5 vs. 1.0; p<0.05) in comparison to the control (Figure 3C). Similarly, FASN protein abundance was enhanced in subcutaneous adipocytes with both doses of IGF-II: 7.5ng/ml (2.7 vs. 1.0; p<0.001) and 62.5ng/ml (4.5 vs. 1.0; p<0.001) (Figure 3D). Insulin-stimulated glucose uptake was significantly increased using the higher dose of IGF-II (p<0.01) (Figure 3E), which further indicates a depot-specific action of IGF-II.

IGF-II treatment for 24 hours down-regulated total insulin receptor, insulin receptor isoform A and GLUT4 mRNA expression and glucose uptake in visceral adipocytes

After investigating the role of IGF-II in differentiation, we examined the acute effect of IGF-II on mature differentiated adipocytes, these cells being differentiated with normal glucose (5mM/L) differentiation media and collected on day 14. These adipocytes were serum-starved for 24 hours followed by 24 hours' treatment with IGF-II (62.5 ng/ml). IGF-II treatment down-regulated the mRNA expression of total insulin receptor levels in visceral adipocytes; the expression was normalized to GAPDH reference gene and relative fold change vs. control was 16.5 vs. 42.07 (p<0.01). Furthermore, there was a reduction in total levels of the insulin receptor mRNA in subcutaneous adipocytes (15.9 vs. 1.0; p<0.05) (Figure 4A). There were no significant changes in IGF-IR mRNA expression after IGF-II treatment for subcutaneous and visceral adipocytes (Figure 4B). When looking specifically at the insulin receptor isoforms, we initially characterized the insulin receptor isoform distribution in preadipocytes (Figure 4C) and adipocytes (Figure 4D): visceral preadipocytes show a predominance of IR-A /IR-B (28.2 vs. 1.5; p<0.001), whereas subcutaneous preadipocytes have higher IR-B in comparison to IR-A (8.9 vs. 1.0; p<0.05). With differentiation, the IR-B ratio has
increased significantly in both fat depots. The relatively high expression of IR-A in visceral compared to subcutaneous preadipocytes was maintained with differentiation to mature adipocytes.

With IGF-II treatment, there was an IGF-II induced down-regulation of insulin receptor isoform B in both visceral and subcutaneous adipocytes vs. control (visceral 5.5 vs. 18.7; p<0.05; subcutaneous 2.1 vs. 14.0; p<0.01) (Figure 4E, 4F). However, insulin receptor isoform A was only significantly down-regulated in visceral adipocytes (1.4 vs. 12.8; p<0.05), consistent with the maintenance of IR-A and maintained sensitivity to IGF-II. IGF-II treatment down-regulated GLUT4 mRNA expression in visceral adipocytes with a fold change in comparison to the control (0.66 vs. 8.13; p<0.001) (Figure 5A); this was also reflected in IGF-II induced reduction in visceral GLUT4 protein abundance (0.4 vs. 0.8; p<0.05) (Figure 5B, 5C). The down-regulation of GLUT4 was associated with a 33.8% reduction in 2DG glucose uptake (1580.5 DPM vs. 2388.8 DPM; p<0.05) (Figure 5D). There were no significant changes in GLUT4 or glucose uptake with IGF-II treatment in subcutaneous adipocytes.
4. DISCUSSION

The IGF system has recognized effects on adipose tissue. IGF-I promotes preadipocyte proliferation and differentiation, as seen in the 3T3-L1 cell line (21) and in primary cultures (4) and, as we previously reported, in primary cultures obtained from children (16). However, IGF-II’s role has been poorly investigated despite its abundance in the human circulation and its predominant local production from adipose tissue, where it exceeds that of IGF-I (17).

A potential role of IGF-II in adipose tissue regulation has emerged because of its association with weight, obesity, and a number of cancers (9). With the increasing prevalence of obesity overall and in childhood, and the association of IGF-II genetic variations with body weight and obesity (8, 32, 38), further understating of IGF-II’s role in adipose tissue is needed.

Because visceral fat accumulation is strongly related to metabolic risk, we investigated IGF-II’s actions using paired visceral and subcutaneous fat biopsies, our data suggesting that IGF-II has depot-specific actions in terms of promoting preadipocyte differentiation. IGF-II treatment with physiological concentrations enhances subcutaneous preadipocyte growth to mature adipocytes and fat deposition but, in contrast, has a restricting effect on visceral preadipocyte maturation. This effect was seen with an increase in the differentiation markers PPARγ and adiponectin in subcutaneous preadipocytes. Interestingly, IGF-II specifically increased PPARγ2 expression, one of the two alternatively spliced forms of PPARγ, indicating that IGF-II alters the splicing preferences of another key adipocyte protein. This may be important as although PPARγ1 and PPARγ2 are involved in adipogenesis in vitro, PPARγ2 is more related to the nutritional status and maintenance of insulin sensitivity (25). It would be interesting to examine whether proteins involved in alternative splicing are differentially regulated in subcutaneous and visceral adipocytes. Differentiation with IGF-II also increased insulin receptor, GLUT4, FASN, and insulin-stimulated glucose uptake in subcutaneous preadipocytes with an opposing effect on visceral preadipocytes.

IGF-II stimulated differentiation of adipose tissue isolated from eyelids in humans in vitro (24), but there is limited literature pointing to any fat depot-specific effects. However, in mammals, similar findings on fat depot differences due to the actions of IGF-II have been reported in fetal baboons, with higher lipid deposition being seen in subcutaneous fat in comparison to visceral fat (41). The depot-
specific pattern of action of IGF-II has been described in human genetic studies, with higher methylation of IGF2/H19 imprinting control region (ICR) in young adults at the age of 17 being associated with higher IGF-II expression and an increase in subcutaneous fat but not increased waist circumference or visceral fat accumulation (20). Furthermore, analysis of the East Hertfordshire cohort study reported that individual variance in the genetic region IGF2-INS-TH is related to body weight. Individuals with an IGF2-INS-TH *5 haplotype, which involves the IGF2 Apal A allele together with allele 9 of TH01 and a subset of class I alleles of INS VNTR as a gene cluster, and who are known to have higher IGF-II levels (32), had lower associated waist circumference, hip-to-waist ratio, and BMI in comparison to non *5 haplotype individuals (36, 37). Controversially, other studies have suggested that increased IGF-II levels are positively associated with central adiposity (28) and overall weight gain (38). However, ethnic differences may be important in IGF-II weight-related effects (10, 35).

We considered that receptor distribution differences might contribute to these IGF-II depot-specific actions (27). IGF-IR abundance decreases with preadipocyte differentiation (47), while insulin receptor abundance increases and visceral fat has higher insulin receptor abundance than subcutaneous fat (27). Insulin receptor isoform distribution—which can markedly alter tissue-specific biological responses to IGF-II—shows a higher distribution of IR-B in tissues primarily responsive to insulin action, such as adipocytes, muscle, and liver, whilst IR-A is more abundant in fetal and some cancer cells where mitogenic responses are important (11, 31), consistent with the reported mainly mitogenic signaling activated by IR-A. Our data indicate that IR-A is the predominant isoform in visceral preadipocytes, whilst IR-B predominates in subcutaneous preadipocytes. Although the IR-B/IR-A ratio increased with differentiation, IR-A remains significantly higher in visceral adipocytes in comparison to subcutaneous adipocytes, rendering them potentially more IGF-II responsive.

Additionally, we investigated how acute IGF-II treatment affected mRNA expression of the IGF-IR, insulin receptor and its isoforms in adipocytes. As predicted, our results indicate that IGF-II exposure has a minimal effect on the IGF-IR in adipocytes. However, IGF-II caused down-regulation of total insulin receptor mRNA levels, particularly with respect to IR-A in visceral adipocytes. This down-regulation was associated with a lowering of GLUT4 (insulin sensitive glucose transporter) and glucose uptake into the cell. The reason that IGF-II actions were more profound in visceral adipocytes...
may be the higher ratio of IR-A in visceral adipocytes in comparison to subcutaneous fat, the higher affinity for IGF-II binding to the IR-A (6), and the fact that IR-A has a higher internalization rate than IR-B (43). It has previously been reported that IGF-II can activate the insulin receptor in human adipocytes at physiological levels (2) and that the ability of IGF-II to modulate glucose uptake is mediated through insulin receptors (40).

The ability of IGF-II to regulate IR-A expression in visceral fat might be of clinical importance, because insulin resistance has been linked to higher mRNA expression of insulin receptor isoform A (33). Furthermore, IGF-II has been reported to have an anti-inflammatory effect by reducing TNF-α, associated with obesity and insulin resistance (30). However, the relationship between obesity and IGF-II is far from clear (5). Although a high fat diet has been reported to cause down-regulation of IGF-II expression from adipocytes and up-regulation of IGF-IIR expression, leading to a decrease in tissue availability with obesity (30), another conflicting study has indicated that IGF-II secretion and bioavailability are increased in obese individuals, particularly from visceral fat (18). However, whether this obesity-related increase in IGF-II is a compensatory mechanism associated with obesity or a cause of obesity requires further investigation and understanding.

Overall, our results indicate that IGF-II might have a role as a physiological regulator of preadipocyte growth and metabolism and may play a protective role in regulating body fat composition. Interestingly, IGF-II’s actions are not only limited to fat, with IGF-II also previously reported to be a regulator of muscle mass and skeletal muscle cell differentiation (45). IGF-II could also restrain metabolic risk by favoring muscle formation over that of fat. In conclusion, our data suggest a novel role for IGF-II in adipocyte regulation in childhood because it acts in a depot-specific manner to buffer excess visceral fat accumulation.
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6. REFERENCES


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Abbreviations: BMI: body mass index; SDS: standard deviation score.
Figure 1. Characterization of subcutaneous and visceral preadipocyte fat biopsies from prepubertal children. (A) Photomicrographs of human subcutaneous and visceral preadipocytes (day 0) displaying a fibroblastic morphology and differentiated adipocytes (day 14) stained with Oil Red O. Magnification at (X10). (B) Quantitative absorbance analysis of Oil Red O staining showing a significant increase in fat deposition in mature adipocytes in comparison with preadipocytes. (C) Western blotting of the differentiation markers (PPAR-γ, adiponectin) in subcutaneous and visceral preadipocytes and in differentiated adipocytes (day 14). β-actin was used as a loading control. (D) Quantitative densitometry analysis of western blot indicating an increase in differentiation marker expression. (E) Relative mRNA expression of differentiation markers using qPCR, indicating an increase in differentiation. GAPDH used as reference gene. Data expressed as the mean ± SEM of duplicate runs for absorbance analysis and qPCR; each western blot densitometry is representative of experiments performed in triplicate from four individual biopsies (n=4). Statistical analysis performed using one-way ANOVA (* p<0.05, ** p<0.01 ***p<0.001).

Figure 2. IGF-II promoted differentiation of subcutaneous but not visceral preadipocytes. Subcutaneous and visceral preadipocytes were differentiated for 14 days in the presence or absence of IGF-II at 7.5 ng/ml or 62.5 ng/ml. (A) Micrograph of differentiated preadipocytes stained in Oil Red O at day 14 of differentiation. Magnification at (X10). (B) Quantitative Oil Red O staining absorbance analysis of (A) showing a reduction in Oil Red O absorbance in visceral adipocytes (p<0.05) and an increase in absorbance in subcutaneous differentiated adipocytes (p<0.01) with IGF-II treatment (62.5 ng/ml). (C) Western blot of the differentiation markers PPARγ and adiponectin. Densitometry of (D) adiponectin and (E) PPARγ western blot showing protein abundance after normalization to the reference protein β-actin. Data expressed as mean ± SEM of duplicate runs for absorbance analysis; each western blot densitometry is representative of experiments performed in triplicate from three individual biopsies (n=3). Statistical analysis performed using one-way ANOVA on day 14 of differentiation (* p<0.05, ** p<0.01).

Figure 3. IGF-II treatment reduced fat deposition in visceral adipocytes after 14 days of differentiation. (A) Western blotting of paired subcutaneous and visceral preadipocytes differentiated for 14 days with IGF-II (7.5 ng/ml or 62.5 ng/ml) showing insulin receptor, GLUT4 and FASN protein
abundance. Densitometry analysis of (B) insulin receptor protein abundance (C) GLUT4 and (D) FASN. β-actin was used to ensure equal loading of samples. Densitometry represents the mean ± SEM of three experimental repeats from three individual biopsies (n=3). (E) Insulin stimulated H3 - 2deoxy glucose uptake for visceral and subcutaneous adipocytes following differentiation with IGF-II (n=3). Statistical analysis performed using one-way ANOVA on day 14 of differentiation (* p<0.05, ** p<0.01, ***p<0.001).

Figure 4. Effect of IGF-II treatment on receptor expression in differentiated adipocytes. (A) Relative mRNA expression of the insulin receptor (B) IGF-IR following 24 hours IGF-II (62.5 ng/ml) treatment was performed using qPCR showing down-regulation of the IR in visceral adipocytes. Insulin receptor isoform distribution in (C) preadipocytes and (D) adipocytes. (E) relative mRNA expression of the insulin receptor isoforms IR-A and IR-B in (E) subcutaneous and (F) visceral adipocytes showing down-regulation of IR-A and IR-B with IGF-II treatment in visceral adipocytes and IR-B expression in subcutaneous adipocytes. In all experiments, expression was normalized to the reference gene GAPDH; data represented as the mean ± SEM of experiments performed in duplicate runs from individual biopsies (n=6-4). Statistical analysis performed using one-way ANOVA (* p<0.05, ** p<0.01).

Figure 5. Effect of IGF-II treatment on glucose transporter 4 and glucose uptake in differentiated adipocytes. (A) Relative mRNA expression levels of GLUT4 in subcutaneous and visceral adipocytes measured by qPCR and normalized to GAPDH reference gene. (B) Western blot showing GLUT4 protein abundance; GAPDH used a loading control. (C) Densitometry analysis of (B) indicating a reduction in GLUT4 protein abundance in visceral adipocytes. (D) [3H]2-Deoxy glucose uptake following IGF-II treatment in visceral and subcutaneous adipocytes. Data expressed as the mean ± SEM of duplicate runs for qPCR; each western blot densitometry is representative of experiments performed in triplicate from three individual biopsies (n=3). Statistical analysis performed using one-way ANOVA (* p<0.05, ** p<0.01 ***p<0.001).