0021-972X/00/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 85, No. 11
Printed in U.S.A.

Insulin/Insulin-Like Growth Factor I Hybrid Receptors Overexpression Is Not an Early Defect in Insulin-Resistant Subjects

DANIELA SPAMPINATO, GIUSEPPE PANDINI, ANTONIO IUPPA, VINCENZO TRISCHITTA, RICCARDO VIGNERI, AND LUCIA FRITTITTA

Istituto di Medicina Interna, Endocrinologia e Malattie del Metabolismo, Ospedale Garibaldi, Università di Catania (D.S., G.P., R.V., L.F.), 95123 Catania; Clinica Chirurgica, Ospedale Vitt. Emanuele, Università di Catania (A.I.), 95123 Catania; and Divisione ed Unità di Ricerca di Endocrinologia, Istituto Scientifico Casa Sollievo della Sofferenza, San Giovanni Rotondo (V.T.), 71013 Foggia, Italy

ABSTRACT

Hybrid receptors (HRs), insulin receptor (IR)/insulin-like growth factor I receptor (IGF-I-R) heterodimers have been reported increased in skeletal muscle of obese and type 2 diabetic patients and to contribute to the patient insulin resistance. To investigate whether or not the increased expression of hybrid receptors is an early defect (probably genetic) of insulin resistance, we measured by specific enzyme-linked immunosorbent assays both IR, IGF-I-R, and HR content in skeletal muscle of healthy nonobese, nondiabetic subjects either insulin sensitive or insulin resistant, and also in patients with moderate obesity.

IR content was significantly reduced in insulin-resistant subjects both nonobese and obese, compared with insulin-sensitive subjects (2.32 ± 0.26, 2.36 ± 0.18, and 3.45 ± 0.42 ng/mg protein, respectively, $P = 0.002$). In contrast, IGF-I-R content was similar in the three groups. Muscle HR content was not different in insulin-sensitive *vs.* insulin-resistant subjects (both nonobese and obese) (4.90 ± 0.46, 4.69 ± 0.29, and 4.91 ± 0.25 ng/mg protein, respectively, P = not significant). These studies indicate that, in insulin-resistant subjects without diabetes or severe obesity, muscle IR content but not IGF-I-R or HR content is reduced. They do not suggest, therefore, a primary (genetic) role of increased HR as a cause of IR decrease and insulin resistance. (*J Clin Endocrinol Metab* **85:** 4219–4223, 2000)

HYBRID RECEPTORS (HRs), formed by assembling one insulin receptor (IR) $\alpha\beta$-heterodimer and one type I insulin-like growth factor receptor (IGF-I-R) $\alpha\beta$-heterodimer, are present in tissues and cells coexpressing both IR and IGF-I-R (1–3). These HRs behave like IGF-I-R rather than IRs because they bind to and are activated by IGF-I with an affinity similar to that of typical IGF-I-R, whereas they bind insulin with a much lower affinity (3, 4).

Although the mechanisms regulating HR formation are not known, random assembly of heterogeneous dimers, made available during IR and IGF-I-R syntheses, is believed to be the most likely evidence. If this is the case, factors affecting availability of either IR or IGF-I-R heterodimers will influence the cell/tissue abundance of HRs (5).

Clinical interest for HRs has been raised by recent observations that the relative abundance of HRs is increased in skeletal muscle tissue of patients with type 2 diabetes with respect to control subjects (6). In these studies, HRs were measured as the ^{125}I-IGF-I fraction that was first bound to a solid phase anti-IGF-I-R antibody and then immunoadsorbed with an anti-IR antibody. Data were expressed as the fraction of total labeled IGF-I binding (to both typical IGF-I-Rs and to HR). An increased formation of HRs was hypothesized to contribute to the insulin resistance by drawing away $\alpha\beta$-heterodimer to the formation of IR homodimers and consequently reducing the cell IR content, cell insulin binding, and responsivity (6, 7). Chronic hyperinsulinemia has been hypothesized as a possible cause of increased HR formation in type 2 diabetic patients (7). This hypothesis was supported by the observation that patients with hyperinsulinemia due to insulinoma had a relative abundance of HR higher than normal subjects (7). In insulinoma patients, however, hyperinsulinemia does not reflect that observed in type 2 diabetic patients both because insulin plasma levels are usually more elevated and because hyperinsulinemia caused by insulinoma is a primary phenomenon and not the consequence of insulin resistance as it is in diabetic patients. The reasons and the role of increased HRs in insulin resistance, therefore, remain unclear.

To investigate this problem, we first set up direct measurements of IR, IGF-I-R, and HR by specific enzyme-linked immunosorbent assays (ELISAs) and then measured HR content in muscle tissue of healthy nonobese, nondiabetic subjects either insulin sensitive or insulin resistant. In these insulin-resistant individuals, insulin resistance precedes more relevant metabolic abnormalities like obesity and/or diabetes and is believed to be mostly genetically determined (8, 9). We measured IR, IGF-I-R, and HR also in muscle tissue of moderately obese, nondiabetic patients, a model in which acquired insulin resistance may be considered a predominant feature, in addition to the genetic component. The results indicate that: 1) the HR content in human muscle tissue is higher than expected by the random assembly model; 2)

Received November 22, 1999. Revision received May 8, 2000. Accepted July 19, 2000.

Address correspondence and requests for reprints to: Lucia Frittitta, M.D., Endocrinologia, Ospedale Garibaldi, P.zza S. M. Gesù, 95123 Catania, Italy. E-mail segmeint@mbox.unict.it.

HR content is not increased in muscle tissue of insulin-resistant subjects who are not severely obese or diabetic; and 3) the moderate chronic hyperinsulinemia typical of insulin resistance does not cause HR increase. Therefore, HRs do not seem to contribute to insulin resistance in these subjects.

Materials, Subjects, and Methods

Materials

Triton X-100, bacitracin, phenylmethyl sulfonyl fluoride (PMSF), and BSA were purchased from Sigma (St. Louis, MO). The following monoclonal antibodies to the IR were used: MA-20, which reacts with an α-subunit epitope close to the insulin binding site (10) and does not recognize HR; αCT-1, which reacts with β-subunit at residues 1344–1355 (11); and 83–7, which reacts with an epitope at the α-subunit (12) and recognizes both IR and HR. Monoclonal antibodies to the IGF-R were: αIR3, which reacts with the α-subunit at residues 223–274 and recognizes typical IGF-1-R (13); and 17–69, which reacts with the α-subunit residues 440–586 and recognizes both the IGF-1-R and HR (14, 15). Antibody MA-20 was kindly provided by Dr. I. D. Goldfine (University of California–San Francisco, San Francisco, CA). Antibodies αCT-1, 83-7, and 17-69 were kindly provided by Dr. Siddle (University of Cambridge, Cambridge, UK). Monoclonal antibody αIR3 was purchased from Calbiochem Oncogene Research Products (Cambridge, MA). For immunoblotting, the following antibodies were used: an anti-IR β-subunit polyclonal antibody (Transduction Laboratories, Lexington, KY) and a chicken polyclonal antibody against the α-subunit of IGF-I-R (UBI, Lake Placid, NY). Plasma insulin and IGF-I levels were measured (by immunoradiometric assay) with commercially available kits (for insulin: IMx system insulin, Abbott Laboratories, Daimabot, Tokyo, Japan; for IGF-I: Diagnostics Systems Laboratories, Inc., Webster, TX).

Subjects

Fourteen moderately obese [body mass index (BMI), 27.5–35.2 kg/m^2] patients and 21 nonobese (BMI, <27) subjects undergoing elective abdominal surgery were studied after giving informed consent, according to the Declaration of Helsinki. All subjects were not diabetic as evaluated by oral glucose tolerance test, according to WHO criteria. In all of them "homeostasis model assessment" (HOMA), an index of insulin sensitivity, was calculated according to the formula: insulin/(22.5e-^{ln} glucose) (16). The 21 nonobese subjects were subdivided according to HOMA values into 11 insulin-sensitive (having HOMA <1.4, the median value) and 10 insulin-resistant (HOMA, >1.4) subjects. Moreover, in 8 obese patients and in 13 nonobese individuals who gave informed consent, insulin-mediated whole-body glucose utilization was also determined by the euglycemic hyperinsulinemic clamp technique after an overnight fast. Insulin-stimulated glucose disposal was evaluated with an insulin infusion at a constant rate (1.0 mU/kg^{-1}·min^{-1}) and a variable glucose infusion to maintain plasma glucose within 10% of the baseline value, as described previously (17).

All subjects were fed a weight-maintaining diet (50% carbohydrate, 30% lipid, and 20% protein) for the 8 days preceding the study.

Tissue specimens

Human muscle tissue specimens were obtained from the human rectus abdominus skeletal muscle during elective abdominal surgery from all subjects studied, 3–5 days after insulin sensitivity studies. After adipose tissue was dissected and blood was removed, muscle specimens were immediately frozen in liquid nitrogen. Soluble extracts were prepared from frozen muscle tissue as described previously (18). Briefly, muscle tissue (~150 mg) was pulverized under liquid nitrogen and then homogenized in a 2-mL buffer [50 mmol/L HEPES, 150 mmol/L NaCl, and 2 mmol/L PMSF (pH 7.6)] at 4 C using a polytron homogenizer for 10 sec at medium speed. Triton X-100 was added to a final concentration of 1%, and the homogenates were solubilized for 60 min at 4 C. The material was then centrifuged at 100,000 × g for 60 min at 4 C, and the supernatants were used for the IR, IGF-I-R, and HR content measurement. The protein content was measured by the Bradford method (19).

IR, IGF-I-R, and HR measurements

By ELISA—IR ELISA. IR muscle content was measured by a specific ELISA as described previously (20). Briefly, wells in Maxisorb plates (Nunc, Roskilde, Denmark) were precoated (overnight incubation at 4 C) with anti-IR antibody MA-20 (2 μg/mL). After washing to remove unbound antibody, wells were blocked with 1% BSA and then ~100–200 μg protein extracts were added to each well, and IR was allowed to bind overnight at 4 C. After extensive washing, biotinylated anti-IR antibody α-CT-1 was added [0.3 μg/mL in 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.05% Tween 20, 1 mM PMSF, 1 mg/mL bacitracin, and 1% BSA). After 2 h at 22 C, the peroxidase-streptavidin was added, and 30 min later wells were washed again, and then 100 μL biotinyl-tyramide solution were added. After a 15-min incubation at 22 C, wells were washed with TBST (20 mM Tris, 150 mM NaCl, and 0.05% Tween 20) and streptavidin-horseradish peroxidase solution was added (30 min at 22 C). After further extensive washing, the peroxidase activity was determined colorimetrically by adding 3.3′.5.5′-tetramethylbenzidine at a concentration of 0.4 g/L in an organic base, and the absorbance was measured at 451 nm.

The IR standard was purified from NIH-3T3 cells transfected with human IR complementary DNA, as described previously (20). No appreciable interference was caused by adding IGF-I-R or IR/IGF-I-R to the IGF-I-R standard curve (20).

IGF-I-R ELISA. IGF-1-R was measured by immunocapturing receptors with anti-IGF-I-R αIR-3 monoclonal antibody (2 μg/mL) and revealed by biotinylated anti-IGF-I-R 17-69 monoclonal antibody (20). The IGF-I-R standard was obtained from Chinese hamster ovary cells transfected with human IGF-I-R complementary DNA, as described previously (21). No appreciable interference was caused by adding IR or HR to the IGF-I-R standard curve (20). About 200–400 μg protein extracts were added to each well, and the assay was performed as described above.

Assay sensitivity was linear from 0.125–1.0 ng/well, intra- and interassay coefficients of variation were less than 10% (20).

HR ELISA. HRs were measured by immunocapturing receptors with anti-IR 83-7 monoclonal antibody (2 μg/mL) and using biotinylated anti-IGF-I-R 17-69 monoclonal antibody (0.3 μg/mL) as a revealing second antibody (20). The HR standard was obtained from human placenta, as described previously (3). About 100–300 μg protein extracts were added to each well, and the assay was performed as described above. A typical standard curve for HR hybrid ELISA using a purified HR preparation is shown in Fig. 1. The minimal detectable amount of hybrids was 0.125 ng/100 μL (1.25 ng/mL). The assay was linear from 0.125–1.0 ng/well. No appreciable interference was caused by adding 1 ng IR or IGF-1-R to the HR standard curve (20). Intra- and interassay coefficients of variation were less than 10%. Multiple dilution of solubilized muscle tissue produced a dose-response curve parallel to that obtained with the purified HR standard (Fig. 1).

IR, IGF-I-R, and HR content was normalized for protein content (19).

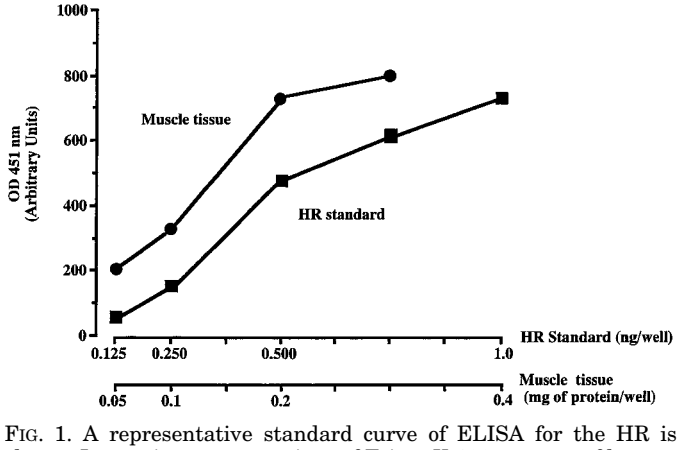

FIG. 1. A representative standard curve of ELISA for the HR is shown. Increasing concentrations of Triton X-100 extracts of human muscle tissue elicit dose-response curves that are parallel to those obtained with purified HR standard.

By Western blot. To validate the ELISA methods, IR, IGF-I-R, and HR content were measured by Western blot analysis in muscle tissues from six subjects showing a wide range of both IR and HR content as measured by ELISA. Muscle extracts (1.5 mg protein) were immunoprecipitated (2 h, 4 C) with anti-IR antibody MA-20 (4.0 μg/sample) or anti-IGF-I-R antibody α-IR3 (4.0 μg/sample) for IGF-I-R or anti-IGF-I-R receptor antibody 17-69 (4 μg/sample) for HR. Samples were then precipitated with rabbit antimouse Ig-G prebound to protein A Sepharose and subjected to SDS-PAGE in a 7.5% polyacrylamide gel under reducing conditions. Proteins were transferred (3 h at 4 C) to nitrocellulose membranes that were first blocked with 10% BSA, then washed with TBST buffer (10 mM Tris, 150 mM NaCl, and 0.5% Tween 20), and incubated with an anti IR β-subunit polyclonal antibody (1 μg/mL) for IR and HR or with a polyclonal antibody against the α-subunit of the IGF-I-R for IGF-I-R. After 16 h at 4 C, membranes were incubated for 1 h with a rabbit antimouse serum conjugated with horseradish peroxidase, and the reaction was developed according to an enhanced chemiluminescence detection system. The specific signaling was revealed by autoradiography.

Predicted value of IR/IGF-I-R and statistical analysis

If we assume that HRs are formed by random assembly of $\alpha\beta$-heterodimers of IR and IGF-I-R, before they are assembled with homologous $\alpha\beta$-heterodimers to form typical IR and IGF-I-R, the predicted HR formation can be calculated on the basis of the cell/tissue IR and IGF-I-R content. If the total concentrations of IR and IGF-I-R half receptors are I and G, respectively, and these half receptors combine randomly, then the predicted relative concentrations of HRs would be I^2:G^2:2IG (22). Thus, on the basis of random assembly, the expected HR content of each specimen can be calculated as equal to $2.\sqrt{\text{IR}}.\sqrt{\text{IGF-I-R}}$ (22).

Unpaired Student's *t* test and ANOVA were used to compare mean values. Linear regression analysis was used to calculate numerical correlation between variables.

Results

Subjects studied

The clinical characteristics of the 35 subjects studied are shown in Table 1. According to HOMA values, the 21 nonobese subjects were divided as insulin sensitive and insulin resistant. Age, gender, and fasting plasma glucose (FPG) were neither different in these two groups nor in comparison with the 14 obese patient group. BMI was similar in the two groups of nonobese subjects. Obese subjects had a significantly higher BMI ($P < 0.001$) and were insulin resistant by HOMA ($P < 0.01$) with respect to the 11 nonobese insulin-sensitive subjects. This difference in insulin sensitivity was confirmed by the euglicemic hyperinsulinemic clamp in randomly selected individuals from each group: the M value was significantly ($P < 0.01$) lower in nonobese insulin-resistant (by HOMA) individuals (n = 5; M = 4.4 ± 0.2) and obese patients (n = 7; M = 4.1 ± 0.7) compared with the eight insulin-sensitive subjects (M = 6.8 ± 0.6). FPG was significantly higher ($P < 0.01$) in obese patients compared with both insulin-sensitive and insulin-resistant nonobese subjects. Fasting plasma insulin concentrations were significantly higher in insulin-resistant subjects (both nonobese and obese) compared with insulin-sensitive subjects ($P < 0.004$). The average plasma IGF-I concentration was similar in the three groups (Table 1).

IR and IGF-I-R content in skeletal muscle tissue

Skeletal muscle IR content was significantly reduced in insulin-resistant subjects (both nonobese and obese) with respect to insulin-sensitive subjects (2.32 ± 0.26, 2.36 ± 0.18,

TABLE 1. Clinical characteristics of the subjects studied

Subjects	Gender (m/f)	Age (yr)	BMI (kg/m^2)	FPG (mmol/L)	FIRI (pmol/L)	IGF-I (nmol/L)	HOMA
Insulin sensitive (n = 11)	8/3	51 ± 4.3 (21–74)	23.6 ± 0.9 (18.5–26.7)	4.7 ± 0.1 (4.3–5.3)	33.2 ± 3.6 (16.5–46.6)	29.2 ± 4.7 (10.9–52.7)	1.0 ± 0.1 (0.5–1.3)
Insulin resistant (n = 10)	5/5	43 ± 3.3 (28–64)	25.1 ± 0.4 (23.4–26.5)	4.8 ± 0.1 (4.3–5.3)	76.5 ± 7.7[a] (55.2–126.3)	32.5 ± 2.9 (18.8–45.4)	2.3 ± 0.2[b] (1.5–3.8)
Obese (n = 14)	7/7	45 ± 3.5 (22–62)	29.9 ± 0.7[c] (27.5–35.2)	5.3 ± 0.2[c] (4.3–6.4)	96.9 ± 17.2[a] (43.0–269.8)	27.7 ± 3.9 (12.3–41.4)	3.3 ± 0.7[b] (1.3–10.7)

Data are expressed as mean ± SEM, with the range in *parentheses*; FIRI, Fasting immunoreactive insulin.
[a] $P < 0.004$ *vs.* insulin-sensitive subjects.
[b] $P < 0.01$ *vs.* insulin-sensitive subjects.
[c] $P < 0.001$ *vs.* insulin-sensitive and -resistant subjects.

and 3.45 ± 0.42 ng/mg protein, respectively; $P = 0.002$). In contrast, skeletal muscle IGF-1-R content was similar in the three groups (0.66 ± 0.06, 0.71 ± 0.09, and 0.82 ± 0.08 ng/mg of protein, respectively; P = not significant). When all subjects were considered together, muscle IR content was inversely correlated with fasting plasma insulin levels ($r = -0.37$; $P = 0.03$) and muscle IGF-I-R content was inversely correlated with plasma IGF-I levels ($r = -0.45$; $P < 0.05$).

HR content in skeletal muscle tissue

Skeletal muscle HR content ranged from 2.7–8.0 ng/mg protein and was not different in insulin-resistant subjects (both nonobese and obese), when compared with insulin-sensitive nonobese subjects (4.69 ± 0.29, 4.91 ± 0.25, and 4.90 ± 0.46 ng/mg protein, respectively; P = not significant) (Table 2).

When we calculated the predicted HR content in each muscle tissue specimen on the basis of the specific content of IR and IGF-I-R, and assuming that HR assembly occurs at random, the measured HR content in skeletal muscle was significantly higher than the predicted value in all instances, although it was significantly correlated with the predicted value ($r = 0.47$; $P < 0.05$). The average HR content directly measured in the 35 specimens studied was 4.81 ± 0.19 ng/mg protein. In contrast, according to the muscle IR and IGF-I-R content, the expected HR value was 2.73 ± 0.14 ng/mg protein ($P < 0.01$, *vs.* the value measured). In muscle tissue, therefore, at variance with other human tissues (20, 22, 23), a preferential formation or a decreased degradation of HR occurs. This difference between measured and expected HR content persisted also when subjects were subdivided in the groups of insulin-sensitive and insulin-resistant (nonobese and obese) subjects (Table 2).

Skeletal muscle HR content was significantly correlated with IGF-I-R content ($r = 0.45$, $P < 0.01$) while the correlation with IR content did not reach the significance level ($r = 0.31$, $P = 0.06$). No correlation was observed between either plasma insulin ($r = 0.18$, $P = 0.3$) or plasma IGF-I concentrations and HR content ($r = 0.10$, $P = 0.6$).

Western blot studies

To validate the ELISA immunoassay, quantitation of IR, IGF-I-R, and HR was assessed also by Western blot. A single band of ~97 kDa corresponding to the β subunits of the IR and the HR and a single band of ~130 kDa corresponding to the α subunits of IGF-I-R were revealed (Fig. 2). The intensity of the bands was quantitated by scanning densitometry and was significantly correlated to results obtained in the same six subjects with the ELISA measurements of IR ($r = 0.94$; $P < 0.02$), HR ($r = 0.8$; $P < 0.05$), and IGF-I-R ($r = 0.92$; $P < 0.001$).

Discussion

Insulin resistance is a common clinical condition, the etiology of which is poorly understood and for which there is no effective treatment. Because insulin resistance is a major feature of obesity and type 2 diabetes, its reversal could potentially either prevent, delay, or ameliorate obesity and hyperglycemia. Recent studies, by indirect measurement of HRs, have suggested that an increased HR content in skeletal muscle of obese and type 2 diabetic patients is a possible cause of insulin resistance. This hypothesis is based on the assumption that an increased HR formation would decrease the availability of IR homodimers and, therefore, insulin responsiveness. However, it is not clear whether the increased HR content is the cause or a consequence of the metabolic alterations that characterize insulin-resistant states like obesity and diabetes. At variance with previous studies where HRs were measured by indirect methods, in the present study we measured skeletal muscle HR content using a novel, direct ELISA measurement. In addition to this technical advancement, we also evaluated skeletal muscle HR content in insulin-resistant individuals who do not have the metabolic and endocrine abnormalities due to severe obesity and/or diabetes. Lean, healthy subjects with insulin resistance are believed to represent a model of insulin resistance with a strong genetic component. These subjects are still able to compensate the tissue insulin resistance by increasing insulin secretion (8, 24, 25). Patients with moderate obesity and more pronounced insulin resistance were also studied. In all these subjects, in addition to HRs we measured also muscle tissue IR and IGF-I-R contents.

No difference in muscle HR content was observed in the three groups of subjects, despite the observation that both lean insulin-resistant individuals and obese patients were clearly hyperinsulinemic and had a significantly reduced IR

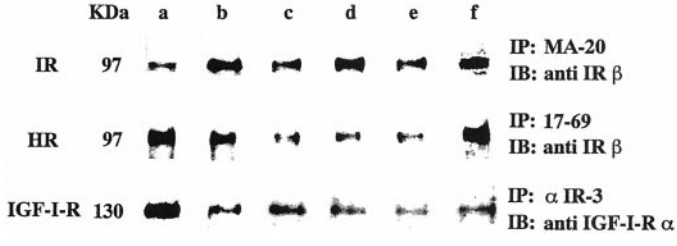

FIG. 2. Quantification of IR, IGF-I-R, and HR as assessed by Western blot. Muscle extracts, from six subjects with a wide range of IR and HR content as measured by ELISA, were immunoprecipitated with MA-20, αIR3, and 17-69 monoclonal antibodies, respectively, for IR, IGF-I-R, and HR. The immunoblotted antibodies precipitated molecular species of approximately M_r 97,000 corresponding to the β-subunits of IR and HR and M_r 130,000 corresponding to the α-subunits of IGF-I-R.

TABLE 2. IR, IGF-I-R, HR, and HR predicted in skeletal muscle

Subjects	IR (ng/mg prot)	IGF-I-R (ng/mg prot)	HR (ng/mg prot)	HR predicted (ng/mg prot)
Insulin sensitive (n = 11)	3.45 ± 0.42 (1.6–6.1)	0.82 ± 0.08 (0.5–1.3)	4.90 ± 0.46 (2.9–8.0)	3.28 ± 0.29[a] (2.0–4.7)
Insulin resistant (n = 10)	2.32 ± 0.26[b] (1.0–3.3)	0.66 ± 0.06 (0.4–1.0)	4.69 ± 0.29 (2.7–6.4)	2.39 ± 0.17[a,b] (1.3–3.1)
Obese (n = 14)	2.36 ± 0.18[b] (1.3–4.1)	0.71 ± 0.09 (0.2–1.4)	4.91 ± 0.25 (3.5–6.4)	2.53 ± 0.20[a,b] (1.7–3.8)

Data are expressed as mean ± SEM, with the range in *parentheses*; prot, Protein.
[a] $P < 0.01$ *vs.* measured HRs.
[b] $P < 0.05$ *vs.* insulin-sensitive subjects.

content in muscle, a likely consequence of a down-regulation effect (26, 27) as indicated by the significant inverse correlation between plasma insulin levels and muscle IR content.

In lean or moderately obese insulin resistant subjects, therefore, insulin resistance and hyperinsulinemia are associated with (and probably cause) reduced muscle IR content but are not associated with changes of either IGF-I plasma levels or IGF-I-R content in skeletal muscle. In these subjects, the muscle HR content is not altered, arguing against the possibility of a primary role of increased HR formation (and IR $\alpha\beta$ heterodimer sequestration) as a cause of insulin resistance. This conclusion is also supported by the consideration that a 30% decrease of muscle IR content is unlikely to cause insulin resistance because of the presence of spare receptors: a more marked IR reduction may be necessary to become a limiting step for insulin action (28); more so when hyperinsulinemia is present. Therefore, although the HR/IR ratio is slightly increased in subjects with insulin resistance, the present findings do not support a clinical relevance of this condition. Finally, our data also argue against the possibility that the moderate hyperinsulinemia (2- to 3-fold increase) secondary to insulin resistance may *per se* cause an increased HR formation in human muscle tissue.

Differences between the present data and previous data may be explained both by differences in the technique used to measure HR in the type of patients investigated. The ELISA assays that we have used provide a direct measurement of muscle receptor protein content and very well reflect measurements obtained by Western blot studies. The coprecipitation method, instead, provides HR measurement data as the percentage of bound ^{125}I-IGF that is immunoprecipitated by an anti-IR antibody. By calculating total IGF-I binding by competition/inhibition studies, it is then possible to extrapolate the HR aliquot. Of course the two methods rely on different assumptions and do not necessarily provide similar results.

Relevant differences between the present study and previous studies also regard the subjects studied. Severely obese (average BMI, >40) and diabetic patients were investigated in previous studies. In those patients, complex metabolic and hormone abnormalities are present and may contribute to abnormalities in muscle HR content. For instance, an important decrease of IGF-I plasma levels and the consequent up-regulation of IGF-I-R muscle content was observed in those patients. Increased IGF-I-R synthesis may provide overabundant was $\alpha\beta$ heterodimers that become available for HR formation.

Additional information obtained in the present study concerns the HR formation in of IR and IGF-I-R $\alpha\beta$ heterodimers. In human muscle tissues this may well be the case because the measured value of HR content was directly correlated with the value that can be predicted on the basis of IR and IGF-I-R content in each muscle specimen. Measured values, however, were always higher than predicted values, suggesting that in muscle, at variance with other tissues (20, 22, 23), some mechanisms may provide a more efficient assembly of available IR and IGF-I-R $\alpha\beta$ heterodimers into HR rather than into typical IRs or IGF-I-Rs. Alternatively, in muscle tissue, HR degradation and recycling may be slower than that of IR and IGF-I-R, resulting in an overall HR content higher than expected. The biological relevance of this observation, if any, remains to be understood.

References

1. **Moxham CP, Duronio V, Jacobs S.** 1989 Insulin-like growth factor-I receptor β-subunit heterogeneity. J Biol Chem. 264:13238–13244.
2. **Soos MA, Whittaker J, Lammers R, Ullrich A, Siddle K.** 1990 Receptors for insulin and insulin-like growth factor-I can form hybrid dimers. Biochem J. 270:383–390.
3. **Kasuya J, Paz BI, Maddux BA, Goldfine ID, Hefta SA, Yamaguchi YF.** 1993 Characterization of human placental IGF-I/insulin hybrid receptors by protein microsequencing and purification. Biochemistry. 32:13531–13536.
4. **Soos MA, Field CE, Siddle K.** 1993 Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I but not insulin with high affinity. Biochem J. 290:419–426.
5. **Treadway JL, Morrison BD, Goldfine ID, Pessin JE.** 1989 Assembly of insulin/insulin-like growth factor-I hybrid receptors *in vitro*. J Biol Chem. 264:21450–21453.
6. **Federici M, Zucaro L, Porzio O, et al.** 1996 Increased expression of insulin/insulin-like growth factor-I hybrid receptors in skeletal muscle of noninsulin-dependent diabetes mellitus subjects. J Clin Invest. 98:2887–2893.
7. **Federici M, Lauro D, D'Adamo M, et al.** 1998 Expression of insulin/insulin-like growth factor-I hybrid receptors is increased in skeletal muscle of patients with chronic primary hyperinsulinemia. Diabetes. 47:87–92.
8. **Reaven G.** 1988 Role of insulin resistance in human disease. Diabetes. 37:1595–1607.
9. **Trischitta V, Frittitta L, Vigneri R.** 1997 Early molecular defects in human insulin resistance: studies in healthy subjects with low insulin sensitivity. Diabetes Metab Rev. 13:147–162.
10. **Roth RA, Cassel DJ, Wong KY, Maddux BA, Goldfine ID.** 1982 Monoclonal antibodies to the human insulin receptor block insulin binding and inhibit insulin action. Proc Natl Acad Sci USA. 79:7312–7316.
11. **Ganderton RH, Stanley KK, Field CE, Coghlan MP, Soos MA, Siddle KA.** 1992 Monoclonal anti-peptide antibody reacting with the insulin receptor β-subunit. Biochem J. 288:195–205.
12. **Soos MA, Siddle K, Baron MD, et al.** 1986 Monoclonal antibodies reacting with multiple epitopes on the human insulin receptor. Biochem J. 235:199–208.
13. **Kull FC, Jacobs S, Su YF, Svoboda ME, Van Wyk JJ, Cuatrecasas P.** 1983 Molecular antibody to receptor for insulin and Somatomedin C. J Biol Chem. 258:6561–6566.
14. **Gustafson TA, Rutter WJ.** 1990 The cysteine-rich domains of the insulin-like growth factor I receptors are primary determinants of hormone binding specificity. Evidence from receptor chimeras. J Biol Chem. 265:18663–18667.
15. **Soos MA, Field CE, Lammers R, et al.** 1992 A panel of monoclonal antibodies for the type I insulin-like growth factor receptor. J Biol Chem. 267:12955–12963.
16. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Tuner RC.** 1985 Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentration in man. Diabetologia. 28:412.
17. **DeFronzo RA, Tobin JD, Andres R.** 1979 Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 237:E214–E223.
18. **Frittitta L, Youngren J, Vigneri R, Maddux BA, Trischitta V, Goldfine ID.** 1996 PC-1 content in skeletal muscle of non-obese, non-diabetic subjects: relationship to insulin receptor tyrosine-kinase and whole body insulin sensitivity. Diabetologia. 39:1190–1195.
19. **Bradford MM.** 1978 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem. 72:248–254.
20. **Pandini G, Vigneri R, Costantino A, et al.** 1999 Insulin and insulin-like growth factor-I (IGF-I) receptors overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. Clin Cancer Res. 5:1935–1944.
21. **Le Bon TR, Jacobs S, Cuatrecasas P, Kathuria S, Fujita-Yamaguchi Y.** 1986 Purification of insulin-like growth factor-I receptor human placental membranes. J Biol Chem. 261:7685–7689.
22. **Bailyes EM, Nave BT, Soos MA, Orr SR, Hayward AC, Siddle K.** 1997 Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting. Biochem J. 327:209–215.
23. **Federici M, Porzio O, Zucaro L, et al.** 1997 Distribution of insulin/insulin-like growth factor-I hybrid receptors in human tissues. Mol Cell Endocrinol. 129:121–126.
24. **Martin BC, Warram JH, Rosner B, Rich S, Soeldner JS, Krowleski AS.** 1992 Familial clustering of insulin sensitivity. Diabetes. 41:850–854.
25. **Beck-Nielsen H, Groop LC.** 1994 Metabolic and genetic characterization of prediabetic states. J Clin Invest. 94:1714–1721.
26. **Olefsky JM.** 1976 Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. J Clin Invest. 57:1165–1172.
27. **Okabayashi Y, Maddux BA, McDonald AR, Logsdon CD, Williams JA, Goldfine ID.** 1989 Mechanisms of insulin-induced insulin-receptor down-regulation. Decreased of receptor biosynthesis and mRNA levels. Diabetes. 38:182–187.
28. **Freidenberg GR, Suter SL, Henry RR, Reichart D, Olefsky JM.** 1991 *In vivo* stimulation of the insulin receptor kinase in human skeletal muscle. Correlation with insulin-stimulated glucose disposal during euglicemic clamp studies. J Clin Invest. 87:2222–2229.