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THE OVEREXPRESSION OF INSULIN RECEPTOR MAKES CHO CELLS RESISTANT TO THE ACTION OF IGF-1: ROLE OF IRS-1

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We investigated the influence of the relative abundance of insulin and IGF-1 receptors on cellular growth, by measuring the stimulation of c-fos expression and of H^3-thymidine incorporation into DNA by insulin and IGF-1 in CHO cells overexpressing Insulin Receptor (CHO-IR). We found that CHO-IR cells were resistant to the action of IGF-1, but were more responsive to insulin, compared to parental clone. This result suggested the presence of a limiting step, distal to the IGF-1 receptor, in the transduction pathway. To address this question we measured the IGF-1 effect on c-fos expression in CHO-IR cells, transiently transfected with the cDNA for IRS-1, the common intracellular substrate for both insulin and IGF-1 receptors (CHO-IR/IRS-1 cells). In these cells IGF-1 stimulated a 10 fold higher c-fos expression compared to CHO-IR cells. These results suggest that the abundance of IRS-1, relative to the number of insulin and IGF-1 receptors, represents a limiting step for the intracellular transduction of insulin and IGF-1 biological messages.

Insulin is regarded as a major regulator of cellular carbohydrate, lipid and protein metabolism, while IGF-1 is considered as a regulator of cell growth and differentiation; however, both insulin and IGF-1 have been shown to regulate similarly metabolic and mitogenic responses (1-4). These hormones and their receptors show a high degree of homology and share the early steps of intracellular signal transduction. Both receptors are tyrosine kinases and become autophosphorylated upon ligand binding (5). This process is believed to be essential for their biological activity (6-8). It has been suggested that, following tyrosine phosphorylation, these receptors physically interact and phosphorylate an intracellular protein, named thereafter the Insulin Receptor Substrate 1 (9). This docking protein is the iuxtamembrane initial step of a

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ABBREVIATIONS: CHO-IR, CHO cells overexpressing the Insulin Receptor; CHO-neo, CHO cells expressing the G418 resistance; CHO-IR/IRS-1, CHO-IR cells transiently transfected with human IRS-1 cDNA; IRS-1, Insulin Receptor Substrate 1; IR, Insulin Receptor; IGF-1 R, IGF-1 Receptor; RT-PCR, Reverse Transcriptase - Polymerase Chain Reaction.

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complex framework that leads to the biological effects of insulin and IGF-1, through a cascade of phosphorylation and dephosphorylation reactions. (9,10)

Aim of this study was to investigate the effect of overexpression of IR on the regulation of cell growth by IGF-1 and Insulin, in CHO cells. Cell growth activation has been evaluated by measuring c-fos expression (11,12) and thymidine incorporation into DNA. Here we report experimental evidences indicating that 1) the relative abundance of IRs and IGF-1Rs expressed by CHO cells is a critical determinant of the cellular responsiveness to these two ligand, 2) the availability of common intracellular effectors is limiting for signal transduction mediated by either receptors.

MATERIALS AND METHODS

Materials. Anti human IR, anti phosphotyrosine and anti IRS-1 antibodies were from UBI; anti rabbit or anti mouse Ig horseradish peroxidase linked antibodies and ECL reagents were from Amersham. Plasmids: IRS1 cDNA cloned in pcDNA I amp (Invitrogen) was kindly provided by G. Sesti (Univ. Tor Vergata Roma) and D. Accili (NIH Bethesda USA), pRSVneo was from ATCC. MLV- Reverse Transcriptase, polynucleotide kinase, Rnasin, Rnazol were from Promega; Taq polymerase was from Boehringer; P^{32}-γATP was from NEN-Dupont. All other reagents were from Sigma.

Cell cultures and transfection procedures. CHO-K1 cells, grown in DMEM medium with 10 % fetal calf serum , were cotransfected with a cDNA coding for human IR, in $pCMV_2$ plasmid (13) , and a cDNA, in pRSV plasmid, carrying G418 resistance. CHO-IR cells, stably overexpressing IR, were selected by binding studies. CHO-neo cells were used as negative control. Transient expression of human IRS1 in CHO-IR was obtained by transfection with a full lenght IRS1 cDNA. Cells were used for experimental procedures 48 h later.

Quantitative RT-PCR. After serum starvation for 16 h at 37°C, CHO-IR and CHO-neo cells were incubated with insulin or IGF-1(100 ng/ml) for 30 min at 37°C. Total RNA was extracted by RNAzol and 1 µg was reverse transcribed for 1 h at 37 °C in 20 µl of reaction volume containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2 , 50 mM KCl, 1 u/µl RNAsin, 1 pmol/µl oligo dT. RT reaction was terminated by heating at 96 °C for 5 min then samples were subjected to PCR amplification following addition of 20 pmoles of 5' end labeled upstream and downstream primers. β-Actin primer sequences were the following: upstream GAATGGGTCAGAAGGACTCC, downstream GCATGGTGTC CGTAACACTACC; c-fos primer sequences were: upstream CTATCTCCTGAA GAGGAAGAGAAACG, downstream CGTTCTAGGGGCTATGGAA. Amplification was carried out for 25 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and extension at 72°C for 1 min following the initial denaturation step at 95°C for 10 min. A 10 min elongation period at 72°C was added at the end of the cycles. PCR products were separated on a 10% acrylamide gel which was subsequently subjected to autoradiography. Bands of interest were excised and radioactivity was determined in a scintillation counter.

H^3Thymidine incorporation into DNA. CHO-IR and CHO-neo were grown to 70% confluence in 24 wells, starved for 24 h in DMEM 0.1% BSA, then incubated with indicated concentrations of insulin or IGF1 for 16 h at 37°C. Cells were then pulsed with methyl H^3 thymidine (1 µC/well) for 4 h, washed with cold PBS, fixed by TCA 10% on ice, solubilized with 1% SDS and counted for radioactivity.

Protein analysis by immunoblotting. CHO-IR and CHO-neo, serum starved for 16 h, were incubated with the indicated concentrations of insulin or IGF1 for 10 min at 37°C. Incubation was stopped by quickly removing medium and freezing cells with liquid

nitrogen. Cells were lysed in 50 mM Hepes buffer pH 7.4 containing 2% Triton X 100, 5mM EDTA, 5mM EGTA, 20 mM sodium pyrophosphate, 1mM sodium orthovanadate, 20 mM NaF, 1mM PMSF and 20 μg/ml each of aprotinin and leupeptin. Insoluble material was removed by centrifugation at 15000 xg for 30 min at 4°C . IRS-1 and IR were immunoprecipitated from supernatant with anti IRS-1 antibodies (5 μg/ml) or anti insulin receptor antibodies (1 μg/ml). An equal amount of immunoprecipitated protein were separated by SDS-PAGE and transferred to nitrocellulose for 1 h at 100 V. Non specific protein binding sites were blocked in 10% BSA. The nitrocellulose was incubated with antiphosphotyrosine antibody (1μg/ml) for 1h at room temperature. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG followed by ECL detection according to manufacturer's instructions.

Binding Studies. Cells grown in six-well plates were washed twice with PBS and incubated in 25 mM Hepes buffer, pH 7.8, in the presence of increasing amounts of insulin (0-100 ng/ml) and ^{125}I-Insulin for 3 h at 16°C. The cells were washed three times with cold PBS and solubilized in 0.1% SDS. The non specific binding, determined in the presence of 1 μg/ml insulin, was substracted to each point. From competition curves, Scatchard analysis of the data was obtained and the binding parameters were calculated.

RESULTS AND DISCUSSION

Overexpression of receptors for growth factors (insulin, IGF-1, EGF) in different cell lines renders these cells more responsive to the specific ligands, compared to the parental ones (14-17). These findings imply that components, located downstream the receptor, of the machinery responsible for the specific cellular responses are available in the transfected cells. Recently the intracellular pathways of receptors containing tyrosine kinase activity have been, at least partially, unravelled. Receptors with intrinsic tyrosine kinase activity interact with several proteins containing SH2 domains directly (EGF, PDGF receptors) or through intermediate proteins (IRS-1 in the case of IR and IGF-1R) (18). Experiments of IRS-1 overexpression (19,20) or functional black-out (21), have demonstrated that IRS-1 is important to activate cellular mitogenesis, stimulated by insulin and IGF-1. Since IR and IGF-1R are highly homologous and share the first effector protein inside the cell (IRS-1), we were interested in investigating whether IR overexpression could affect the cellular response to IGF-1. To address this question we used two cell clones, obtained by stable transfection: CHO-IR, cotransfected with the human IR cDNA and the neomycine resistance cDNA and, as a control, CHO-neo, transfected with the neomycine resistance cDNA only. These two clones, derived from the parental CHO-K1 cells, differ from each other only in the number of IRs expressed on the cell membrane. As shown in Tab. 1, CHO-neo and CHO-IR cells express 1.8 x 10^3 and 2.7 x 10^5 IRs/cell, respectively. Both cell clones express the same number of IGF-1Rs (0.4 x 10^5/cell). Fig. 1 shows that transfected IRs are functional: they can autophosphorylate and are able to phosphorylate endogenous substrates, in this case IRS-1.

TABLE 1
Comparative receptor parameters for CHO-neo and CHO-IR cells

CELL CLONE	K_D (mol/L)	R_0/cell
INSULIN RECEPTOR		
CHO-neo	-	1.8×10^3
CHO-IR	1.11×10^{-9}	2.7×10^5
IGF-1 RECEPTOR		
CHO-neo	1.1×10^{-8}	0.4×10^5
CHO-IR	1.1×10^{-8}	0.4×10^5

Studies for binding of ^{125}I-Insulin and ^{125}I-IGF-1 were performed on monolayer cultured cells as described in Methods. The receptor dissociation constant and the receptor number have been estimated by Scatchard analysis of data.

Expression of c-fos mRNA and the H^3-thymidine incorporation into DNA are markers of cell growth replication. The ability of insulin and IGF-1 to stimulate these two activities was then compared in both cell clones. As expected, in CHO-neo cells insulin stimulation shows a barely detectable effect on c-fos expression, while IGF-1 is able to induce a five fold increase in c-fos mRNA (fig. 2A). Interestingly, CHO-IR are more responsive to insulin, displaying a five fold increase in c-fos expression over basal levels, while appear to be less responsive to IGF-1, compared to CHO-neo (IGF-1 induces only a two fold increase in c-fos mRNA). Consistent with the effect on c-fos expression over basal levels, insulin stimulates an increase in H^3-thymidine uptake three fold higher in CHO-IR than CHO-neo cells , whereas the former cell clone appears to be two fold less responsive to IGF-1 than the latter (fig. 2B). These findings suggest that IR overexpression is able to make CHO cells more sensitive to insulin, but, at the same time, renders them resistant to IGF-1. Recently, it has been shown that in cultured bovine fibroblasts preincubation with insulin inhibits the mitogenic effects of IGF-1, without affecting IGF-1 binding (22). It is known that insulin and its own receptor are internalized more rapidly and effectively than IGF-1 and its own receptor (23) and therefore it could be speculated that insulin receptors interact with common intracellular effectors and, by a competitive mechanism, blunt the IGF-1

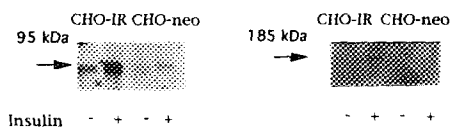

Fig. 1.
CHO-IR and CHO-neo cells were treated with insulin (100 ng/ml) for 10 min. Immunoprecipitation was carried out with anti-IR (left) or anti-IRS-1 (right) antibodies , protein were resolved on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and detected with antiphosphotyrosine antibody.

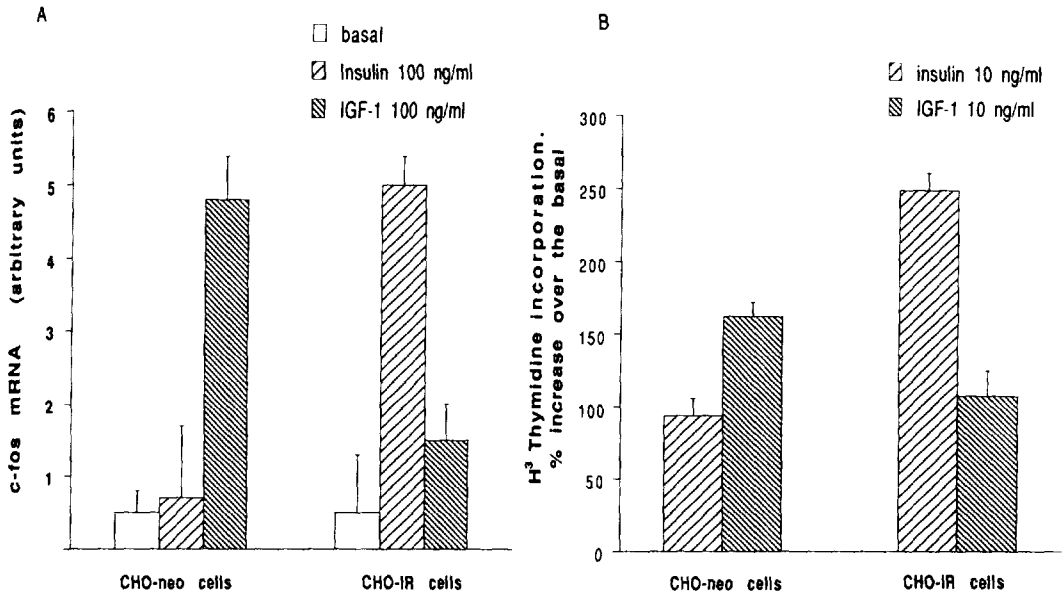

Fig. 2.
A. One microgram of total RNA prepared from CHO-neo and CHO-IR cells, treated with insulin (100 ng/ml) or IGF-1 (100 ng/ml) for 30 min at 37°C, was reverse transcribed into cDNA, subjected to PCR, using ^{32}P-labeled primers for c-fos and β-actin, and resolved on 10% polyacrylamide gel. Bands corresponding to c-fos and β-actin amplified fragments were excised and the associated radioactivity quantitated by Cerenkov counting. The data are presented as arbitrary units and are expressed as mean ± SEM. **B.** Confluent monolayers of CHO-neo and CHO-IR cells were cultured in serum-free medium for 24 h at 37°C. Cells were then incubated for 16 h in medium with Insulin or IGF-1. 1 μCi of ^{3}H-thymidine was then added for 4 h. Finally cells were rinsed and solubilized as described in Methods. Data were normalized for 10^5 cells. Data are represented as percent of control and expressed as mean ± SEM.

action. In contrast with our results, two independent studies reported that the overexpression of IR did not affect the IGF-1 action in NIH3T3 fibroblasts. The different kind of cells used could explain this discrepancy: in fact, NIH-3T3 display levels of IGF-1Rs five fold higher than CHO cells (16,17), which may be associated with a greater availability of intracellular substrates necessary for IGF-1 action. These findings, together with the present results, suggest that one or more steps distal to the insulin and IGF-1 receptors are limiting for the signal transduction to the nucleus.

IRS-1 plays an important role in the process of intracellular signal transduction of insulin and IGF-1 (10). Experimental evidences suggest that IRS-1 could represent a limiting step in this pathway, since cells overexpressing both IR and IRS-1 are more responsive to insulin than the cells overexpressing IR, only. (19). To test the hypothesis that the relatively limited abundance of IRS-1 could be the cause of the IGF-1 resistance in CHO-IR cells, we measured the effect of the transient expression of IRS-1 in CHO-IR on the IGF-1 stimulated c-fos expression. As shown in fig. 3, in

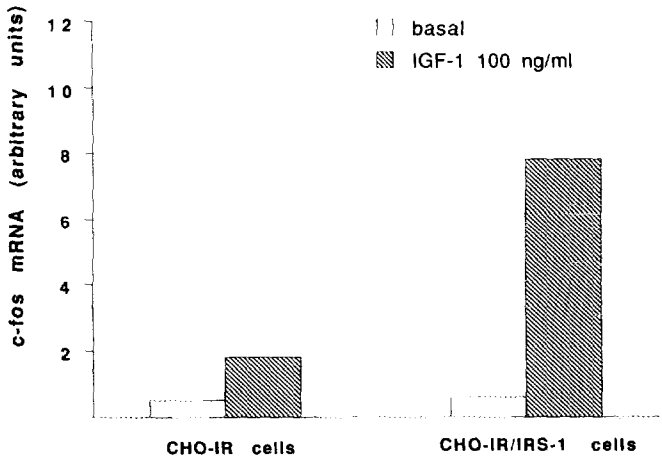

Fig. 3.
CHO-IR cells, transiently transfected with a cDNA for human IRS-1, were incubated with IGF-1 (100 ng/ml) for 30 min. RT-PCR was conducted exactly as described in the legend to Fig. 2A.

CHO-IR cells transiently expressing the human IRS-1, IGF-1 (at 100 ng/ml) stimulates a ten-fold increase in c-fos expression. This finding suggests that, not only the relative abundance of receptors for insulin and IGF-1 expressed by CHO cells, but also the relative amount of IRS-1 is critical for the cellular sensitivity to these growth factors.

In conclusion, we have demonstrated that CHO cells overexpressing the human insulin receptor are resistant to IGF-1 stimulation of cell growth. We propose that IRS-1 abundance in CHO-IR cell clone is limiting for IGF-1 action. The limited availability of IRS-1, relative to the number of receptors for insulin and IGF-1, could represent a mechanism of control of insulin and IGF-1 action.

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REFERENCES

1. Kahn, C.R. (1994) Diabetes 43, 1066-1084.
2. Froesch, E.R., Schmid, C., Schwander, J., Zapf, J. (1985) Annual Rev. Physiol. 47, 443-467.
3. Kolaczynsky, J.W., Caro, J.F. (1994) Ann. Intern. Med. 120, 47-54.
4. Pillemer, G., Lufasi-Evgi, H., Scharovski, G., Naor, D. (1992) Int. J. Cancer 50, 80-85.
5. Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., Fujita-Yamaguchi, Y. (1986) EMBO J. 5, 2503-2512.

6. Rosen, O.M. (1987) Science, 237:1452-1458.
7. Kato, I., Faria, N.T., Stannard B., Roberts C.T., LeRoith D. (1994) Mol. Endocrinol. 8, 40-50.
8. Gronborg, M., Wulff, B.S., Rasmussen, J.S., Kjeldsen, T. and Gammeltoft, S. (1993) 268, 23435- 23440.
9. Sun, X.J., Rothemberg, P., Kahn, R.C., Backer J.M., Araki E., Wilden P.A., Cahill D.A., Goldstein B.J., White M.F. (1991) Nature 352, 73-77.
10. Meyers M.G., Sun X.J., White M.F. (1994) TIBS 19, 289-293.
11. Herschman H.R. (1991) Annu.Rev.Biochem. 60, 281-319.
12.Stumpo, D.J., Blackshear, P.J. (1986) Proc. Natl. Acad. Sci. USA 83, 9453-9457.
13. Andersson, S., Davis, D.N., Dahlback, H., Jornvall, H. and Russell, D.W. (1989) J. Biol. Chem. 264, 8222-8229.
14. Osterop, A. P. R. M., Medema, R. H., Ouwens, D. M., Van der Zon, G. C. M., Moller, W. and Maassen, J. A. (1994) 33, 7453-7459.
15. Giorgino, F., Belfiore, A., Milazzo, G., Costantino, A., Maddux, B., Whittaker, J., Goldfine, I.D., Vigneri, R. (1991) Mol. Endocrinol. 5, 452-459.
16. Hofmann, C. , Goldfine, I.D., Whittaker, J. (1989) J. Biol. Chem. 264, 8606-8611.
17. Mastick, C.C., Kato, H., Roberts, C., LeRoith, D., Saltiel, A.R. (1994) Endocrinology 135, 214- 222.
18. White, M.,Kahn, C.R. (1994) J.Biol.Chem. 269, 1-4.
19. Sun, X.J., Miralpeix, M., Myers, M.G. Jr., Glasheen, E.M., Backer, J.M., Kahn, C.R., and White, M. (1992) J. Biol. Chem. 267, 22662-22672.
20. Myers, G.M. Jr., Sun, X.J., Cheatham, B., Jachna, B.R., Glasheen, E.M., Backer, J.M. and White, M.F. (1993) Endocrinology 132, 1421-1430.
21. Waters, S.B., Yamauchi, K. and Pessin, J.E. (1993) J. Biol. Chem. 268, 22231-22234.
22. Conover, C.A., Clarkson, J.T., Bale, L.K. (1994) Diabetes 43, 1130-1137.
23. Zapf, A., Hsu, D., and Olefsky, J.M. (1994) Endocrinology 134, 2445-2452.