Differential Half-Maximal Effects of Human Insulin and Its Analogs for In Situ Glucose Transport and Protein Synthesis in Rat Soleus Muscle

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Analogs of human insulin have been used to discriminate between responses of metabolic and mitogenic (growth-related) pathways. This study compared the stimulatory effects of human insulin (HI) and 2 analogs (X2, B-Asp^{9}, B-Glu^{27} and H2, A-His^{8},B-His^{4},B-Glu^{10}, B-His^{27}) on glucose uptake and protein synthesis in rat soleus muscle in situ. Glucose uptake, estimated by intramuscular (IM) injection of 2-deoxy[1,2-^{3}H]glucose with or without insulin, was maximally increased at 10^{-6} mol/L for HI and X2 and 10^{-7} mol/L for H2. HI had a larger effect (318%) than either X2 (156%) or H2 (124%). The half-maximal effect (ED_{50}) values for HI, X2, and H2 were 3.3×10^{-8} mol/L, 1.7×10^{-7} mol/L, and 1.6×10^{-9} mol/L, respectively. Protein synthesis, estimated by protein incorporation of [^{3}H]phenylalanine injected into muscles with or without insulin, was maximally increased at 10^{-5} mol/L for HI and 10^{-6} for X2 and H2. HI had a larger effect in stimulating protein synthesis (34%) than either X2 (25%) or H2 (19.8%). The ED_{50} values for HI, X2, and H2 were 3.0×10^{-7} mol/L, 3.2×10^{-7} mol/L, and 1.0×10^{-9} mol/L, respectively. The biological potency of each analog (ED_{50}insulin/ED_{50}analog) showed X2 to be less potent than HI for both glucose uptake (0.2) and protein synthesis (0.9), whereas H2 is more potent than HI with ratios of 20 and 300, respectively. These data suggest that this approach for studying insulin responsiveness in a single muscle in situ may be a useful tool for investigating insulin signaling in muscle in vivo.

HUMAN INSULIN and 6 of its recombinant DNA analogs have been used to compare metabolic and mitogenic potencies.[1] Hansen et al estimated metabolic potency by measurement of 3-O-methylglucose transport into rat adipocytes and mitogenic potency by incorporation of thymidine into DNA of Chinese hamster ovary (CHO) cells.[1] Several of these analogs showed a greater ratio of mitogenic to metabolic potency when compared with human insulin. These ratios of mitogenic to metabolic potency correlated inversely, on an exponential plot, with the dissociation rate constant (K_d) for the binding of the analog to the insulin receptor. The mitogenic potency was particularly increased with analog H2 (A-His^{8},B-His^{4},B-Glu^{10}, B-His^{27}). The H2 analog had a ratio of mitogenic to metabolic potency of 6.8 in accord with a very slow rate constant that was just 1.5% of the value determined in CHO cells for human insulin. In contrast, analog X2 (B-Asp^{9},B-Glu^{27}) showed a dissociation rate constant that was 39% faster than for human insulin and a mitogenic potency similar to the metabolic potency (ratio of 1.3). The authors also demonstrated that extended binding of the analogs, such as H2, also prolonged activation of the insulin receptor tyrosine kinase, receptor autophosphorylation and phosphorylation of Shc, whereas phosphorylation of insulin receptor substrate-1 (IRS-1) decayed. Therefore, the signal transduction pathway associated with Shc phosphorylation seemed linked to the especially elevated mitogenic potency.

In previous studies, we have considered responses of carbohydrate and protein metabolism in soleus muscle to insulin.[2-7] As an extension of other studies, we have evaluated the potential utility of analogs H2 and X2, compared to human insulin, to distinguish between insulin effects on a metabolic (glucose uptake) and a growth-related process (protein synthesis). This has been accomplished by attempting to reproduce in situ the qualitative differences in responses to these analogs as was obtained using in vitro methodology. To this end, we determined the half-maximal effect (ED_{50}) values for human insulin and the 2 analogs for uptake of 2-deoxy[1,2-^{3}H]glucose and incorporation of [^{3}H]phenylalanine into protein by the soleus muscle, and compared them to the ED_{50} values obtained for glucose uptake by adipocytes (metabolic process) and DNA synthesis (mitogenic/growth-related process) as described above.[1] As noted, the analogs selected bind much more strongly (66-fold) or more weakly (30% lower), respectively, than human insulin.[1]

MATERIALS AND METHODS

Animals

Procedures were approved by the University of Arizona Animal Care and Use Committee. Juvenile female Sprague-Dawley rats (~100 g; National Cancer Institute, Frederick, MD) were used and maintained on food and water ad libitum. Soleus muscles were studied for consistency with previous studies from this laboratory.[8-10]

In Situ Incubation

As described previously,[9] to inject the soleus, a 5-mm incision was made in the outer side of the shaved, ethanol-swabbed hindlimb of rats anesthetized with xylazine (8 mg/kg) plus ketamine (63 mg/kg). After the underlying fascia was cut, the soleus was exposed by gently

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hooking it with fine forceps. The belly of the muscle was injected, as described below, using a 10-μL Hamilton syringe equipped with a 26-gauge bevel-tipped needle. Incisions were covered by gauze wetted with warm (37°C) isotonic saline solution. Rats were then placed under an incandescent light bulb to help maintain their body temperature during the incubation period.

Uptake of 2-deoxyglucose was estimated in situ as described previously.[8] Contralateral muscles were injected (4 μL/100 g body weight) with a solution containing 20 mmol/L 2-deoxy[1,2-^{3}H]glucose (10 μCi/mL; New England Nuclear, Boston, MA), 0.1% bovine serum albumin (BSA), 150 mmol/L NaCl, and ^{14}C-mannitol (1 μCi/mL; New England Nuclear) in the absence or presence of human insulin or one of its analogs at the indicated concentrations. These analogs included X2 (B-Asp^9,B-Glu^{27}) and H2 (A-His^8,B-His^4,B-Glu^{10}, B-His^{27}) (Novo-Nordisk, Bagsvaerd, Denmark). In each rat, the left soleus served as the control (without insulin), while the right muscle received hormone treatment.

In separate experiments, protein synthesis was estimated from the incorporation of [^{3}H]phenylalanine into total protein in situ using the intramuscular (IM) flooding dose approach.[8] Muscles were injected (4 μL/100 g body weight) with a solution containing 84 mmol/L L-[2,6-^{3}H]phenylalanine (104 μCi/mL; Amersham, Piscataway, NJ), 0.1% BSA, and 110 mmol/L NaCl in the absence or presence of human insulin or one of its analogs at the indicated concentrations. These analogs included X2 and H2. Again, in each rat, the left soleus served as the control (without insulin), while the right muscle received hormone treatment.

Twenty minutes after IM injection, animals were killed by cervical dislocation and the middle approximately two thirds of the soleus excised. Previous experiments determined that 89% ± 3% of injected radioactivity resided in this portion of the muscle. Consequently, excision of the entire muscle would underestimate the process being measured.[8]

Analysis of Treated Muscles

After excision, muscles injected with 2-deoxyglucose were immediately frozen in liquid nitrogen, homogenized in 1 mL of 10% trichloroacetic acid (TCA), and then centrifuged for 5 minutes at 3,000 × *g*. A 700-μL aliquot of the supernatant was added to 5 mL of Ecolume (ICN, Aurora, OH) and analyzed for ^{3}H and ^{14}C radioactivity. The protein pellet was washed once with 2 mL of 10% TCA and once with 2 mL of ethanol:ether (1:1). After drying and then solubilizing the protein pellets in 1 mL of 0.5 N NaOH, total protein was assayed by the Biuret procedure.[11]

For protein synthesis measurements, after excision, muscles were homogenized in 1 mL of 10% TCA and then centrifuged for 5 minutes at 3,000 × *g*. The protein pellet was washed once with 2 mL of 10% TCA and once with 2 mL of ethanol:ether (1:1). After drying and then solubilizing the protein pellets in 1 mL of 0.5 N NaOH, a 500-μL aliquot of the solubilized pellet was added to 5 mL of Ecolume and analyzed for ^{3}H radioactivity. Total protein of the remaining solubilized pellet was assayed by the Biuret procedure.[11]

Analysis of Data

Uptake of 2-deoxyglucose was calculated as pmol/mg protein/20 minutes.[8] 2-Deoxy[1,2-^{3}H]glucose in the interstitial fluid was corrected using the ^{14}C-mannitol data. The rate was then determined using the specific activity of the injected 2-deoxy[1,2-^{3}H]glucose.

Protein synthesis was calculated as dpm/mg protein/20 minutes. We did not calculate rates using intracellular specific radioactivity because in other experiments we found that the intracellular specific activity for phenylalanine, with the flooding dose technique, is similar in contralateral muscles. In a typical experiment, the values were 4.37 ± 0.59 and 4.31 ± 0.48 dpm/μmol for contralateral soleus muscles of 5 animals. Similar intracellular specific activity is achieved because the amount of radioactive phenylalanine injected into the muscle (~40 nmol/mg protein) is much greater than the concentration of intracellular phenylalanine (~1.6 nmol/mg protein) that we have measured. Since the current experiments compared the effects of insulin in paired muscles, the percent stimulation would be no different for data expressed simply as radioactive incorporation versus fractional rates of synthesis.[8] Hence we chose the simpler approach for this study.

A paired Student's *t* test was used to compare the results for contralateral muscles within each treatment group. Analyses were performed with Statview 5.0 (SAS Institute, Chicago, IL). Data for percent effects on 2-deoxyglucose uptake and phenylalanine incorporation are presented as the mean ± SEM. The few data points, of the more than 100 collected in 2-deoxyglucose uptake and protein synthesis experiments, that were more than 1.75 SD above the mean (outside 99% confidence interval) were excluded because these outlying points resulted from abnormally low baseline rates, as determined by averaging all of the baseline values. Because the percent hormone effects were determined individually for each animal through paired analysis, an abnormally low basal value would increase the stimulation value by several-fold, thus abnormally skewing the data point for that concentration. No points below the mean exceeded 1.75 SD. The injected concentration that elicited half the maximum effect (ED_{50}) was estimated from a log-log plot versus the mean percent of maximum stimulation at each injected concentration. Analysis of variance (ANOVA) was performed for each condition.

RESULTS AND DISCUSSION

Uptake of 2-Deoxyglucose

Initial studies determined the concentration at which a maximal response to insulin or its analogs could be observed. Because injections of human insulin and X2 at 10^{-5} mol/L produced a smaller response than at 10^{-6} mol/L (data not shown), effects at the latter concentration were assumed to be maximally effective. Such inhibitory effects of high concentrations of insulin on glucose uptake have been observed by us in vitro as well.[2] Because the effect of H2 at 10^{-7} mol/L and 10^{-6} mol/L were similar, the lower concentration was used as the maximally effective dose.

The stimulatory effect of 10^{-6} mol/L human insulin on 2-deoxyglucose uptake was 318% ± 75%, which was used as the maximal response value (Fig 1A). At 10^{-7} mol/L, this effect declined by about one third to 206% ± 88%. At concentrations of 10^{-11} to 10^{-9} mol/L, there were no significant differences in the hormone effect, with values averaging between 43% to 59%. When these data for 10^{-11} to 10^{-6} mol/L were plotted as a percent of maximal stimulation, an ED_{50} of 3.3×10^{-8} mol/L was obtained (Fig 1B).

Similar experiments were conducted with the analogs of human insulin. The analogs all showed smaller maximal percent effects than did human insulin. Analog X2, which binds more weakly than human insulin,[1] had a maximal effect of 156% ± 24% at 10^{-6} mol/L (Fig 1C). For X2, the initial 10-fold decline in concentration lowered its effect by nearly half to 82% ± 13%. Unlike human insulin, which was still effective at 10^{-11} mol/L, X2 showed no significant effect at 10^{-9} mol/L (3% ± 2%). When these data were plotted as a percent of maximal stimulation an ED_{50} of 1.7×10^{-7} mol/L was obtained (Fig 1D). H2, which binds most strongly,[1] showed no difference in

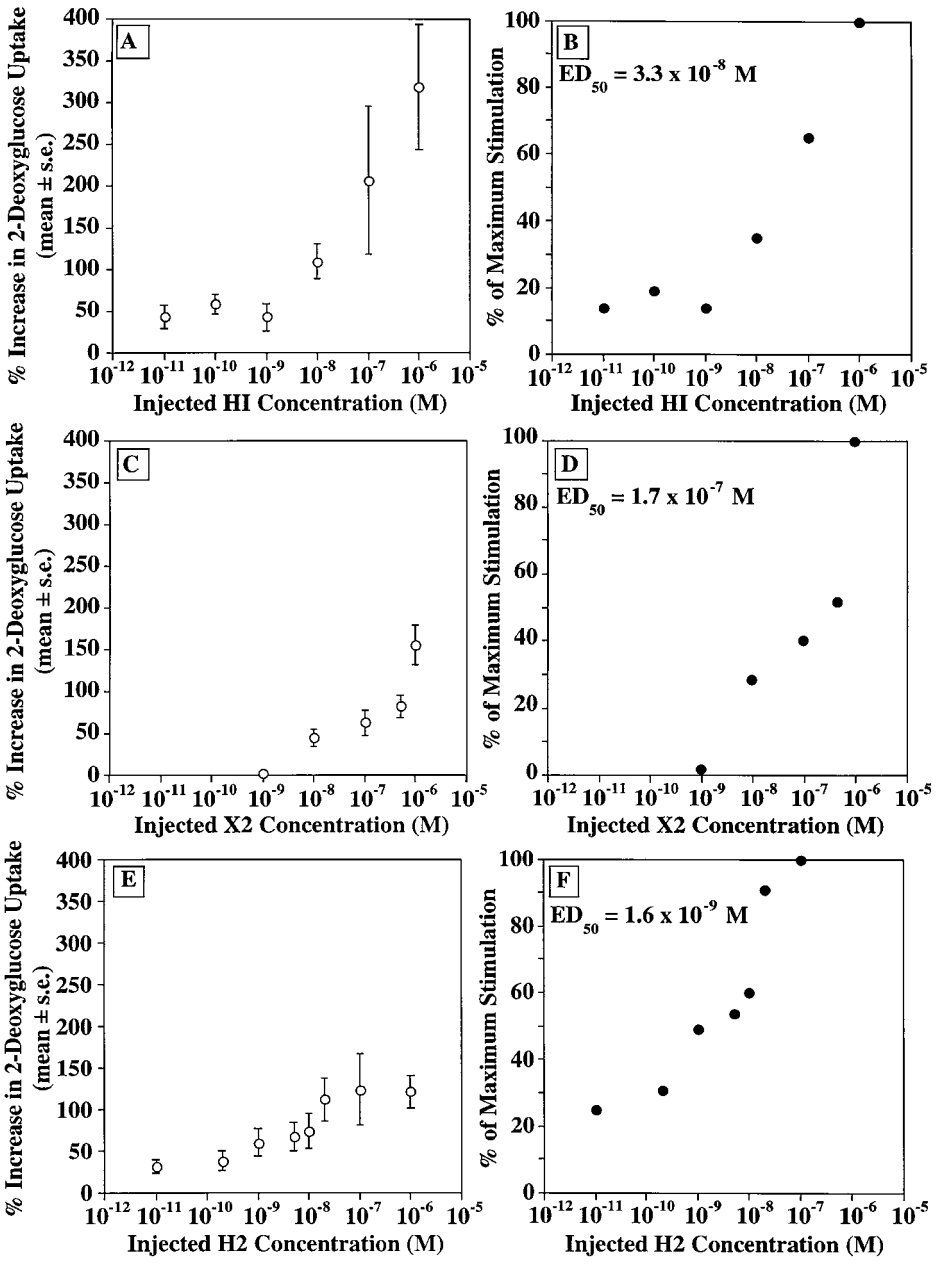

Fig 1. Response of 2-deoxyglucose uptake to human insulin (HI) and analogs X2 and H2. (A, C, E) Data are the percent increase in 2-deoxyglucose uptake due to hormone administration. Experiments were conducted as described in the Methods. (B, D, F) Data are the percent of maximal stimulation at each concentration of hormone. (B) The ED_{50} was calculated from a plot of log-log data where log [HI] = -15.694 + 4.835 · log [percent maximal stimulation]; R^2 = .866, P < .01. Four to 18 rats were tested at each concentration. More animals were used at 10^{-6} mol/L because this concentration was tested in more experiments for determining the concentration for the maximal response. A concentration of 10^{-5} mol/L had a lesser stimulatory effect on 2-deoxyglucose uptake than did 10^{-6} mol/L (not shown). Thus 10^{-6} mol/L was chosen as the maximally effective dose. (D) The ED_{50} was calculated from log [X2] = -9.73 + 1.74 · log [percent maximal stimulation]; R^2 = .843, P < .05. A concentration of 10^{-5} mol/L had a lesser stimulatory effect on 2-deoxyglucose uptake than did 10^{-6} mol/L (not shown) and thus 10^{-6} mol/L was used as the maximally effective concentration. Five or 6 rats were used at each concentration. Only 3 animals were tested at the lowest concentration, which elicited no response. (F) The ED_{50} was calculated from log [H2] = -18.78 + 5.88 · log [percent maximal stimulation]; R^2 =.936, P < .001. The stimulatory effects at 10^{-7} mol/L and 10^{-6} mol/L were the same (124% *v* 121%) so 10^{-7} mol/L was used as the maximally effective concentration. Four to 7 rats were used at each concentration.

the percent effect at 10^{-7} mol/L (124% ± 43%) and at 10^{-6} mol/L (121% ± 19%) (Fig 1E). Thus, H2 produced its maximal effect at least at a 10-fold lower concentration than did human insulin or the other analogs tested. Like human insulin, H2 still had a significant effect at 10^{-11} mol/L, which was still 25% of maximum (Fig 1F), while human insulin at this concentration produced an effect just 14% of maximum (Fig 1B). Accordingly, H2 had an ED_{50} more than 10-fold lower (1.6×10^{-9} mol/L) than that estimated for human insulin (Fig 1F).

Phenylalanine Incorporation

Initial studies determined the concentration at which a maximal response to insulin or its analogs could be observed for phenylalanine incorporation. X2 at 10^{-5} mol/L produced a smaller response than at 10^{-6} mol/L. Protein synthesis effects were greatest at 10^{-5} mol/L for human insulin and 10^{-6} mol/L for H2.

The effect of 10^{-5} mol/L human insulin on phenylalanine incorporation was 33.9% ± 5.5%, which was used as the maximal response value (Fig 2A). At 5×10^{-6} mol/L this effect declined by about one third to 23.9% ± 3.8%. At 10^{-8} mol/L the hormone effect was 9.2% ± 2.0%. When these data for 10^{-8} to 10^{-5} mol/L were plotted as a percent of maximal stimulation, an ED_{50} of 3.0×10^{-7} mol/L was obtained (Fig 2B).

Similar experiments were conducted with 2 analogs of human insulin, X2 and H2. Both analogs showed smaller maximal

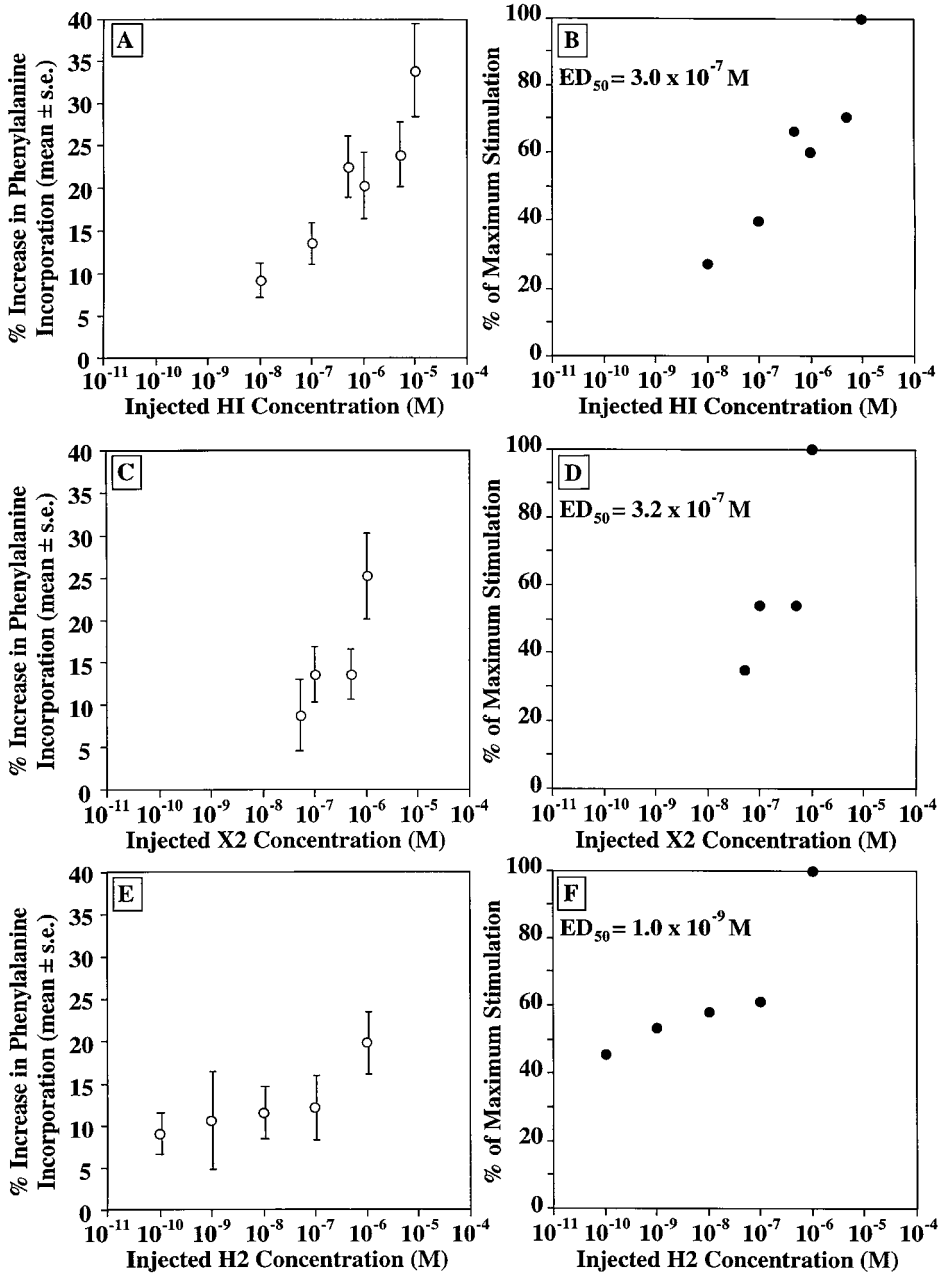

Fig 2. Response of phenylalanine incorporation into protein to human insulin and analogs X2 and H2. Experiments were conducted and data presented as in Fig 1. (B) The ED_{50} was calculated from log [HI] = -15.65 + 5.37 · log [percent maximal stimulation]; R^2 = .936, P < .01. The effect at 10^{-5} mol/L was used as the maximally effective concentration. Three to 12 rats were used at each concentration. (D) The ED_{50} was calculated from log [X2] = -6.94 + 0.26 · log [percent maximal stimulation]; R^2 = .117, P < .05. The effect at 10^{-6} mol/L was used as the maximally effective concentration. Six to 16 rats were used at each concentration. (F) The ED_{50} was calculated from log [H2] = -28.17 + 11.28 · log [percent maximal stimulation]; R^2 = .838, P < .05. The effect at 10^{-6} mol/L was used as the maximally effective concentration. Seven to 13 rats were used at each concentration.

percent effects than did human insulin. Analog X2, which binds more weakly than human insulin,[1] had a maximal effect of 25.2% ± 5.0% at 10^{-6} mol/L (Fig 2C). For X2 a 5-fold decline in concentration lowered its effect by nearly half to 13.6% ± 3.0%. At 5×10^{-8} mol/L the hormone effect was 8.7% ± 4.2%. When these data were plotted as a percent of maximal stimulation an ED_{50} of 1.6×10^{-7} mol/L was obtained (Fig 2D). H2, the analog which binds most strongly,[1] produced its maximal effect at 10^{-6} mol/L (Fig 2E). H2 had an ED_{50} more than 100-fold lower (1.0×10^{-9} mol/L) than that estimated for human insulin (Fig 2F).

It is noteworthy that in vivo studies by other investigators failed to demonstrate insulin stimulation of muscle protein synthesis in humans[12,13] or in fed rats.[14] However, insulin did stimulate protein synthesis in muscle of fasted rats.[14,15] In both a previous[7] and the current study, we have been able to demonstrate insulin stimulation of protein synthesis in vivo in muscle of fed rats. The discrepancy may reflect a major difference in the experimental technique. The studies of others introduced insulin by infusion, which produces appropriate systemic responses of other hormones and factors. We instead have studied the insulin effect on muscle protein synthesis directly by IM injection. Hence we introduce no additional systemic changes that might offset the direct effect of insulin. This is not to say one approach is better than another, but reflects the different objectives of these studies.

Table 1. Relative ED_{50} Values for Stimulation of Glucose Transport and Growth-Related Pathways Compared for In Situ Studies in Skeletal Muscle and In Vitro Studies Using Primary Adipocytes or CHO Cells

	Relative ED_{50} (HI ED_{50}/analog ED_{50})				
	Glucose Transport		Growth-Related Pathways		
Analog	In Situ	In Vitro	In Situ	In Vitro	Relative K_d (%)
X2	0.2	0.4	0.9	0.6	139
H2	20.6	4.3	300.0	29.5	1.5

NOTE. In situ muscle data are taken from Figs 1 and 2. In vitro data using primary adipocytes (glucose transport, uptake of 3-O-methylglucose) and CHO cells (growth-related, thymidine incorporation into DNA or relative K_d, analog K_d/HI K_d) are shown for these same analogs.[1]

Comparison to In Vitro Data

These data demonstrate that it is possible to compare in skeletal muscle in situ the relative ED_{50} values for glucose uptake and protein synthesis for human insulin with analogs that possess varying receptor binding affinities. Qualitatively, our data agree with the study of glucose uptake in primary rat adipocytes.[1] The relative ED_{50} values in our study parallel the analog receptor binding as analyzed by the relative K_d values. Thus, the H2 analog, which has a much smaller K_d (1.5% of human insulin),[1] had an ED_{50} for glucose uptake and protein synthesis that was 20- to 30-fold lower, respectively, than that measured for human insulin in our study. Glucose uptake and protein synthesis in muscle in situ were far more sensitive to H2 than was glucose uptake and DNA synthesis measured in vitro (Table 1). In contrast, X2 in muscle in situ produced relative effects on glucose uptake and protein synthesis that were similar to those seen for glucose uptake and DNA synthesis. A possible explanation for this observation could be differential binding of the analogs X2 and H2 to different isoforms of the insulin receptor. Indeed there is direct evidence for structural variation of the human receptor based on cDNA[16,17] and mRNA data.[18,19] Studies of the rat insulin receptor show 2 forms as well and these authors concluded that subtle variances in the primary structure of the receptor might exhibit property differences between species.[20] Since our in situ data compares different tissues, processes, and sources, it would not be unexpected that there are receptor differences that might be reflected in their responses to analogs with widely different binding characteristics.

In adipocytes the ED_{50} for glucose uptake for human insulin was 1.03×10^{-10} mol/L,[1] a value 2 orders of magnitude lower than we determined in situ (3.3×10^{-8} mol/L; Fig 1). It is noteworthy that our ED_{50} value is based on the concentration injected. Dilution by the interstitial space alone lowers the concentration by at least an order of magnitude. Additional important differences in the studies are in situ versus in vitro, uptake of 2-deoxyglucose versus 3-O-methylglucose, and intact muscle tissue versus adipose cells. The importance of differences in cells and processes studied is evidenced by the fact that the ED_{50} for human insulin for thymidine incorporation into DNA in CHO cells was 20-fold greater than for glucose uptake by adipocytes.[1]

Future Studies

Future studies will use these analogs to differentiate between metabolic (glucose uptake) and growth (protein synthesis) responses of the soleus muscle in different physiological or pathological states. In particular, we plan to evaluate mechanisms of altered insulin responsiveness in unweighting atrophying. Unweighting of the juvenile soleus muscle causes increased insulin sensitivity of glucose transport[2,3] due to retention of insulin receptors[2] and GLUT-4 transporter protein.[21] This response to unweighting is associated, in vitro, with increased effects of insulin on protein metabolism[4] and on the transport activity and metabolism of glucose.[2-6,21,22]

In vivo studies support most of these observations. Unweighting of the soleus produced a marked increase in uptake of 2-deoxy[1,2-^{3}H]glucose in response to IM injection of insulin.[7] Other studies suggested that in vivo, as in vitro, protein degradation is more responsive to insulin due to the relative increase in insulin binding.[2,8,9] In contrast, in vivo protein synthesis in the soleus seems resistant to the expected increased responsiveness to insulin[7] that is observed in vitro.[4,9] Use of these analogs may help to identify the mechanisms of this anomalous response of protein synthesis.

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