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Growth promoting and metabolic activity of the human insulin analogue [Gly^{A21},Arg^{B31},Arg^{B32}]insulin (HOE 901) in muscle cells

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Abstract

[Gly^{A21},Arg^{B31},Arg^{B32}]insulin (HOE 901) represents a biosynthetic human insulin analogue that, due to its isoelectric point, precipitates at neutral tissue pH leading to a retarded absorption rate and a corresponding longer duration of action. In the present investigation we have evaluated the growth promoting and metabolic activity of this analogue in muscle tissue using exponentially growing H9c2 cardiac myoblasts and adult rat ventricular cardiomyocytes. Equilibrium binding studies of ^{125}I-labelled IGF-I (insulin-like growth factor I) to differentiating myoblasts revealed the presence of 7×10^3 IGF-I receptors per cell. In contrast, no specific binding of insulin could be detected. Competition binding experiments showed a slightly higher affinity of HOE 901 for the IGF-I receptor when compared to regular human insulin with IC_{50} (half-inhibitory concentration) values of 70 and 101 nM, respectively. However, the supermitogenic insulin analogue [Asp^{B10}]insulin competed significantly more efficiently for IGF-I binding (IC_{50}: 44 nM). Maximum growth promoting activity of the peptides was then determined in serum-starved myoblasts by an incubation with the peptides (5×10^{-7} M) for 16 h in the presence of [^{3}H]thymidine. [Asp^{B10}]Insulin produced a stimulation of DNA synthesis (about 3-fold) which was comparable to the effect of IGF-I and significantly ($P < 0.005$) higher than the effect of HOE 901 with the latter being essentially equipotent to native insulin. Comparable results were obtained at lower concentrations of the peptides (10^{-9} to 10^{-8} M). Metabolic activity of HOE 901 was determined by measuring the dose-dependent stimulation of 3-*O*-methylglucose transport in adult cardiomyocytes. Maximum transport stimulation was identical for insulin and HOE 901 with EC_{50} (half-effective concentration) values of 0.7×10^{-10} and 1.9×10^{-9} M, respectively. We concluded that the IGF-I receptor-mediated growth promoting activity of HOE 901 in muscle cells and the maximal metabolic activity of this analogue are not different from those of native human insulin. It is suggested that differential interaction with IGF-I receptors significantly contributes to the action profile of insulin analogues.

Keywords: Insulin analog; Insulin; Mitogenic activity; Cardiomyocyte; Insulin-like growth factor receptor

1. Introduction

Effective glycemic control has been recognized to play a pivotal role in forestalling the chronic microvascular complications of diabetes (The DCCT Research Group, 1993). Unfortunately, currently available insulins are unable to simulate the pattern of endogenous insulin secretion and metabolic action (for review, see Galloway, 1993; Galloway and Chance, 1994). Recombinant DNA technology has permitted the design of novel insulin molecules with altered physico-chemical, biological and pharmacodynamical properties that might serve to substantially improve glycemic control (Galloway, 1993).

Modification of the B-chain of the insulin molecule was shown to produce insulin analogues with reduced self-association (Galloway, 1993; Brange et al., 1988) leading to the development of [Lys^{B28},Pro^{B29}]insulin, a rapidly absorbed analogue of human insulin (Howey et al., 1994). On the other hand, a significant clinical need exists for a long-acting insulin analogue simulating basal insulin secretion (Galloway, 1993). By shifting the isoelectric point of the insulin molecule towards neutral pH a retarded absorption and protracted action can be obtained (Grau, 1985). [Gly^{A21}, Arg^{B27},Thr^{B30}-NH2]insulin (Jorgensen et al., 1989) and diarginylinsulin (Zeuzem et al., 1990; Monti et al., 1992) were reported to fulfill these requirements, however, restricted bioavailability strongly limits the po-

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tential clinical use. [Gly^{A21},Arg^{B31}, Arg^{B32}]Insulin (HOE 901) is a novel human insulin analogue with protracted action. The substitution at position A21 was found to largely increase the bioavailability of this insulin analogue (Hilgenfeld et al., 1992; Seipke et al., 1992) which was reported to exhibit a protracted action profile in dogs and rabbits twice as potently as NPH insulin (Seipke et al., 1992). Recent clinical data suggest that HOE 901 seems to be an ideal candidate for basal insulin substitution with a daily single injection (Talaulicar et al., 1994).

Any modification of the insulin molecule can lead to an altered interaction with the insulin receptor and the structurally homologous IGF-I (insulin-like growth factor I) receptor, probably resulting in an altered action profile. Specifically, the mitogenic activity of insulin analogues requires careful control, since one such analogue, [Asp^{B10}]insulin, has been observed to possess carcinogenic properties in vivo (Dideriksen et al., 1992). In differentiating muscle tissue the mitogenic action of insulin is predominantly mediated by the IGF-I receptor (Bornfeldt et al., 1991). In the present investigation we have therefore evaluated (1) the interaction of HOE 901 with IGF-I receptors on H9c2 myoblasts, (2) the effect of HOE 901 on DNA synthesis in these cells, and (3) the metabolic potency of this insulin analogue in adult cardiomyocytes. The data clearly show that the growth promoting and maximal metabolic activity of HOE 901 is comparable to that of native human insulin.

2. Materials and methods

2.1. Chemicals

3-*O*-[^{14}C]Methyl-D-glucose (54 mCi/mmol), L-[1-^{14}C]glucose (55 mCi/mmol), ^{125}I-labelled human insulin (2000 Ci/mmol), ^{125}I-labelled insulin-like growth factor I (IGF-I) (2000 Ci/mmol) and [*methyl*-^{3}H]thymidine (86 Ci/mmol) were purchased from Amersham (Braunschweig, Germany). Collagenase (EC 3.4.24.3) was a product of Serva (Heidelberg, Germany). Fetal calf serum, Dulbecco's modified Eagle medium (DMEM) and non-essential amino acids were purchased from Gibco (Eggenstein, Germany). Human recombinant IGF-I was obtained from Calbiochem (Bad Soden, Germany). Human insulin, HOE 901 and [Asp^{B10}]insulin were prepared by Hoechst (Frankfurt, Germany) using recombinant DNA techniques. The H9c2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). All other chemicals were of the highest grade commercially available.

2.2. Cell culture and isolation of cardiomyocytes

The spontaneously fusing rat heart muscle cell line H9c2 (Kimes and Brandt, 1976) was kept in monolayer culture in DMEM supplemented with 10% fetal calf serum, non-essential amino acids (1%), streptomycin (100 μg/ml) and penicillin (100 U/ml) in 175-cm^2 flasks in an atmosphere of 5% CO_2 at 37°C. Myoblasts were maintained in continuous passages (< 15) by trypsinization of subconfluent cultures 7 days after plating. The medium was changed every 72 h. Cell number was determined after cell dissociation with trypsin/EDTA at 37°C.

Adult rat cardiomyocytes were isolated by perfusion of the heart with collagenase, as previously described (Eckel et al., 1983). Male Wistar rats weighing 280–320 g were used in all experiments.

2.3. Hormone binding assays

For all experiments on IGF-I and insulin receptor binding, myoblasts were plated at a density of 1×10^5 cells/9.6 cm^2 in multi-well culture dishes. After 48 h in culture, cells were washed two times with phosphate-buffered saline (PBS) and incubated for 60 min at 37°C in DMEM without fetal calf serum containing 0.5% bovine serum albumin. ^{125}I-Labelled IGF-I (1.7×10^{-11} M) or ^{125}I-labelled insulin (5×10^{-11} M) were then added and incubation was continued in the absence or presence of the indicated peptide hormones for 90 min at 37°C. The medium was then removed, the monolayers were washed twice, the cells were lysed with 0.1% sodium dodecylsulfate (SDS), and the radioactivity was determined in a gamma counter. Non-specific binding was quantified in parallel incubations performed in the presence of an excess of unlabelled IGF-I (0.1 μM) or unlabelled insulin (1 μM), respectively.

2.4. Thymidine incorporation

Myoblasts were plated at a density of 1×10^5 cells/9.6 cm^2 in multi-well culture dishes and cultured for 24 h in DMEM containing 10% fetal calf serum. Cells were then serum-starved for 30 h in DMEM containing 0.5% bovine serum albumin. This medium was then removed and replaced by fresh DMEM. [*methyl*-^{3}H]Thymidine (2 μCi/well) was then added and incubation was continued in the absence or presence of 10% fetal calf serum or various concentrations of peptide hormones for 16 h. The cells were washed twice with PBS and solubilized in 0.1% SDS at room temperature. DNA was precipitated with ice-cold 20% trichloroacetic acid and collected on glass fiber filters. The precipitate was washed three times with ice-cold 10% trichloroacetic acid, and the radioactivity associated with the filters was quantified by scintillation counting.

2.5. Assay of 3-O-methylglucose transport

All transport experiments using freshly isolated cells were performed at 37°C in HEPES buffer (composition: NaCl 130 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $MgCl_2$ 1

mM, $CaCl_2$ 1 mM, HEPES 25 mM, D-glucose 5 mM, bovine serum albumin 20 g/l, pH 7.4). The reaction was started by pipetting a 50 μl aliquot of the cell suspension to 50 μl of HEPES buffer containing 3-*O*-[^{14}C]methyl-D-glucose (0.1 μCi, final concentration 100 μM). Carrier-mediated glucose transport was then determined using a 10 s assay period and L-[^{14}C]glucose in order to correct for simple diffusion, as described in earlier reports from this laboratory (Eckel et al., 1983, 1990).

2.6. Presentation of data and statistics

All data analysis was performed using Prism (GraphPad, San Diego, CA, USA) or t-ease (ISI, Philadelphia, PA, USA) statistical software. Data of ^{125}I-IGF-I binding were fitted by non-linear regression analysis using a one-site binding and one-site competition model. EC_{50} (half-effective concentration) values for stimulation of 3-*O*-methylglucose transport were calculated by fitting the data to a sigmoidal dose-response curve. Best of fit was estimated from R^2 and sum-of-squares values. Significance of reported differences was evaluated by using the null hypothesis and *t* statistics for paired data. Corresponding significance levels are indicated in the figures.

3. Results

3.1. Interaction of HOE 901 with IGF-I receptors in H9 myoblasts

Although insulin has been shown to exert growth-promoting effects through its own receptor (Lammers et al., 1989), interaction of insulin with IGF-I receptors may significantly contribute to the mitogenic potency of this hormone, specifically in muscle tissue (Bornfeldt et al., 1991). Equilibrium binding studies of IGF-I to H9 myoblasts cultured for 48 h are presented in Fig. 1. Non-linear regression analysis of the binding data indicated the presence of about 7×10^3 IGF-I receptors per cell with an affinity of 2.6 nmol/l. In contrast, under the same experimental conditions no specific binding of ^{125}I-labelled insulin (50 pM) to myoblasts could be detected. After 10 days in culture (myotubes) a significant amount of specific insulin binding became detectable (Fig. 2). Additional experiments using variations of incubation media and insulin concentrations also failed to show insulin binding to 48 h cultured myoblasts (data not shown). Therefore, at this culture phase IGF-I receptors most probably exclusively mediate insulin action. All subsequent experiments on H9 myoblasts were carried out after 48 h in culture.

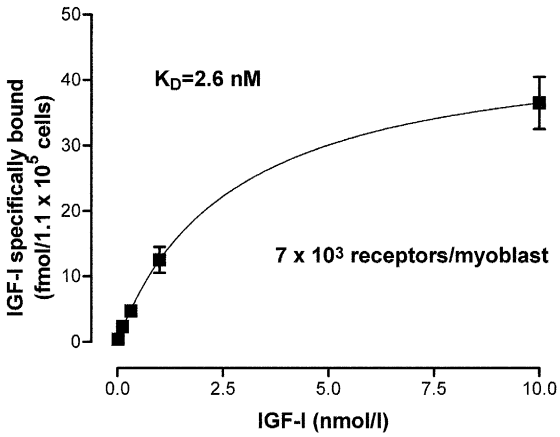

Fig. 1. Concentration dependence of IGF-I binding to H9 myoblasts. Cells were plated at an initial density of 1×10^5 cells/9.6 cm^2 and cultured for 48 h in DMEM plus 10% fetal calf serum. Binding of ^{125}I-labelled IGF-I (1.7×10^{-11} M) was then determined in DMEM in the presence of increasing concentrations of unlabelled IGF-I (10^{-10} to 10^{-8} M) as detailed in Section 2. All data have been corrected for non-specific binding. Cell numbers were determined for each individual culture after cell dissociation with trypsin/EDTA. Data were fitted by non-linear regression analysis with an R^2 value of 0.999. Each point is the mean ± S.E.M. of three separate experiments.

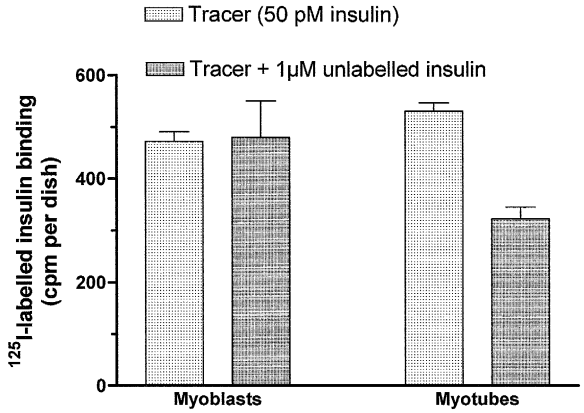

Fig. 2. Determination of ^{125}I-labelled insulin binding to H9 myoblasts and H9 myotubes. Cells (1×10^5 cells/9.6 cm^2) were cultured for 48 h (myoblasts) or 10 days (myotubes) in DMEM plus 10% fetal calf serum and binding of ^{125}I-labelled insulin (5×10^{-11} M) was then quantified as outlined in Fig. 1. Data are mean values ± S.E.M. of three separate experiments.

We then tested the competition of native insulin, HOE 901 and IGF-I (each at 1 nM) for binding of ^{125}I-labelled IGF-I to H9 myoblasts. For comparison, the analogue [Asp^{B10}]insulin, which exhibits a reported higher affinity to the IGF-I receptor than regular insulin (Drejer et al., 1991), was also investigated. As presented in Fig. 3, displacement of labelled IGF-I increased with the following order of potency: native human insulin < HOE 901 < [Asp^{B10}]insulin < IGF-I. Non-linear regression analysis of competition binding experiments using 1.7×10^{-11} M labelled IGF-I and increasing concentrations of the peptides from 10^{-9} to 10^{-6} M indicated a slightly higher affinity of HOE 901 for the IGF-I receptor when compared to insulin with IC_{50} (half-inhibitory concentration) values of 70 and 101 nM, respectively (Fig. 4). [Asp^{B10}]Insulin was more potent with an IC_{50} value of 44 nM.

3.2. Effect of HOE 901 on DNA synthesis

Serum-starved H9 myoblasts responded with a 4–5-fold increase in [^{3}H]thymidine incorporation when stimulated

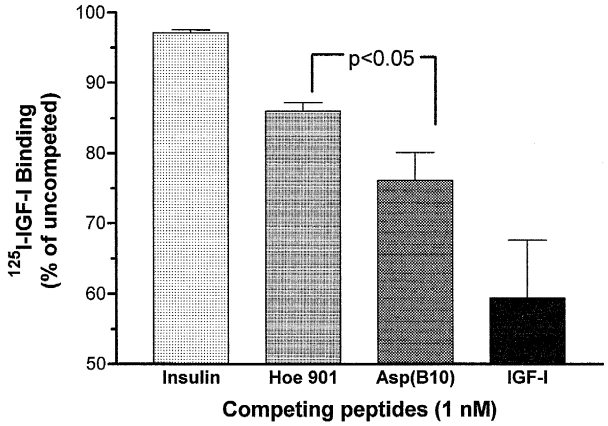

Fig. 3. Competition of insulin and insulin analogues for ^{125}I-labelled IGF-I binding in comparison to unlabelled IGF-I. H9 myoblasts were incubated for 90 min at 37°C in DMEM with ^{125}I-labelled IGF-I (1.7×10^{-11} M) in the absence or presence of the indicated peptide hormones (each at 1 nM). Labelled IGF-I binding was then determined as described in Section 2. All data have been corrected for non-specific binding determined in the presence of 10^{-7} M unlabelled IGF-I. The results shown are mean values ± S.E.M. obtained from 3–4 separate experiments.

with 10% fetal calf serum for 16 h (Fig. 5). Under the same experimental conditions IGF-I (10^{-7} M) was less potent than fetal calf serum with a 2.6-fold stimulation of DNA synthesis. As shown in Fig. 5, [Asp^{B10}]insulin at a maximal concentration of 5×10^{-7} M was equipotent to IGF-I and significantly more effective than HOE 901 in stimulating [^{3}H]thymidine incorporation. Most importantly, the maximum growth promoting activity of HOE 901 was essentially identical to the effect of regular insulin with a 2-fold stimulation of DNA synthesis (Fig. 5, upper

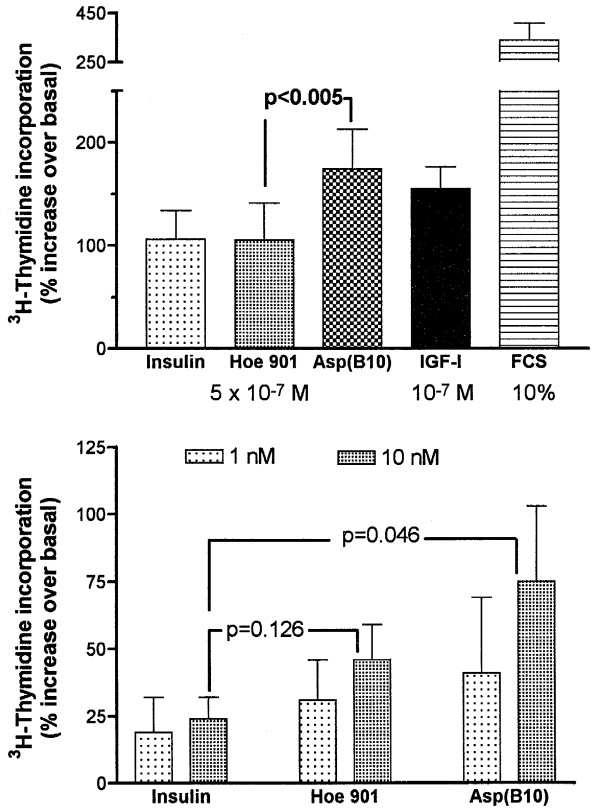

Fig. 5. Effects of insulin, insulin analogues and IGF-I on [^{3}H]thymidine incorporation into DNA in H9 myoblasts. Myoblasts were serum-starved for 30 h in DMEM containing 0.5% bovine serum albumin and subsequently incubated for 16 h with [*methyl*-^{3}H]thymidine (2 μCi/well) in the absence (basal) or presence of the indicated concentrations of peptide hormones or fetal calf serum (FCS) (upper panel) or in the presence of increasing concentrations of insulin analogues (lower panel). Cells were solubilized and DNA was precipitated with trichloroacetic acid, collected by filtration, and counted in a liquid scintillation counter. Data reported are mean values ± S.E.M. of 3–5 separate experiments.

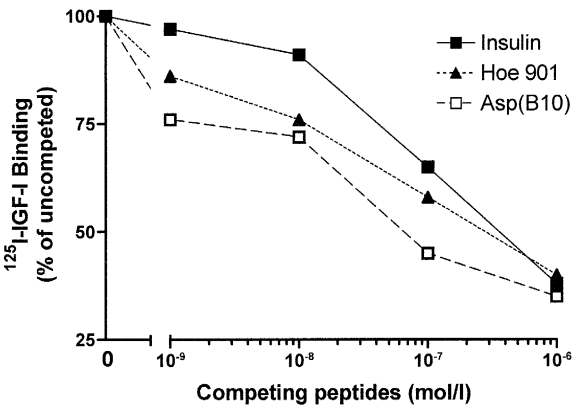

Fig. 4. Displacement of ^{125}I-labelled IGF-I binding by insulin, HOE 901 and [Asp^{B10}]insulin. H9 myoblasts were incubated for 90 min at 37°C in DMEM with ^{125}I-labelled IGF-I (1.7×10^{-11} M) and increasing concentrations of the peptide hormones. Data were analysed by non-linear regression analysis using a one-site competition model for the determination of IC_{50} values. All data have been corrected for non-specific binding determined in the presence of unlabelled IGF-I (10^{-7} M) and represent mean values of three separate experiments.

panel). As outlined above, these effects can be completely attributed to the interaction of insulin and insulin analogues with the IGF-I receptor. It should be noted that the actual increase in cell number produced by IGF-I and [Asp^{B10}]insulin was also higher when compared to that produced by insulin and HOE 901 (data not shown).

We then analysed the mitogenic activity of the three insulins at lower, more physiological insulin concentrations. At concentrations of 1 nM the three peptides produced an increase in DNA synthesis of 19–41% over basal; however, these data did not reach statistical significance (Fig. 5, lower panel). At 10 nM [Asp^{B10}]insulin was significantly more potent than native insulin, at least partly resulting from a higher level of receptor occupancy. HOE 901 and insulin produced a moderate increase in [^{3}H]thymidine incorporation (46 and 24% over basal, respectively); this difference, however, was not statistically significant (Fig. 5, lower panel).

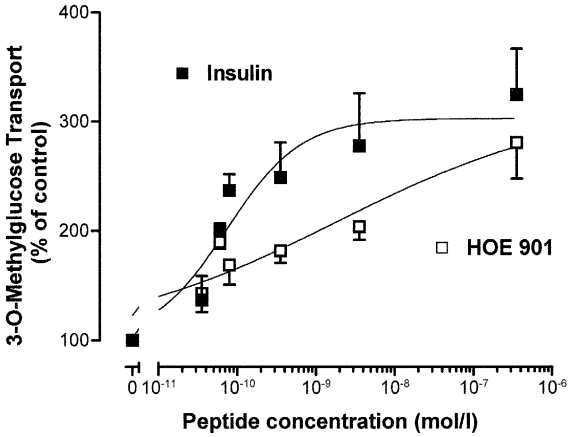

Fig. 6. Dose-response relationship for insulin and HOE 901 stimulated transport of 3-*O*-methylglucose in adult cardiomyocytes. 4×10^5 cells/ml were incubated for 60 min in the absence (control) or presence of increasing concentrations of either human insulin or HOE 901 (3.5×10^{-11} to 3.5×10^{-7} M). Initial rates of 3-*O*-methylglucose transport were then determined over a 10-s assay period as outlined in Section 2. Data were fitted by non-linear regression analysis and are mean values ± S.E.M. ($n = 3$–5).

3.3. Effect of HOE 901 on glucose transport in adult cardiomyocytes

The data obtained so far suggest that mitogenic signalling of insulin and the analogue HOE 901 in muscle cells are superimposable. Adult ventricular cardiomyocytes expressing a high level of insulin receptors and the insulin-sensitive glucose transporter GLUT4 (Eckel et al., 1983, 1990; Kolter et al., 1992; Russ and Eckel, 1995) have then been used to evaluate the metabolic potency of HOE 901 by measuring initial rates of 3-*O*-methylglucose transport. As shown in Fig. 6, glucose transport stimulation by increasing concentrations of native insulin could be fitted to a sigmoidal dose-response curve ($R^2 = 0.914$) with an EC_{50} value of 0.7×10^{-10} M and a maximal transport stimulation of about 3-fold. The same batch of cells was incubated in parallel with increasing concentrations of HOE 901 and 3-*O*-methylglucose transport was then determined (Fig. 6). As can be seen from the data, the maximal transport stimulation remained unaffected, reaching about 3-fold. A comparable maximal stimulation was also detected for [Asp^{B10}]insulin (data not shown). However, we observed a considerable rightward shift of the dose-reponse curve for HOE 901 with an EC_{50} value of 1.9×10^{-9} M (Fig. 6).

4. Discussion

Recombinant DNA technology has provided access to a variety of insulin analogues with altered pharmacodynamical properties (Galloway and Chance, 1994; Galloway, 1993; Brange et al., 1988; Grau, 1985). These novel insulin molecules with either rapid or protracted action profiles represent an essential step towards an optimized insulin therapy and a tight glycemic control (Galloway and Chance, 1994). However, changes of the primary structure of native insulin may lead to altered receptor interactions and a modified action profile (Drejer et al., 1991; Burke et al., 1990; Volund et al., 1991). In light of the potential atherogenic potency of insulin (Stout, 1990), the mitogenic activity of insulin analogues requires specific attention. This hormonal response has been recognised to be mediated by both the insulin receptor and the IGF-I receptor with a variable contribution of each receptor depending on the type of tissue (Bornfeldt et al., 1991; Lammers et al., 1989; Straus, 1989; De Meyts et al., 1993). Using H9 cardiac myoblasts we show here that the long-acting insulin analogue HOE 901 exerts a growth promoting activity essentially identical to that of native insulin.

After 48 h in culture H9 myoblasts were found to express a high number of IGF-I receptors but no detectable amount of insulin receptors. Consistently, a high level of IGF-I binding and a low level of insulin binding have been determined in different myoblast systems like the BC3H-1 and C2 muscle cell lines (Brunetti et al., 1989). Specifically, after 48 h in culture an extremely low level of insulin binding has been observed in BC3H-1 myoblasts with a 5-fold increase after 72 h (Standaert et al., 1984). As shown here, insulin receptors remain undetectable on 48 h cultured H9 myoblasts, making these cells suitable for studies on IGF-I receptor-mediated growth promoting activity of insulin and insulin analogues without overlap from insulin receptors. The analogue HOE 901 exhibited a slightly higher affinity to the IGF-I receptor than regular insulin, most probably resulting from the two additional arginines at the carboxy terminus of the B-chain (Zhang et al., 1994). Further, IGF-I has an acidic amino acid at position B10 with insulin having a basic amino acid (histidine) in this position. Apparently, this position contributes significantly to the recognition by the IGF-I receptor, since [Asp^{B10}]insulin was 2–3-fold more potent than native insulin in competing for ^{125}I-labelled IGF-I binding. Similar findings concerning the binding of this analogue were reported by Bornfeldt et al. (1991) using aortic smooth muscle cells.

A key finding of the present investigation consists of the observation of an identical maximum growth promoting activity of HOE 901 and native insulin both being considerably less potent than IGF-I. The identical mitogenic potency of insulin and HOE 901 is in contrast with the slightly higher affinity of the analogue for the IGF-I receptor. It should be noted, however, that binding experiments were done at 37°C for extended time periods and that additional, possibly non-receptor mediated events cannot be completely excluded. Furthermore, binding of the peptide to the receptor does not imply proper signal transduction and downstream elements have to be considered.

On the other hand, [Asp^{B10}]insulin was equipotent to IGF-I in stimulating DNA synthesis at high concentrations and thus acts as a full agonist. These data suggest that the acidic amino acid at position B10 found in IGF-I (Zhang et al., 1994) may represent an important structural element for IGF-I receptor binding and signalling. In complete agreement with our results [Asp^{B10}]insulin was found to be equipotent to IGF-I with a 60% higher maximal stimulation of DNA synthesis in aortic smooth muscle cells (Bornfeldt et al., 1991). Recent data suggest that the ligand specificity of the IGF-I receptor resides in a cysteine-rich region that is different from the insulin receptor (Kjeldsen et al., 1991). It may be speculated that the interaction of the acidic amino acid at position B10 with this region contributes to subunit-subunit communication and downstream signalling to the beta subunit. Further work will be needed to dissect the molecular differences of IGF-I receptor signal transduction in response to insulin, HOE 901, [Asp^{B10}]insulin and IGF-I.

It may be argued that the analogue HOE 901 could exert enhanced growth promoting activity by an altered interaction with the insulin receptor. Indeed, [Asp^{B10}]insulin was found to be 'supermitogenic' in a murine T-cell lymphoma cell line that is completely devoid of IGF-I receptors (De Meyts et al., 1993; Pillemer et al., 1992). It was shown that this higher mitogenic activity correlates with an increased half-life of the insulin-receptor complex and an impaired or absent regulation of the dissociation rate by negative cooperativity (De Meyts et al., 1993). In contrast to [Asp^{B10}]insulin, HOE 901 was reported to show normal dissociation kinetics from human insulin receptors and an unaltered receptor phosphorylation/dephosphorylation process (Berti et al., 1995). These data in conjunction with our findings on the interaction of HOE 901 with IGF-I receptors support the notion that the mitogenic potency of this insulin analogue is identical to native insulin, making a higher atherogenic risk most unlikely.

As expected, HOE 901 produced full activation of 3-*O*-methylglucose transport in adult cardiomyocytes, a response related to the translocation of the glucose transporter GLUT4 (Kolter et al., 1992). However, we observed a clear reduction in the sensitivity by about one order of magnitude. Since diarginylinsulin has almost identical insulin receptor binding compared to native insulin (Zeuzem et al., 1990) and position A21 is part of the receptor binding region (Pullen et al., 1976), the substitution of Asn^{21} by Gly most probably underlies the reduced EC_{50} of glucose transport stimulation in cardiomyocytes. Slightly reduced activity of HOE 901 has also been observed in isolated adipocytes (G. Seipke, personal communication). It should be noted, however, that insulin analogues with markedly different in vitro potencies resulting from differences in receptor binding affinity were found to be equipotent under in vivo conditions, most probably resulting from different elimination from blood and subsequent degradation (Volund et al., 1991; Ribel et al., 1990; Robertson et al., 1992). Consistently, HOE 901 exhibits full biological potency under in vivo conditions (Seipke et al., 1992).

Our study shows that in muscle tissue the novel long-acting insulin analogue HOE 901 exerts growth promoting and maximal metabolic activity identical to native insulin. We suggest that differential interaction of insulin analogues with both insulin and IGF-I receptors determines the action profiles of these molecules.

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