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Changes in insulin and IGF-I receptor expression during differentiation of human preadipocytes

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Short title: Insulin and IGF-I receptors in preadipocytes

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Abstract

Mature adipocytes originate from fibroblast-like precursor cells, preadipocytes, which differentiate to obtain the characteristics of adipocytes. Our aim was to investigate how differentiation of human preadipocytes affects the distribution of insulin receptors (IR) and IGF-I receptors (IGF-IR) and other cell characteristics. Preadipocytes were differentiated using indomethacine, dexamethasone, isobutyl-methylxantine (IBMX) and high concentration of insulin. Gene expression was quantified by real-time RT-PCR in preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD). The amount of expressed receptor protein was analyzed using receptor specific ELISAs and Western blot. We also studied DNA synthesis with radiolabeled thymidine incorporation and glucose accumulation with radiolabeled glucose. Differentiation of PA increased gene expression of IR but not IGF-IR. GLUT4, growth hormone receptor (GHR) and adiponectin appeared or increased. In PA and dPA only IR-A was expressed whereas also IR-B was detected in mAD. By Western blot and ELISA, IR and IGF-IR were detected in PA, dPA and mAD. During differentiation the ratio of IR to IGF-IR increased severalfold. In PA both the IR and the IGF-IR was phosphorylated by their own ligand at 1 nM and in dPA the activation of both receptors was stimulated by IGF-I, but not insulin, at 1 nM. Accumulation of glucose in PA was increased by insulin at 10 nM and by IGF-I at 1 nM and 10 nM. DNA synthesis was increased by insulin and IGF-I at 10 nM.

In conclusion, both IR and IGF-IR are present in human preadipocytes and adipocytes.

Differentiation is characterized by an increased IR/IGF-IR ratio.

Introduction

Recruitment of new fat cells is an important process when excess food is stored as fat. The adipocytes originate from mesenchymal stem cells which undergo determination to preadipocytes and finally differentiate into mature adipocytes (1). During differentiation the expression of a number of genes is altered. Several studies of both animal and human cells have shown that adipocyte specific genes, as adiponectin and leptin, and genes characteristic for mature adipocytes, as the glucose transporter protein GLUT4 and growth hormone receptor (GHR), are activated or enhanced (2-7). In addition, previous studies of mouse preadipocyte cells show changes in the expressions of insulin receptors (IR) and insulin-like growth factor I receptors (IGF-IR) (8).

The IR and the IGF-IR are closely related sharing a homology of more than 50% (9). They are both covalent dimers consisting of two extracellular α -subunits and two transmembrane β -subunits containing tyrosine kinase domains. Although the two receptors are very similar they have distinct biological effects, as reviewed by Blakesley and colleagues (10). IGF-IR signaling primarily leads to mitogenic effects while IR activation mostly leads to metabolic effects. The mechanisms for the differential signaling are not yet known, but could depend on tissue distribution of the IR and IGF-IR, internalization rate and structural differences. There are also results that indicate differences in tyrosine phosphorylation of receptors (11) and activation of IRS-1 and IRS-2 (12).

IR occur in two different isoforms due to alternative splicing of exon 11 on the IR gene. The isoform lacking the 12 amino acids encoded by exon 11 (IR-A) has been shown to have higher affinity for IGF-II than the receptor containing these amino acids (IR-B) (13). The binding affinity of insulin for IR-A is significantly higher than for IR-B (14). The isoforms display different kinase activity and are localized to different regions of the plasma membrane (15). There are also differences in sensitivity for both the metabolic and mitogenic actions of

insulin. The expression of the two isoforms varies according to type of cells and cell differentiation (13, 14, 16, 17).

Hybrid receptors consisting of one $\alpha\beta$ -subunit from the IGF-IR and one $\alpha\beta$ -subunit from the IR occur and functions mainly as an IGF-IR with approximately 20-fold higher affinity for IGF-I than for insulin (18). Insulin/IGF-I hybrid receptors have been found in several different human tissues, as muscle, fat, kidney (19) and human cell types, including different breast cancer cells (20) and endothelial cells (21, 22).

Most studies on differentiation of preadipocytes are done in animal models and animal cell lines which may not be representative for human cells. There are results indicating species differences, both in IR and IGF-IR distribution (19) and signaling (23). The aim of this study was to characterize changes in IR and IGF-IR abundance and function during human preadipocyte differentiation. Experiments were performed on cultured preadipocytes (PA), on cultured differentiated preadipocytes (dPA) and, for comparison, on freshly isolated mature adipocytes (mAD).

Materials and Methods

Cell culture

Cell culture flasks and plates were purchased from Corning (Schiphol-Rijk, The Netherlands) and all other cell culture materials were purchased from Invitrogen (Paisley, UK) or as indicated in the text.

Human preadipocytes were provided by Professor Gunnar Kratz, Department of Clinical and Experimental Medicine, Linköping University. The cells came from subcutaneous adipose tissue from patients undergoing routine reduction abdominoplasty or reduction mammoplasty (24). The patients had no known diseases and were not on any medication. Mature adipocytes were obtained from subcutaneous adipose tissue from elective abdominal surgery on female

patients, age 30-80 years and a BMI of 24-45 kg/m². Procedures were approved by the local ethics committee.

Preadipocytes were isolated from biopsies which were washed and incubated with collagenase type 1 and the cell suspension was then centrifuged and the pellet was resuspended in cell culture medium and grown in 75 cm² cell culture flasks (24). Adipocytes were isolated by digestion with 300 U/ml collagenase type I (Sigma-Aldrich Sweden AB, Stockholm, Sweden) in a Krebs-Ringer solution (0.12 M NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ and 2.5 mM CaCl) with addition of 25 mM HEPES (Sigma), 2 mM glucose, 200 nM adenosine and 3.5% fatty acid-free BSA. Digested tissue was filtered through gauze to remove connective tissue and cells were washed with Krebs-Ringer with addition of 25 mM HEPES, 2 mM glucose, 200 nM adenosine and 1% fatty acid-free BSA. Isolated cells were incubated overnight in DMEM with addition of 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 µg/ml Fungizone® and 1% fatty acid-free bovine serum albumin. The culture medium used for preadipocyte culture was a mixture of equal amounts of DMEM and Ham's F-12 with 10% newborn calf serum (NCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml Fungizone®. Medium was changed 2-3 times a week and cells were passaged using trypsin-EDTA when near confluent.

To induce differentiation we used DMEM with addition of 10% foetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 µg/ml Fungizone®, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxantine (IBMX), 200 µM indomethacin and 10 µM insulin. Medium was changed 2-3 times a week. Cells were considered differentiated when lipid droplets could be seen with a microscope, after approximately 3-4 weeks.

Real-time RT-PCR

RNA was extracted using E.Z.N.A.[™] Total RNA kit (Omega Bio-tek, Doraville, USA) according to the manufacturer's instructions. Cells were lysed directly in the flasks and RNA samples were stored in -70 °C until further use. 1 µg of RNA was transcribed to cDNA using Superscript II Reverse Transcriptase, purchased from Invitrogen. cDNA was stored at -20°C until use.

Comparative quantitative real-time PCR was performed using primers and probes for IR, IGF-IR, IR isoforms A and B, growth hormone receptor (GHR), GLUT4 and adiponectin, purchased from Scandinavian Gene Synthesis (Köping, Sweden), see Table 1. 20-100 ng cDNA was mixed with 300 nM of sense and antisense primers, 50 nM probe, 12.5 µl 2x TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden) and water to a final volume of 25 µl. DNA amplification was run and measured in an ABI Prism 7500 Fast Sequence Detector System (Applied Biosystems). Data was analyzed using a standard curve method described by Applied Biosystems Bulletin #2.

IGF-IR ELISA

The amount of IGF-IR protein was measured using an enzyme-linked immunosorbent assay (ELISA). Lysate was prepared from near confluent preadipocytes, differentiated preadipocytes or freshly isolated mature adipocytes. The cells were washed twice with cold PBS and then collected in cold PBS. The suspension was centrifuged at 4°C, 700 g for 5 minutes. The pellet was dissolved in lysis buffer (pH 7.5) containing 20 mM TrizmaBase, 150 mM NaCl, 5 mM EDTA, 0.5% sodiumdeoxycholate and 0.5% Triton X-100 with addition of 1 mM phenylmethylsulphonyl fluoride (PMSF), 1.5 µg/ml leupeptin, 1 mM Na₃VO₄ and 1.5 µg/ml aprotinin prior to use. The cells were lysed on ice for 30 minutes and then centrifuged at 4°C, 12000 g for 10 minutes. Supernatant was stored at -70°C until further use. The plate

Table 1. Primers and probes for real-time RT-PCR.

Gene		Sequence	Ref.
IGF-IR	Forward	5'-CGATGTGTGAGAAGACCACCA-3'	(34)
	Reverse	5'-ACATTTTCTGGCAGCGGTTT-3'	(34)
	Probe	5'-(FAM)-CAATGAGTACAACCTACCGCTGCTGGACCA-(TAMRA)-3'	(34)
IR	Forward	5'-AGGAGCCCAATGGTCTGA-3'	(21)
	Reverse	5'-GAGACGCAGAGATGCAGC-3'	(21)
	Probe	5'-(FAM)-ACCATATCGCCGATAACTCACTTCATACAG-(TAMRA)-3'	(21)
IR-A	Forward	5'-GTTTAGGAAGACGTTTGAGGATT-3'	(22)
	Reverse	5'-TCACATTCCCAACATCGCCA-3'	(22)
	Probe	5'-(FAM)-ACAACGTGGTTTTTCGTCCCAGGCCAT-(TAMRA)-3'	(22)
IR-B	Forward	5'-ATTACCTGCACAACGTGGTTTTTC-3'	(22)
	Reverse	5'-GGACCTGCGTTTCCGAGAT-3'	(22)
	Probe	5'-(FAM)-AACCTCTTCAGGCACTGGTGCCGAG-(TAMRA)-3'	(22)
GHR	Forward	5'-CCTTGGCACTGGCAGGATCAA-3'	
	Reverse	5'-TTTGCAGACTCCAGGGTGCTC-3'	
	Probe	5'-(FAM)-CTTTTTCTGGAAGTGAGGCCACAGCAGCTATCC-(TAMRA)-3'	
Adiponectin	Forward	5'-GCTCTGTGCTCCTGCATCTG-3'	(35)
	Reverse	5'-ACGCTCTCCTCCCCATACA-3'	(35)
	Probe	5'-(FAM)-AGGTGGGCGACCAAGTCTGGCTC-(TAMRA)-3'	(35)
GAPDH	Forward	5'-GAAGGTGGAGGTCCGAGTC-3'	
	Reverse	5'-GAAGATGGTGATGGGATTC-3'	
	Probe	5'-(VIC)-CAAGCTTCCCGTTCTCAGCC-(TAMRA)-3'	
GLUT4	Forward	5'-GCTACCTCTACATCATCCAGAATCTC-3'	(36, 37)
	Reverse	5'-CCAGAAACATCGGCCCA-3'	(36, 37)
	Probe	5'-(FAM)-CTGCCAGAAAGAGTCTGAAGCGCCT-(TAMRA)-3'	(36, 37)

used in the assay was a 96-well Maxisorp plates (NUNC, Roskilde, Denmark) which was coated over night at 4°C with monoclonal anti human IGF-IR antibodies (MAB 391) (R&D

Systems, Abingdon, UK) diluted as 2 mg/l in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). Day 2 the wells were washed three times with 0.05% Tween 20 in PBS (PBS-T) to remove unbound fractions of antibody before blocking with 5% BSA and 0.02 % NaN₃ in PBS for 1 hour at RT, followed by another wash with PBS-T. Recombinant human IGF-IR (R&D Systems) was used as standard and was, as well as cell lysate, diluted in 0.1% human serum albumin (HSA) in PBS. Samples and standards were added to the plate and incubated on a shaker at RT for 2 hours and then the unbound fraction was washed away with PBS-T. The secondary antibody was a biotinylated anti human IGF-IR antibody (BAF 391) purchased from R&D Systems. The antibody was diluted to 0.5 mg/l in 0.1% HSA in PBS and then added to each well and incubated on a shaker at RT for 2 hours, followed by washing. The plate was then incubated on a shaker at RT for 20 minutes with horseradish peroxidase (HRP) conjugated streptavidin, diluted 500 times in 0.1% Tween 20 in PBS and washed with PBS-T. Substrate for the reaction with the enzyme was 0.4 mg/l tetramethylbenzidine dihydrochloride dissolved in phosphate citrate buffer (35 mM citric acid, 67 mM Na₂HPO₄) (pH 5.0) with 0.006% H₂O₂. The plate was incubated with the substrate for 30 minutes and then the reaction was stopped by addition of 1 M H₂SO₄ and optical densities were measured at 450 nm.

IR ELISA

The amount of IR protein was measured using Insulin Receptor Immunoassay Kit (BioSource Europe S.A., Nivelles, Belgium) according to the manufacturer's instructions. Cell lysate was prepared as described above.

Total protein

Total protein amounts were measured using a BCATM Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

Phosphorylation

Preadipocytes were starved over night with DMEM containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 µg/ml Fungizone® and 0.1% BSA. Insulin or IGF-I at different concentrations were added to the flasks and they were then incubated at 37°C for 10 minutes. The medium was removed and a lysis buffer (pH 7.5) containing 20 mM TrizmaBase, 150 mM NaCl, 5 mM EDTA, 0.5% sodiumdeoxycholate and 0.5% Triton X-100 with addition of 1 mM phenylmethylsulphonyl fluoride (PMSF), 1.5 µg/ml leupeptin, 1 mM Na₃VO₄ and 1.5 µg/ml aprotinin prior to use was added to the flasks. The cells were lysed on ice for 30 minutes and then harvested by scraping. The lysate was centrifuged at 12000 g for 15 minutes at 4°C and the supernatant was stored at -70°C until use. Phosphorylation of differentiated preadipocytes was induced as described above but with 4 days serum deprivation.

Immunoprecipitation

Lysate from phosphorylation experiments was immunoprecipitated (IP) with either rabbit polyclonal anti-IRβ antibody c-19 or rabbit polyclonal anti-IGF-IRβ antibody c-20 (Santa Cruz Biotechnology, Heidelberg, Germany), diluted as 1:400, and incubated at 4°C for 2 hours. 20 mg/ml Protein A Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in 0.1% BSA in lysis buffer (20 mM Trizma Base, 150 mM NaCl, 5 mM EDTA, 0.5% sodiumdeoxycholate and 0.5% Triton X-100) was then added and the lysate was incubated over night at 4°C with gentle rocking. The next day lysate was centrifuged at 6500 g for 5 minutes, supernatant was removed and the pellet was washed 3 times with lysis buffer. It was

then dissolved in Laemmli sample buffer (6.25 mM TrizmaBase, 1% SDS, 10% glycerol, 0.001% bromophenol) with addition of 2% β -mercaptoethanol and boiled for 3 minutes.

SDS-PAGE and Western blot

Immunoprecipitated samples were loaded on a 7.5% acrylamide gel and proteins were separated at 100 V, 24 mA for about 1 hour. The proteins were then blotted to a polyvinylidene difluoride (PVDF) membrane at 100 V, 250 mA for 1 hour. To avoid unspecific antibody binding the membrane was blocked with 3% bovine serum albumine (BSA) in tris buffered saline (TBS) (50 mM TrizmaBase, 0.15 M NaCl) with 0.1% Tween 20 (TBS-T) for 1 hour. After incubation with mouse monoclonal anti-phosphotyrosine antibody (PY-20) (Santa Cruz Biotechnology) diluted 1:1000 in TBS with 1% BSA and 0.02% NaN_3 over night in 4°C the membranes were incubated with a horse-radish peroxidase conjugated sheep anti-mouse antibody (GE Healthcare) diluted 1:3000 in TBS-T with 2.5% BSA for 1 hour at RT. Separated proteins were detected using HRP-conjugated streptavidin (GE Healthcare) diluted 1:20000 in TBS-T and incubated for 5 min at RT, followed by an enhanced chemiluminescent detection system (ECL plus) (GE Healthcare). Exposure to Hyperfilm ECL (GE Healthcare) revealed autoradiograph patterns. Membranes were stripped in strippingbuffer (62.5 mM Trizma HCl, 2% SDS, 100 mM β -mercaptoethanol) at 56°C for 30 minutes. They were blocked with 5% non-fat milk in TBS-T for 1 hour. To detect IGF-IRs membranes were incubated with rabbit polyclonal anti-IGF-IR β antibody c-20 (Santa Cruz Biotechnology) diluted 1:1000 in TBS with 1%BSA and 0.02% NaN_3 followed by HRP linked goat anti-rabbit antibody diluted 1:10000 in TBS-T with 2.5% non-fat milk. To detect IRs membranes were incubated with rabbit polyclonal anti-IR β antibody c-19 (Santa Cruz Biotechnology) diluted 1:1000 in TBS with 1%BSA and 0.02% NaN_3 followed by HRP

linked goat anti-rabbit antibody diluted 1:10000 in TBS-T with 2.5% non-fat milk. Detection as described above.

[6-³H]-thymidine incorporation

Cultures of hPA were grown in 6-well plates until near confluence. The cells were stimulated with insulin and IGF-I at different concentrations in serum free DMEM over night and then incubated with 1 μ Ci/ml [6-³H]-thymidine for 3 hours at 37°C, 5% CO₂. The wells were washed and DNA was then precipitated with ice cold 5% trichloroacetic acid (TCA) for 15 minutes at 4°C. Incubation with 0.1 M potassium hydroxide (KOH) for 1-2 hours at RT lysed the cells and 800 μ l of the lysate was added to 4 ml of of UltimaGoldTM scintillation fluid (Chemical Instruments AB (CiAB), Sollentuna, Sweden) and the radioactivity was measured in a liquid scintillation counter. Data were expressed as percent of unstimulated control cell radioactivity. EC₅₀ values were calculated with GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA, USA).

D-[U-¹⁴C]-glucose accumulation

Preadipocytes were grown in 6-well plates until near confluence. The cells were serum deprived over night and then incubated for 3 h in serum free DMEM (glucose concentration 1000 mg/l) with addition of 0.2 μ Ci/ml D-[U-¹⁴C]-glucose and insulin or IGF-I at different concentrations. The wells were then washed 3 times with ice cold PBS and lysed with 0.1% SDS for 15 min. 800 μ l of the cell solution was added to 4 ml of UltimaGoldTM scintillation fluid (CiAB) and the radioactivity was measured in a liquid scintillation counter. Data were expressed as percent of unstimulated control cell radioactivity. Accumulation studies on differentiated preadipocytes followed the same protocol after differentiation in 6-well plates

and deprivation of serum, insulin, indomethacine, IBMX and dexamethasone for 4 days. EC₅₀ values were calculated with GraphPad Prism 3.03 (GraphPad Software Inc.).

Statistical analysis

Statistical comparisons were made with SPSS 12.0.1 (SPSS Inc. Headquarters, Chicago, Illinois, USA) by one-way analysis of variance (ANOVA). A *p* value <0.05 was considered statistically significant.

Results

Gene expression

Gene expression of IGF-IR, IR, IR-A, IR-B, GHR, GLUT4 and adiponectin was investigated with real-time RT-PCR in preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD). During differentiation the amount of IR gene expression in PA increased, while the IGF-IR gene was expressed at an approximately constant level when normalized against GAPDH gene expression (Table 2). The results showed an almost 10-fold difference between IR and IGF-IR in mAD but nearly the same amounts in PA. Figure 1 shows the calculated ratio of IR to IGF-IR mRNA when the IGF-IR is given the value 1. The differences in ratios between PA and mAD and between dPA and mAD were statistically significant ($p < 0.001$ and $p < 0.05$ respectively).

The two isoforms of IR, isoform A and isoform B, were differently expressed during differentiation (Fig. 2). IR-A gene expression was found in all three types of cells but was 54 times more abundant in mAD than in PA. Isoform B could only be detected in mAD and at almost 7 times lower levels than IR-A in these cells.

To characterize the cells we looked at genes known to be expressed in adipocytes (Table 3).

GLUT4 expression was found in mAD and at lower levels in the dPA but there was no detectable mRNA in PA. GHR expression increased during differentiation and had very low, but still detectable, levels in PA and high in mAD. Adiponectin is thought to be a true adipokine, only expressed in adipocytes, but low levels of adiponectin mRNA were also found in the PA. Gene expression increased when the cells started to differentiate.

Table 2. Gene expression of insulin receptor (IR) and IGF-I receptor (IGF-IR) in preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD), measured with real-time RT-PCR and expressed in relation to GAPDH mRNA.

	IGF-IR	IR
PA	1.92 ± 0.18	2.06 ± 0.14
dPA	3.63 ± 0.61	14.36 ± 6.84
mAD	2.42 ± 1.26	28.92 ± 16.60

Data are expressed as mean ± SE; n = 3.

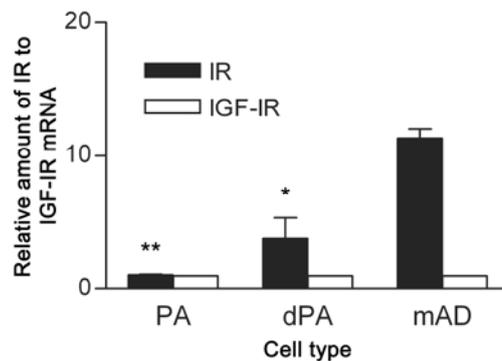


Figure 1: Relative amounts of IR to IGF-IR mRNA

Gene expression of insulin receptor (IR) and IGF-I receptor (IGF-IR) in preadipocytes (PA), differentiated preadipocytes (dPA) and freshly isolated mature adipocytes (mAD) was measured with real-time RT-PCR and results are expressed as the ratio of IR to IGF-IR mRNA (IGF-IR = 1). Bars are mean ± SE from 3 independent experiments with different cell isolations.

* $p < 0.05$ in comparison with mAD

** $p < 0.001$ in comparison with mAD

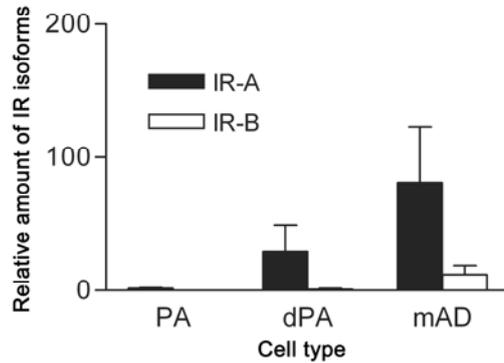


Figure 2: Gene expression of IR-A and IR-B

Gene expression of the two insulin receptor isoforms, IR-A and IR-B, in human preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD). Levels of mRNA were measured with real-time RT-PCR and results are expressed in relation to GAPDH mRNA. Bars are mean \pm SE from 3 independent experiments with different cell isolations.

Table 3. Gene expression of GLUT4, growth hormone receptor (GHR) and adiponectin in preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD), measured with real-time RT-PCR and expressed in relation to GAPDH mRNA.

	PA	dPA	mAD
GLUT4	0	9.9 \pm 5.4	108.6 \pm 51.8
GHR	1.3 \cdot 10 ⁻³ \pm 0.4 \cdot 10 ⁻³	4.6 \pm 2.1	6.1 \pm 2.5
Adiponectin	3.1 \cdot 10 ⁻² \pm 1.0 \cdot 10 ⁻²	4.6 \pm 2.0	16.3 \pm 7.4

Data are expressed as mean \pm SE; n = 3.

Receptor protein

IGF-IR and IR protein was quantified with receptor specific ELISAs in PA, dPA and mAD (Table 4). When total protein was determined in the lysates to normalize the results we found that it was severalfold higher in dPA compared to PA. In mAD there were more than 10-fold higher amounts of IR than IGF-IR expressed as ng receptor protein per 100 μ g total protein. In PA only half as much IR as IGF-IR was found. The differences in calculated ratios between PA and mAD and between dPA and mAD were statistically significant ($p < 0.001$ and $p < 0.05$ respectively) (Fig. 3).

Table 4. Protein amount of insulin receptor (IR) and IGF-I receptor (IGF-IR) in preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD), measured with ELISA and expressed in relation to total protein.

	IGF-IR (ng receptor protein/100 µg total protein)	IR (ng receptor protein/100 µg total protein)
PA	3.33 ± 0.92	1.10 ± 0.09
dPA	1.39 ± 0.55	0.91 ± 0.17
mAD	0.11 ± 0.03	1.13 ± 0.19

Data are expressed as mean ± SE; n = 3.

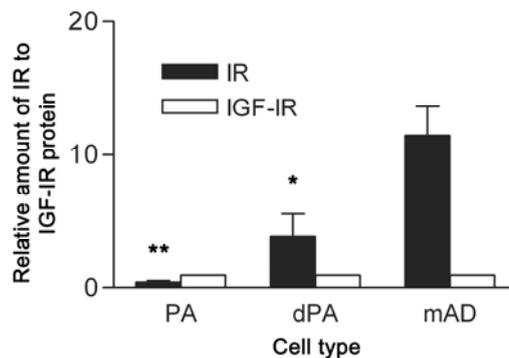


Figure 3: **Relative amounts of IR to IGF-IR protein**

Insulin receptor (IR) and IGF-I receptor (IGF-IR) protein amounts in human preadipocytes (PA), differentiated preadipocytes (dPA) and mature adipocytes (mAD) were measured using receptor specific ELISAs. Results are calculated in relation to total protein amounts and presented as ratios of IR to IGF-IR (IGF-IR = 1). Bars are mean ± SE from 3 independent experiments with different cell isolations.

* $p < 0.05$ in comparison with mAD

** $p < 0.001$ in comparison with mAD

Western Blot

PA and dPA were stimulated with either insulin or IGF-I at 10^{-9} M and after

immunoprecipitation (IP) of the lysates, receptors were analyzed with Western blot. In PA

both the IR and the IGF-IR could be detected (Fig. 4A). After IP with IR antibody we could

also detect IGF-IR by immunoblotting with an IGF-IR antibody and vice versa. This suggests

that some receptors were in the form of hybrid receptors. Both IR and IGF-IR could also be detected in the dPA. (Fig. 4B). In isolated mAD both IR protein and IGF-IR protein could be detected after IP with IR antibody (Fig. 4C). The IGF-IR band was however weak in comparison to the IR band. It is therefore likely that also mAD contain insulin/IGF-I hybrid receptors. When lysate was IP with IGF-IR antibody and blotted with IGF-IR antibody we found a band with the molecular size corresponding to the the molecular size of the IGF-IR β -subunit.

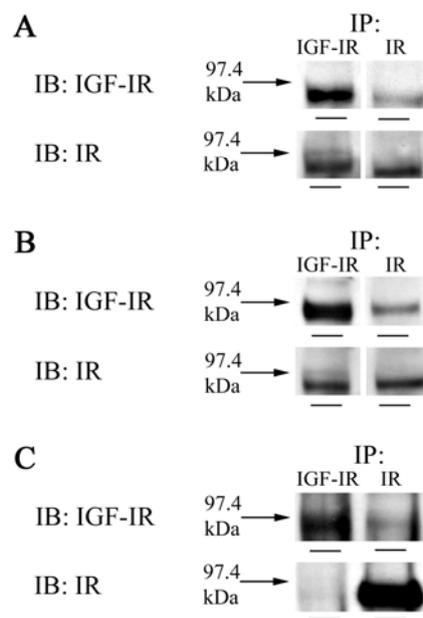


Figure 4: Presence of IGF-I receptor (IGF-IR) and insulin receptor (IR) protein
 Cell lysate was immunoprecipitated (IP) with polyclonal antibodies against either the IGF-IR β -subunit (c-20) or the IR β -subunit (c-19). Immunoblotting (IB) with the same antibodies was used to analyze the presence of receptor protein in A) human preadipocytes (PA), B) differentiated preadipocytes (dPA) and C) mature adipocytes (mAD). Blots are representative of 3 different experiments from different cell isolations.

The activation of IR and IGF-IR was detected by the phosphotyrosine antibody PY-20 in PA after stimulation with insulin or IGF-I at 10^{-9} M. Both IR and IGF-IR were found to be phosphorylated by their own ligand (Fig. 5A). We found no consistent activation of the IR β -subunit by IGF-I. When receptor phosphorylation was studied in dPA after over night serum deprivation there was a strong basal activation of the β -subunits of IR and IGF-IR and no

effect of stimulation by insulin or IGF-I (data not shown). In cells with prolonged serum deprivation for four days and then stimulated by insulin or IGF-I at 10^{-9} M, IGF-IR was phosphorylated by IGF-I and this concentration of IGF-I also weakly activated the IR (Fig. 5B). Neither the IR, nor the IGF-IR was activated by insulin. It should be noted that there was also some activation of the unstimulated controls.

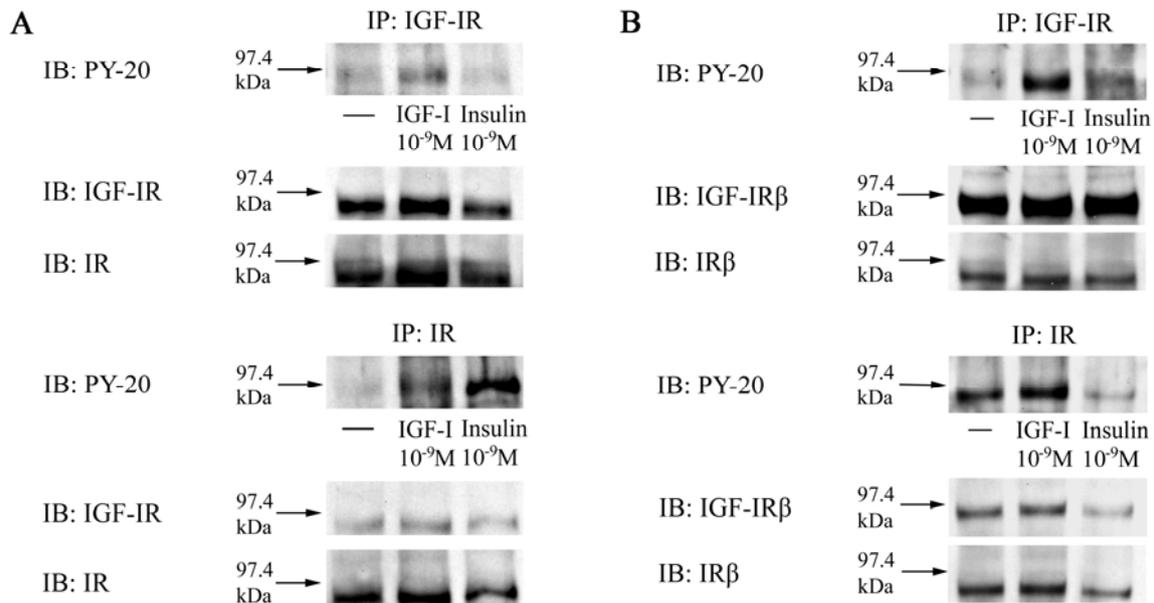


Figure 5: Phosphorylation of IGF-IR and IR

Cell lysate was immunoprecipitated (IP) with polyclonal antibodies against either the IGF-I receptor (IGF-IR) β -subunit (c-20) or the insulin receptor (IR) β -subunit (c-19). Immunoblotting (IB) with a phosphotyrosine antibody (PY-20) was used to analyze the phosphorylation in A) human preadipocytes (PA) and B) differentiated preadipocytes (dPA). Blots are representative of 3 different experiments from different cell isolations.

DNA synthesis

The incorporation of [6-³H]-thymidine into DNA was analyzed to quantify DNA synthesis in PA. The incorporation was stimulated with 10^{-11} - 10^{-8} M insulin or IGF-I. Results showed a significant increase in DNA synthesis, compared to unstimulated control, in PA stimulated with insulin at 10^{-8} M ($p < 0.05$) or with the same concentration of IGF-I ($p < 0.001$) (Fig. 6). A tendency to higher effects of IGF-I than insulin stimulation could be noticed at 10^{-8} M. EC_{50}

was $3.2 \cdot 10^{-9}$ M with a 95% confidence interval of $(6.9 \cdot 10^{-10} - 1.5 \cdot 10^{-8})$ for IGF-I and $1.1 \cdot 10^{-9}$ M, confidence interval $(1.9 \cdot 10^{-10} - 6.2 \cdot 10^{-9})$, for insulin.

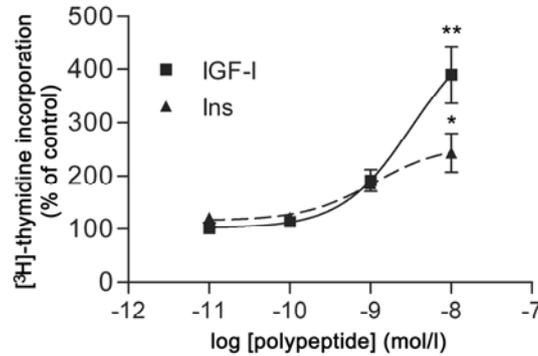


Figure 6: Effects of insulin and IGF-I on the incorporation of $[6\text{-}^3\text{H}]\text{-thymidine}$
Near confluent cultured human preadipocytes (PA) were stimulated with insulin or IGF-I in serum-free DMEM over night and then incubated with $[6\text{-}^3\text{H}]\text{-thymidine}$ for 3 hours. Results are mean \pm SE from 5 independent experiments with different cell isolations.

* $p < 0.05$ in comparison with unstimulated control

** $p < 0.001$ in comparison with unstimulated control

Glucose accumulation

To investigate the biological effect of insulin and IGF-I on glucose metabolism in PA accumulation of D-[U- ^{14}C]-glucose was studied. The cells were stimulated with either insulin or IGF-I at concentrations of 10^{-11} - 10^{-8} M. When compared with unstimulated controls significant differences could be seen for IGF-I at 10^{-9} M and 10^{-8} M ($p < 0.001$) and for insulin at 10^{-8} M ($p < 0.05$) (Fig. 7A). The effects also had a tendency to be higher for IGF-I than for insulin. EC_{50} was $2.3 \cdot 10^{-10}$ M with a 95% confidence interval of $(5.9 \cdot 10^{-11} - 9.3 \cdot 10^{-10})$ for IGF-I and $6.4 \cdot 10^{-10}$ M, confidence interval $(9.0 \cdot 10^{-11} - 4.5 \cdot 10^{-9})$, for insulin.

When glucose accumulation was measured in dPA after over night serum deprivation there were no effects of insulin or IGF-I (data not shown). After four days serum deprivation glucose accumulation increased with increasing concentrations of both insulin and IGF-I, though IGF-I stimulated effects were higher (Fig. 7B). However, none of these effects were

statistically higher than the unstimulated control. EC₅₀ for IGF-I was $6.9 \cdot 10^{-10}$ M with a confidence interval (95%) of ($5.5 \cdot 10^{-11}$ - $8.5 \cdot 10^{-9}$). EC₅₀ for insulin could not be calculated.

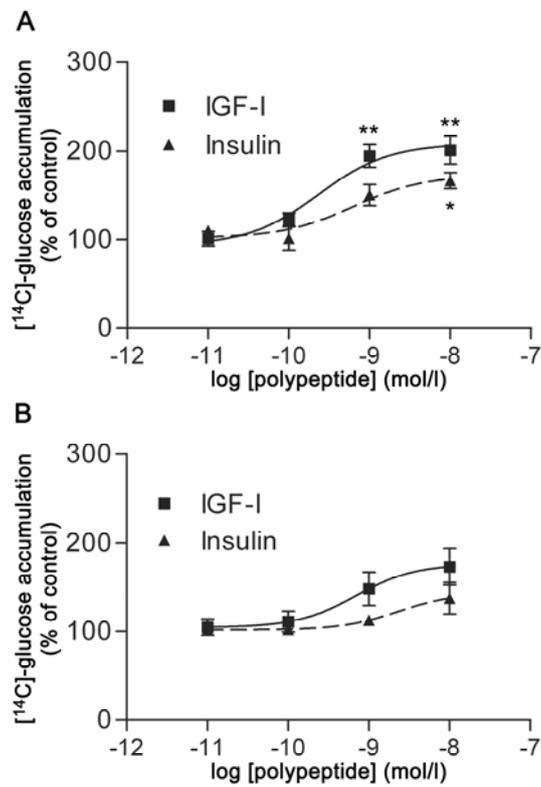


Figure 7: Effects of insulin and IGF-I on the accumulation of D-[U-¹⁴C]-glucose
 A) Near confluent human preadipocytes (PA) were incubated at 37°C for 3 hours with D-[U-¹⁴C]-glucose and polypeptides. Results are mean ± SE from 5 independent experiments. B) Cultured PA were differentiated for 3-4 weeks and serum deprived for 4 days. The differentiated preadipocytes (dPA) were then incubated at 37°C for 3 hours with D-[U-¹⁴C]-glucose and polypeptides. Results are mean ± SE from 3 independent experiments with different cell isolations.

* $p < 0.05$ in comparison with unstimulated control

** $p < 0.001$ in comparison with unstimulated control

Morphology

Looking at the differentiating cells in a microscope revealed great morphological changes (data not shown). After addition of the differentiation cocktail the fibroblast-like preadipocytes started to contract and became more round. After approximately one week the cells also started to accumulate fat in the form of small lipid droplets.

Discussion

During differentiation of human preadipocytes we found a relative increase in IR mRNA and protein levels compared to IGF-IR. This was accompanied by an increase in the expression of genes characteristic for adipocytes although the PA were not differentiated into mAD. At the differentiation stage obtained in dPA the cells were still more sensitive to IGF-I than to insulin.

Differentiation of human PA caused a change in the abundance of IGF-IR and IR mRNA and protein. Compared to IGF-IR the IR gene and protein levels increased more than 10-fold during differentiation. In absolute values IR gene expression increased while the mRNA for the IGF-IR remained almost constant and protein expression for IGF-IR decreased while IR remained constant. These differences are probably due to the normalization procedures.

Receptor gene expression was normalized against one housekeeping gene, GAPDH, and receptor protein was normalized against total protein. During differentiation the amounts of total protein increased severalfold in the cells. If the receptor proteins didn't increase proportionally this could explain the somewhat puzzling results of decreasing IGF-IR and constant IR levels. It should be pointed out that if quotients are calculated the effect of normalization disappears. The experiments were done on isolated human PA before and after differentiation and these results were then compared with isolated mAD. Human PA and mAD have, to our knowledge, never been used to investigate the presence of both the IR and the IGF-IR and we have therefore no other species-specific results to compare our findings with but our results are consistent with results found in animal cells. Entingh-Pearsall and Kahn (8) studied the presence of IR and IGF-IR on immortalized brown PA from mice with binding studies and immunoblotting. They found a 6-fold increase of the IR and almost constant levels of IGF-IR during differentiation. This could also be seen in the PA cell line

3T3-L1 (25). Smith and colleagues detected twice as much IGF-IR as IR in PA when looking at binding sites. After differentiation IR had increased 25-fold while IGF-IR concentration remained constant. Shimizu found an increase in IR and a decrease in IGF-IR binding sites on TA1 PA during differentiation (26).

The human PA were exposed to a combination of insulin, IBMX, dexamethasone and indomethacine, a mixture commonly used to induce differentiation in adipocyte cell lines (27). The protocol we used is a model to look at initial differentiation. It is not optimized to follow the complete differentiation process since the fully differentiated cells detach from the flasks and are washed away when the medium is changed and approximately 30% of the cells don't differentiate. Even if our model is not optimized to obtain fully differentiated fat cells the results clearly showed a change in gene expression towards a more adipocyte-like phenotype. Apart from the changes in IR and IGF-IR expression, genes expressed in mAD, as GLUT4, GHR and adiponectin appeared or increased during differentiation. These results are in agreement with results in both human and animal cells. GHR mRNA has been seen to be upregulated in murine 3T3-L1 and human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes (7, 28), adiponectin mRNA increases during differentiation of 3T3-L1 and human preadipocyte primary cultures (6, 29) and gene expression of GLUT4 is upregulated in differentiating 3T3-L1 cells (30).

We were also interested in the expression of IR isoforms and the change in expression during differentiation since there is little information about human preadipocytes/adipocytes. In PA and dPA only IR-A mRNA could be found whereas mAD also expressed IR-B, but at much lower levels than IR-A. IR-A gene expression increased more than 50-fold during differentiation. In rat preadipocytes Serrano and co-workers could detect IR-B mRNA and the

levels increased during differentiation (16). In contrast, the expression of IR-A decreased. IR-A mRNA were the most abundant isoforms both prior to and after differentiation. The expression of IR-A and IR-B varies between tissues. Hematopoietic and neuronal cells express only IR-A, tissues such as placenta, kidney, adipose tissue and skeletal muscle express both isoforms, whereas liver predominantly express IR-B (13, 14, 17). Our results indicate that IR-A is the predominant isoform in human preadipocytes/adipocytes. This may have two implications. As mentioned in the introduction IR-A has a higher affinity for insulin than IR-B and thus makes the adipocytes more sensitive to insulin (13, 31). In contrast to rodents, IGF-II is expressed after birth in man and occurs in high concentrations in plasma. Since IR-A has a high affinity for IGF-II it is conceivable that IGF-II has effects on adipocytes in vivo (13) and that IR isoforms expression regulate IGF-II effects.

Insulin/IGF-I hybrid receptors are present in many different human cell types and tissues (19-22) and in this study we also found hybrid receptors in human PA and dPA as both the IR and the IGF-IR could be detected on the same Western blot membrane after immunoprecipitation against either receptor. Hybrid receptors have previously been demonstrated in human adipose tissue (19). Since hybrid receptors have high affinity for IGF-I and low affinity for insulin (18) incorporation of IR into hybrid receptors leads to insulin resistance.

There has been some discussion whether or not IGF-IR are present in human mAD. Our results show the presence of IGF-IR in mature adipocytes both with western blot analysis and ELISA analysis in agreement with the findings of Fischer-Posovszky and co-workers (32).

We tested the effect of insulin and IGF-I in PA at a concentration (10^{-9} M) where the peptides don't cross-react with each others receptors (21). Both insulin and IGF-I activated their cognate receptor. For both insulin and IGF-I the activation was accompanied by biological

effects in the form of glucose accumulation and DNA synthesis. The effect of IGF-I and insulin on glucose was moderate in PA compared to what has been reported for mature adipocytes (33). This is probably due to glucose transport by GLUT1 while insulin sensitive GLUT4 transporters are poorly developed as suggested by the low gene expression. Entingh and Kahn studied receptor activation in brown PA from mice but they found no IR or IGF-IR phosphorylation by either insulin or IGF-I at 10^{-9} M (8). Shimizu studied deoxy-glucose uptake in TA1 cells (26). Uptake in preadipocytes was stimulated by IGF-I with a half maximal effect at 10^{-9} M. Insulin was approximately 30-fold less potent. In dPA the effect of insulin was slightly higher than the effect of IGF-I.

In dPA we found a strong basal activation of the IR and no effect of insulin on glucose accumulation. If the cells were starved for four days without serum, insulin, indomethacine, IBMX and dexamethasone the basal activity decreased, the receptors were activated and we tended to have an effect on glucose accumulation. However, activation was only found after stimulation with IGF-I indicating that the effect was mediated by IGF-IR and insulin/IGF-I hybrid receptors.

In conclusion, insulin and IGF-I mRNA and protein are expressed in human preadipocytes and mature adipocytes. During the differentiation process there is a 10-fold increase in the ratio of IR to IGF-IR mRNA and protein. Insulin receptor isoforms A is the predominant isoform in mature adipocytes and is the only isoform expressed in preadipocytes.

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