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REGULATION OF HUMAN INSULIN RECEPTOR RNA SPLICING IN HEPG2 CELLS: EFFECTS OF GLUCOCORTICOID AND LOW GLUCOSE CONCENTRATION

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Human insulin receptor RNA is expressed in two alternatively spliced forms (Ex. 11- and Ex. 11+) encoding protein isoforms with discrete functional differences. In vivo, the splicing varies between tissues and changes concomitantly with the control of glucose homeostasis. Recently, dexamethasone has been described to increase the relative expression of Ex. 11+ RNA molecules. In this study, we confirm that dexamethasone dose-dependently increases inclusion of exon 11, and demonstrate that low concentrations of glucose decrease inclusion. No effect was observed of either insulin or IGF1. These results suggest that hormonal as well as metabolic factors regulate the splicing of insulin receptor RNA in HepG2 cells.

Alternative splicing of pre-mRNA contributes to protein isoform diversity and provides means for fine tuning of the relative abundance of gene products (1-3). Splicing patterns may vary in a tissue-specific, developmental, or temporal fashion (2). One of the most complicated cases of alternative splicing is "exon skipping", resulting in the inclusion or exclusion of an internal exon, where the regulation may involve a number of individual splice sites as well as interactions among multiple sites (2). Insulin receptor pre-mRNA is spliced in two alternative pathways differing by the inclusion or exclusion of the 36-nucleotide long exon 11 (4, 5). This results in two receptor isoforms with discrete functional characteristics (6-10). The relative abundances of the RNA variants and the corresponding protein isoforms vary coordinately and tissue-specifically (4, 5, 11, 12). Besides this variation, a number of studies have presented evidence pro and con an altered ratio of the splice variants in tissue samples from NIDDM

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patients (11, 13-17). Recently, dexamethazone was demonstrated to increase insulin sensitivity and concomitantly increase the inclusion of exon 11 in a human hepatoma cell line, HepG2 (18). In this study, we confirm the effect of dexamethasone on the alternative splicing in HepG2 cells and investigate effects of glucose, insulin, and IGF1.

Materials and Methods

Cell culture media and reagents were from Gibco (Life Technologies, Scotland) besides RPMI 1640 without glucose, which was from SVA (Sweden). Plastic materials were from Costar (USA). The HepG2 cells were cultured at 37°C and 5% CO_2/95% air in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum, 90 µg/ml penicillin, and 150 µg/ml streptomycin (complete medium). Confluent HepG2 cells in monolayer were detached from culture flasks using 0.25% trypsin in 0.02% EDTA, washed with HANK's balanced salt solution, and plated in complete medium on 60 mm petri dishes (approx. 0.5 x 10^6 cells/dish). The following day, cells were washed twice with MEM or RPMI 1640 without additives, before addition of specific medium. Exposures to IGF1 (1.35 nM recombinant human IGF1, Kabi Pharmacia, Sweden) or insulin (1.75 nM porcine insulin, Nordisk Insulin, Denmark) were performed in complete medium without fetal calf serum, but supplemented with 1% bovine serum albumin (Sigma, USA). Exposures to a synthetic glucocorticoid, dexamethasone (1.0 pM to 1.0 µM, Decadron, MSD, The Netherlands), were performed in complete medium. Exposures to varying glucose concentrations (0 - 15 mM) were performed in RPMI 1640 supplemented with 0.5% fetal calf serum, 2 mM L-glutamin, 90 µg/ml penicillin, and 150 µg/ml streptomycin. Specific medium was replaced every 48 h. After a total of four (IGF1 or insulin exposure) or six days (dexamethasone and glucose exposure) incubation, cells were washed twice with cold PBS, and RNA isolated essentially as described (19).

The protocol for determination of the alternative splicing has been described elsewhere (14). All primers were synthesized on a Gene Assembler Plus oligonucleotide synthesizer (Pharmacia, Sweden). Primers #1 and #2 were originally described by Seino et al. (4) and primer #4 by Moller et al. (5). Primers #1 and #4 were fluorescence labeled. Nucleotides are numbered according to reference 20. Essentially, cDNA was synthesized in 20 µl from approx. 2 µg of RNA with 5 nM of primer 2 (nucleotides 9290-9270, exon 14) using BRL SuperScript (Life Technologies, USA). Portions of the cDNA reactions corresponding to 0.1 - 0.4 µg RNA were subjected to PCR amplification in a final volume of 50 µl containing 50 mM KCl, 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 10 mM Tris-HCl (pH 8.4 at 70°C), 0.1% Tween 20, 0.5 µM primers #1 and #4, and 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, USA). The PCR profile consisted of 6 min at 94°C followed by 25 cycles of 1 min at 94°C, 30 s at 55°C, and 3 min at 72°C (DNA Thermal Cycler, Perkin-Elmer/Cetus, USA). Two µl of the PCR reactions were analyzed by separation on a 6% polyacrylamide gel containing 7 M urea using an A.L.F. DNA Sequencer (Pharmacia, Sweden). The PCR products corresponding to the two splice variants were quantified using the Fragment Manager software (Pharmacia,

Sweden). The method was validated by exploration of the linearity and kinetics of the amplification (14). In the present investigation, multiple parallel incubations in separate culture dishes were performed for each condition. The mean deviation from the arithmetic mean percentage of Ex. 11- RNA molecules for each incubation condition presented in Fig. 1 - 4 was 2.1% (n = 54), and the largest deviation 6.8%.

Results

Regulation of the alternative splicing of human insulin receptor RNA was studied by cDNA-mediated PCR amplification of RNA extracted from a human hepatoma cell line, HepG2. A thorough investigation of linearity and kinetics of the method permitted detection of small alterations in the relative abundance of the two splice variants, Ex. 11- and Ex. 11+ (14). In all conditions tested, 26 - 88% of the insulin receptor RNA molecules encoded the Ex.11- variant, and 74 - 12% encoded the Ex. 11+ variant (Fig. 1 - 4). Since the most significant functional distinction described between the protein isoforms is the difference in affinity to insulin and IGF1 (6, 8, 10, 21), the effects of exposure to high physiological levels of these hormones were examined (Fig.1 and Fig. 4A). Multiple separate incubations disclosed no significant difference between samples incubated in the absence or presence of these hormones. In contrast, both glucocorticoid (dexamethasone) and glucose appreciably affected the splicing. Dexamethasone, in the range from 1.0 pM to 1.0 μM, decreased the relative abundance of Ex. 11- RNA from 69 to 26% in a dose-dependent manner (Fig. 2 and Fig. 4B). The effect of glucose was not as pronounced. Severe hypoglycemia, albeit with retained growth rate of the cells, caused a sharp increase in Ex.11- RNA, while varying the glucose concentration in the range 3 - 15 mM had no significant effect (Fig. 3 and Fig. 4C).

Discussion

Multiple studies have demonstrated tissue-specific variation in the alternative splicing of insulin receptor RNA (4, 5, 11, 12). Within a single tissue, alterations in the splicing ratio have been described in NIDDM patients (11, 13-17). We have recently described an NIDDM patient displaying changes of the ratio between the two splice variants, in four consecutive skeletal muscle biopsies, concomitant with changes in glucose homeostasis (17). No genomic deletion or base substitution in either the coding region or exon/intron borders were identified, and both alleles were found to be equally expressed and spliced. However, it was not possible to

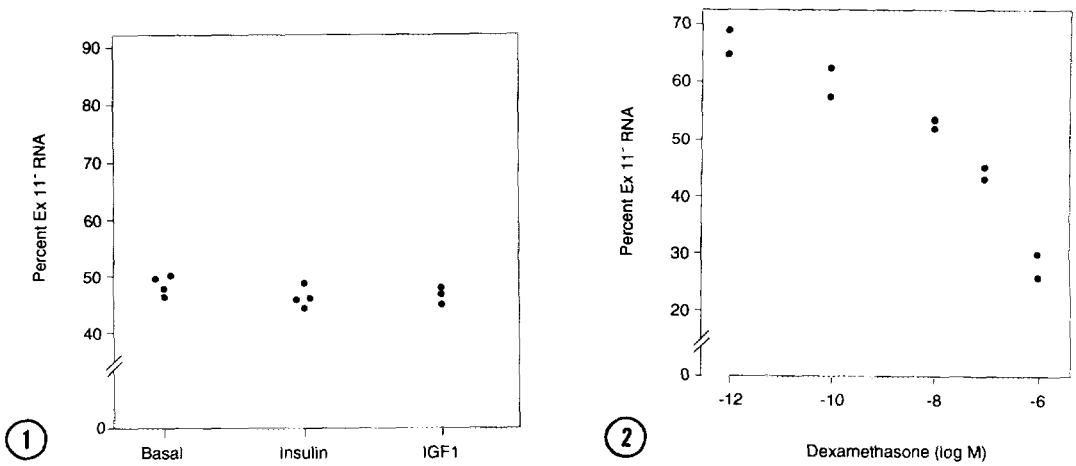

Figure 1.

Alternative splicing of insulin receptor RNA in HepG2 cells incubated in the absence or presence of 1.75 nM insulin or 1.35 nM IGF1. Each determination represents one separate four-day incubation in complete medium without fetal calf serum but supplemented with 1% bovine serum albumin. Medium was replaced every 48 h. Basal values represent parallel incubations without the addition of insulin or IGF1.

Figure 2.

Effect of dexamethasone on the alternative splicing of insulin receptor RNA in HepG2 cells. Each determination represents one separate six-day incubation. Complete medium (10% fetal calf serum) supplemented with dexamethasone was replaced every 48 h.

identify any specific factor of the in vivo metabolic milieu responsible for the splicing pattern in this patient.

In the present investigation, we describe a model for in vitro studies of alternative splicing. Contrary to other cell lines (4, 5), this subline of HepG2 cell was capable of splicing in both pathways, inclusion and exclusion of exon 11. Recently, dexamethasone, a synthetic glucocorticoid, was shown to increase inclusion of exon 11 in HepG2 and 3T3 L1 cells, possibly as a result of differentiation (18). Also in this study, the most effective regulator of alternative splicing was dexamethasone, which decreased the relative abundance of the Ex. 11- RNA in a dose-dependent manner. As determinations of the ratio between the splice variants has been a matter of great controversy (11, 13-17), the uniformity of the determinations observed by Kosaki et al. (18) and in the present study is remarkable. In addition to the effect of dexamethasone, hypoglycemia was identified to promote exclusion of exon 11. Starvation has been reported to specifically increase the insulin receptor RNA levels in HepG2 cells (22,

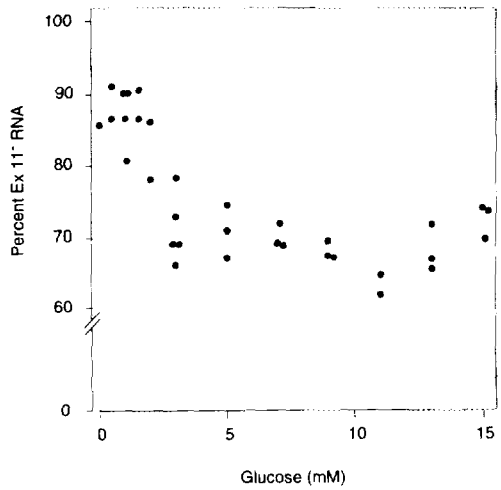

Figure 3.
Effect of glucose on the alternative splicing of insulin receptor RNA in HepG2 cells. Each determination represents one separate six-day incubation. Increasing concentrations of glucose were added to RPMI 1640 (supplied without glucose) supplemented 0.5% fetal calf serum. Medium was replaced every 48 h.

23). Thus in response to hypoglycemia, the cells not only increase the receptor biosynthesis, but also change the alternative splicing to produce more of the Ex.11- variant, which displays a higher affinity for insulin than Ex. 11+ (6, 8, 10, 21). Also dexamethasone increase insulin receptor RNA expression in HepG2 cells (24-26), and it has been speculated that this increased overall expression of insulin receptor RNA might cause the altered splicing ratio (18). However, eventhough both dexamethasone and starvation (22, 23) increase the RNA levels, the effects on the ratio between the splice variants differ. Thus the splicing alterations are not simply caused by a limited supply a specific factor required for splicing.

The trans-acting factor(s) involved in the post-transcriptional processing of insulin receptor RNA remains to be identified. However, the effects of dexamethasone and hypoglycemia may be exerted by regulation of an "exclusion factor" or "inclusion factor" (27, 28). As the effects also may result from opposite effects on differentiation, rather than specific effects on the insulin receptor RNA splicing process, putative in vivo effects of dexamethasone and hypoglycemia merit further investigation. Possibly, the drastically higher fraction of Ex.11- RNA in skeletal muscle of the very insulin resistant NIDDM patient that we have previously described was secondary to intracellular starvation (17). The fact that insulin and IGF1 did not

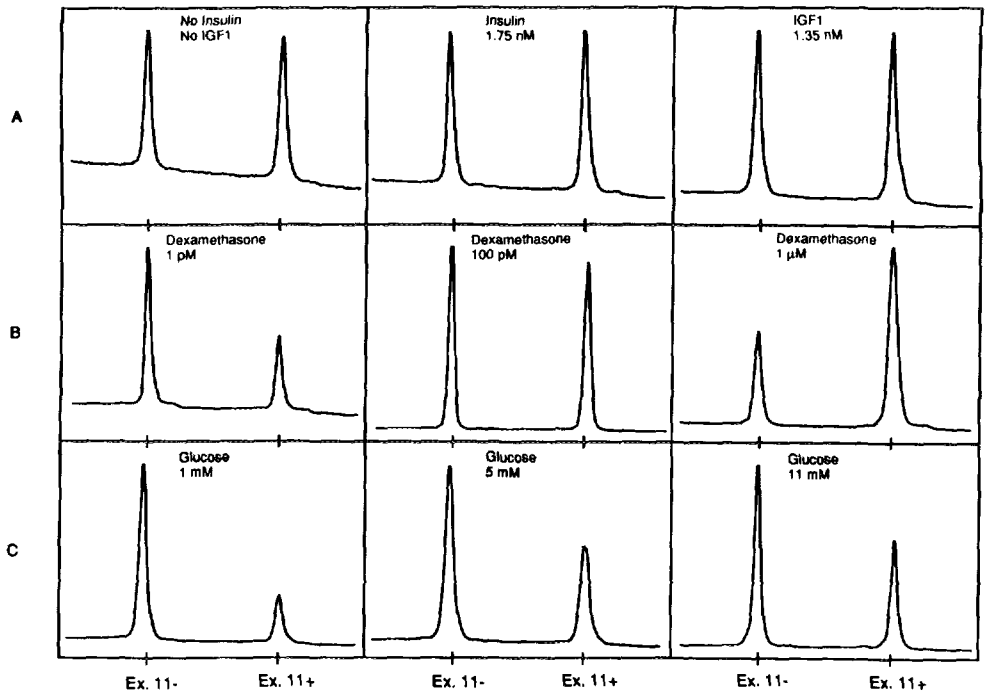

Figure 4.

cDNA-mediated PCR analysis of the insulin receptor RNA splice variants in HepG2 cells. Fluorograms represent analysis of incubations, A, with and without insulin or IGF1; B, with increasing concentrations of dexamethasone ; and, C, with increasing concentrations of glucose. The positions of the Ex. 11- and Ex. 11+are denoted below.

alter the splicing ratio is intriguing, since the protein isoforms differ in their affinities to these hormones (6-10). This also argues against the hypothesis that the low fraction of Ex. 11- RNA in the liver is caused by the high insulin concentration reaching the liver through the portal vein (6).

In conclusion, we have described a system for investigation of in vitro regulation of insulin receptor RNA splicing. Dexamethasone and low glucose concentration were demonstrated to effect the alternative splicing, while no effect of either insulin or IGF1 was observed.

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References

1 Smith, C. W. J., Patton, J. G., and Nadal-Ginard, B. (1989) Annu. Rev. Genet. 23, 527-577.

2 Green, M. R. (1991) Annu. Rev. Cell. Biol. 7, 559-599.

3 McKeown, M. (1992) Annu. Rev. Cell. Biol. 8, 133-155.

4 Seino, S. and Bell, G. I. (1989) Biochem. Biophys. Res. Commun. 159, 312-316.

5 Moller, D. E., Yokota, A., Caro, J. F., and Flier, J. S. (1989) Mol. Endocrinol. 3, 1263-1269.

6 Mosthaf, L., Grako, K., Dull, T. J., Coussens, L., Ullrich, A., and McClain, D. A. (1990) EMBO J. 9, 2409-2413.

7 Kellerer, M., Lammers, R., Ermel, B., Tippmer, S., Vogt, B., Obermaierkusser, B., Ullrich, A., and Häring, H. U. (1992) Biochemistry 31, 4588-4596.

8 Yamaguchi, Y., Flier, J. S., Yokota, A., Benecke, H., Backer, J. M., and Moller, D. E. (1991) Endocrinol. 129, 2058-2066.

9 Vogt, B., Carrascosa, J. M., Ermel, B., Ullrich, A., and Häring, H. U. (1991) Biochem. Biophys. Res. Commun. 177, 1013-1018.

10 Yamaguchi, Y., Flier, J. S., Benecke, H., Ransil, B. J., and Moller, D. E. (1993) Endocrinol. 132, 1132-1138.

11 Benecke, H., Flier, J. S., and Moller, D. E. (1992) J. Clin. Invest. 89, 2066-2070.

12 Norgren, S., Arner, P., and Luthman, H. (accepted) J. Clin. Endocriol. Metabol.

13 Mosthaf, L., Vogt, B., Häring, H. U., and Ullrich, A. (1991) Proc. Natl. Acad. Sci. USA 88, 4728-4730.

14 Norgren, S., Zierath, J., Galuska, D., Wallberg-Henriksson, H., and Luthman, H. (1993) Diabetes 42, 675-681.

15 Sesti, G., Marini, M. A., Tullio, A. N., Montemurro, A., Borboni, P., Fusco, A., Accili, D., and Lauro, R. (1991) Biochem. Biophys. Res. Commun. 181, 1419-1424.

16 Kellerer, M., Sesti, G., Seffer, E., Obermaier-Kusser, B., Pongratz, D. E., Mosthaf, L., and Häring, H. U. (1993) Diabetologia 36, 628-632.

17 Norgren, S., Zierath, J., Wedell, A., Wallberg-Henriksson, H., and Luthman, H. (in press) Proc. Natl. Acad. Sci. USA.

18 Kosaki, A. and Webster, N. J. G. (1993) J. Biol. Chem. 268, 21990-21006.

19 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.

20 Seino, S., Seino, M., and Bell, G. I. (1990) Diabetes 39, 123-128.

21 McClain, D. A. (1991) Mol. Endocrinol. 5, 734-739.

22 Hatada, E. N., McClain, D. A., Potter, E., Ullrich, A., and Olefsky, J. M. (1989) J. Biol. Chem. 264, 6741-6747.

23 Levy, J. D., Krystal, G., Glickman, P., and Davstan, F. (1991) Diabetes 40, 58-65.

24 Rouiller, D. G., McKeon, C., Taylor, S. I., and Gorden, P. (1988) J. Biol. Chem. 263, 13185-13190.

25 McDonald, A. R., Maddux, B. A., Okabayashi, Y., Wong, K. Y., Hawley, D. M., Logsdon, C. D., and Goldfine, I. D. (1987) Diabetes 36, 779-781.

26 McDonald, A. R. and Goldfine, I. R. (1987) J. Clin. Invest. 81, 499-504.

27 Moore, M. J., Query, C. C., and Sharp, P. A. (in press) RNA World, Cold Spring Harbour Press.

28 Nordqvist, K., Öhman, K., and Akusjärvi, G. (in press) Mol. Cell. Biol.