Activation of the Insulin Receptor by Insulin and a Synthetic Peptide Leads to Divergent Metabolic and Mitogenic Signaling and Responses*‡

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Recently, single chain peptides have been designed that target the insulin receptor and mimic insulin action. The aim of this study is to explore if activation of the insulin receptor with such an optimized peptide (S597) leads to the same activation of signaling pathways and biological endpoints i.e. stimulation of glycogen synthesis and cell proliferation as stimulation with insulin. We find that surface activation of the insulin receptor A-isoform with S597 leads to activation of protein kinase B (PKB) and glycogen synthesis comparable to activation by insulin, even though the level of insulin receptor phosphorylation is lower. In contrast, both Src homology 2/α collagen-related (Shc) and extracellular signal-regulated kinase (ERK) 2 activation are virtually absent upon stimulation with S597. Cell proliferation is only stimulated slightly by S597, suggesting that it depends on signals from Shc and ERK. The differences in signaling response could explain both the earlier reported differences in gene expression, and the reported differences in cell proliferation and glycogen synthesis induced by insulin and S597. In conclusion, despite binding equipotency, insulin, and S597 initiate different signaling and biological responses through the same insulin receptor isoform. We show for the first time that it is possible to design insulin receptor ligand mimetics with metabolic equipotency but low mitogenicity.

The insulin receptor is a disulfide linked heterodimeric protein consisting of two 135-kDa extracellular α-subunits and two 95-kDa transmembrane spanning β-subunits, containing the intracellular tyrosine kinase domain that is activated upon ligand binding (1, 2). Binding of insulin to the receptor results in a wide range of metabolic and mitogenic responses initially mediated by phosphorylation of tyrosines on several intracellular protein substrates, including insulin receptor substrates (IRS)β 1 and 2 and Shc (3). IRS1 and IRS2 are the major insulin receptor substrates leading to glucose homeostasis and have distinct and overlapping roles in diverse organs. Furthermore alterations in both IRS1 and IRS2 have been shown to be strongly involved in the development of diabetes mellitus (4, 5). The finding of specific functions of IRS1 and IRS2 in the murine pre-muscle cell line (L6 cells) are summarized by Thirone et al. (6) but the relative contributions of IRS1 and IRS2 to insulin signaling and to the development of insulin resistance is not yet conclusive. The majority of the published literature in this field suggests that IRS1 is the major substrate leading to stimulation of glucose transport in muscle and adipose tissues, whereas in liver IRS1 and IRS2 have complementary roles in insulin signaling and metabolism. In contrast Shc does not appear to be directly involved in metabolic signaling of insulin but plays a critical role in insulin-induced mitogenesis (6, 7). Both IRS and Shc initiate the mitogen-activated pathway kinase/extracellular-regulated kinase (MAPK/ERK) pathway, including activation of ERK1/2 itself leading to gene transcription and protein translation and cell growth. The other major signaling pathway activated by IRS proteins is the phosphatidylinositol 3-kinase (PI 3-kinase) pathway that includes activation of PKB and leads to activation of glycogen synthase and other enzymes/proteins necessary for the acute metabolic effects of insulin (3).

Synthetic peptides that bind to the insulin receptor have been identified by investigation of phage display libraries (ranging in size from 20 – 40 amino acids) (8). The identified peptides bound to three spots on the insulin receptor (sites 1, 2, and 3). The two sites (1 and 2) seem to overlap with the two insulin binding sites on the insulin receptor, whereas the site 3 insulin receptor-binding peptides seem to bind somewhere near the site 2 peptides. Recently homodimers and heterodimers of the site 1 and 2 peptides have been generated. Many of these can activate the insulin receptor with high potency and specificity, possibly through a mechanism similar to the binding of insulin (9). One of these peptides was furthermore found to lower the blood glucose level in rats with equipotency to insulin. Because of the smaller size and simpler structure of these insulin mimetic peptides, they are good candidates for drug development with potential use in the treatment of diabetes as a replacement for, or in combination with, insulin, and as templates for the design of novel peptidomimetics.

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§ The abbreviations used are: IRS, insulin receptor substrate; PKB, protein kinase B; Shc, Src homolog 2/α collagen-related; ERK, extracellular signal-regulated kinase; PI 3-kinase, phosphatidylinositol 3-kinase; MAPK, mitogen-activated pathway kinase; HRP, horseradish peroxidase.
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In related work, we have studied such an optimized peptide (S597) in detail regarding its insulin receptor binding properties, gene expression profile and mitogenicity. We found that S597 binds with a similar affinity as insulin but seems to interact with the insulin receptor in a dissimilar way with positive cooperativity between the two ligands, suggesting that they can coexist on the receptor (supplemental Fig. A). Furthermore we found significant differences between the gene expression patterns induced by these two ligands and a lower mitogenicity of S597. This suggests that S597 is able to induce a different response through the insulin receptor than insulin.

In this study we have studied for the first time the downstream signaling pathways in cells expressing the insulin receptor when stimulated with S597 in comparison with insulin and found some very interesting differences helping to elucidate some of the activation mechanisms of signaling pathways utilized by the insulin receptor and explaining the previously obtained results with S597.

EXPERIMENTAL PROCEDURES

Ligands—The insulin mimetic peptide, S597, with the sequence Ac-SLEEEWAQIECVYGRGCPSFESDFWERQLamide, was synthesized by standard solid phase peptide synthesis using Fmoc chemistry and purified by RP-HPLC after formation of the disulfide bridge. Insulin was obtained from Novo Nordisk, Denmark and IGF-1 from GroPep, Adelaide, Australia.

Antibodies—Anti-phosphotyrosine, 4G10 (UBS 05–321, diluted 1:500), anti-phospho-PKB T308 (Cell Signaling 9275, diluted 1:1000), anti-phospho-Shc (Tyr239/40) (Cell Signaling 2434, diluted 1:1000), anti-dual phospho MAPK 44/42 (Protech V667A, diluted 1:5000), anti-IRS-1 (UBI 06-248); anti-dual phospho MAPK 44/42 (Protech V667A, diluted 1:5000), anti-IRS-1 (UBI 06-248); anti-dual phospho MAPK 44/42 (Protech V667A, diluted 1:5000), anti-IRS-1 (UBI 06-248); anti-IRS-2 (UBI 06-506) both for immunoprecipitation, 5/200 μg cellular extract. Secondary antibodies: goat anti-mouse HRP (Bio-Rad 170-6516), goat anti-rabbit HRP (Bio-Rad 170-6515), both diluted 1:3000.

Cells—Cell line and culture conditions: The rat myoblast cell line stably transfected with the human insulin receptor (hIR) isoform A (L6-hIR) was kindly provided by Bo Falck Hansen, Novo Nordisk, Denmark. These cells express about 100,000 insulin receptors per cell. The L6-hIR cells and the L6 cells were cultured in Dulbecco’s modified Eagle’s medium 21885-025 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycine. Furthermore 0.5 mg/ml Geneticin was added to the L6-hIR medium (all from Invitrogen). The cells were grown at 37 °C in 5% CO2 humidified atmosphere and passaged 2–3 times a week.

Western Blots and Immunoprecipitations—Cells were stimulated as indicated, the media removed, and the cells immediately frozen by pouring liquid N2 in to the well. The cells were solubilized in lysis buffer by scraping of the cells at 4 °C, and the cellular lysate cleared by centrifugation. Phosphorylation of IR and IRS measured by Western blots after stimulation with insulin or S597 measured by analyzing the 95-kDa and 180-kDa band on a tyrosine phosphor Western blot. For immunoprecipitation cell lysates were incubated with antibody against IRS-1 or IRS-2 (5 μg) overnight at 4 °C with rotation. Antigen antibody complexes were absorbed on protein-A-Sepharose for 1 h with agitation, and collected by centrifugation. Beads were washed three times in phosphate-buffered saline, and the complexes solubilized in SDS-PAGE loading buffer. Cellular lysates (equal amounts of protein) or immunoprecipitates (from equal amounts of protein) were separated by SDS-PAGE (6–12% gradient gels) followed by transfer to PVDF membrane (Immobilon, Millipore). The membrane was blocked in TBS with albumin (2%) for 1 h at room temperature, followed by incubation with primary detection antibody overnight. The membrane was washed three times 5 min at room temperature, incubated with polyclonal anti-mouse or rabbit secondary antibody labeled with HRP for 1 h, and again washed four times in TBS and incubated with HRP substrate and visualized on a Phosphorimager (Fujilas 3000). The intensity of the bands was quantified with the Phosphorimager software. Representative Western blots and immunoprecipitations are shown in supplemental figures.

Internalization Assay using Acid Wash—Cells were grown in 24-well plates (150,000 cells/well) overnight, washed with Hepes binding buffer (HBB: 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1 mM EDTA, 100 mM glucose, 15 mM sodium acetate, 100 mM Hepes and 1% bovine serum albumin, pH 7.6) and incubated with 20,000 cpm 125I-labeled insulin or S597 for 1 h at 4 °C. Unbound tracer was removed by washing the cells with HBB and the cells incubated at 37 °C at different times, as indicated. After incubation, 1 ml of HBB pH 3.5 was added to half of each plate and incubated for additional 20 h at 4 °C (to remove surface-bound ligand), the other half was left without buffer. 500 μl of trypsin-EDTA were added to each well and the detached cells transferred to tubes and counted in a Packard gamma counter. There were four replicates for each condition, and the experiments were repeated three times.

Decreasing the incubation time in buffer pH 3.5 to 8 min in the experiments with 125I-insulin gave the same results as incubation for 20 h at 4 °C but S597 was not removed by this treatment (data not shown). Furthermore trichloroacetic acid precipitation was performed on both the incubation buffer used in the 37 °C incubation step and 20 h of incubation at 4 °C to detect if the obtained results were due to extracellular degradation of the tracer. No degradation of the tracer could be detected in any of the experiments (data not shown).

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Tyrosine Phosphorylation of the Insulin Receptor—The ability of S597 to tyrosine-phosphorylate the insulin receptor compared with insulin was examined by incubating L6 cells expressing the human insulin receptor (100,000 receptors/cell) with increasing amounts of insulin or S597 for various times. Compared with the basal level (set equal to 1) stimulation for 10 min with increasing concentrations of S597 lead to increasing tyrosine phosphorylation of the insulin receptor but only up to ~60% of the level induced by 100 nM insulin (Fig. 1A). Using 1 or 10 nM insulin or 100 nM S597 for various times shows that at all time points S597 gave only ~50% insulin receptor tyrosine phosphorylation compared with 10 nM insulin and roughly twice of that induced by 1 nM insulin. The kinetics of insulin receptor tyrosine phosphorylation by maximal S597 resembled that of the lower insulin concentration with the absence of a peak at 5 min seen with high insulin (Fig. 1B). Similar results were also obtained with other insulin peptide mimetics (data not shown).

The difference in receptor phosphorylation by insulin and S597 could be due to S597 phosphorylating a lower number of insulin receptors than insulin, or S597 phosphorylating the same number of receptors but to a lower degree. To elucidate which one was the case, we stimulated cells and performed immunoprecipitations with anti-phosphotyrosine antibody followed by Western blotting with insulin receptor antibody to bring down the activated receptors and measured the corresponding amount of tyrosine phosphorylation of the receptor by S597 and insulin without immunoprecipitation. White bars, insulin; black bars, S597. Representative Western blots are shown in supplemental Fig. B.

In the present work, we investigated for the first time the differential activation of the insulin receptor by S597 and insulin to phosphorylate the insulin receptor is measured using Western blots by incubating L6 hIR cells with: A, increasing amounts of insulin (-) and S597 (-); B, constant concentrations of S597 (100 nM) and insulin (10 nM); C, measuring the corresponding amount of tyrosine phosphorylation of the receptor by S597 and insulin without immunoprecipitation. White bars, insulin; black bars, S597. Representative Western blots are shown in supplemental Fig. B.

In the present work, we investigated for the first time whether the activation of the insulin receptor by insulin or the peptide mimetic S597 results in activation of the same downstream signaling mechanisms. Furthermore we studied if S597 had the same biological properties as insulin with respect to stimulation of glycogen synthesis and cell proliferation.

RESULTS

In the present work, we investigated for the first time whether the activation of the insulin receptor by insulin or the peptide mimetic S597 results in activation of the same downstream signaling mechanisms. Furthermore we studied if S597 had the same biological properties as insulin with respect to stimulation of glycogen synthesis and cell proliferation.

Glycogen Synthesis—The effect of the test compound on glucose incorporation into glycogen is determined by measuring incorporation of radiolabeled d-[U-14C]glucose into L6 hIR cells (10). Briefly, the starved cells were incubated with and without compound in KRH-buffer (5 mM glucose, 20 mM mannitol) for 120 min and the last 60 min with tracer amounts of radiolabeled d-[U-14C]glucose. After the incubation, the cells were washed in buffer and solubilized by heating 30 min in 1 M NaOH at 90 °C. The glycogen was precipitated with 96% ethanol in the cold in the presence of carrier glycogen and dissolved in water, scintillation liquid was added, and radioactivity was counted. The results are the mean of three independent experiments, made in duplicates.

Tyrosine Phosphorylation of the Insulin Receptor—The ability of S597 to tyrosine-phosphorylate the insulin receptor compared with insulin was examined by incubating L6 cells expressing the human insulin receptor (100,000 receptors/cells) with increasing amounts of insulin or S597 for various times. Compared with the basal level (set equal to 1) stimulation for 10 min with increasing concentrations of S597 lead to increasing tyrosine phosphorylation of the insulin receptor but only up to ~60% of the level induced by 100 nM insulin (Fig. 1A). Using 1 or 10 nM insulin or 100 nM S597 for various times shows that at all time points S597 gave only ~50% insulin receptor tyrosine phosphorylation compared with 10 nM insulin and roughly twice of that induced by 1 nM insulin. The kinetics of insulin receptor tyrosine phosphorylation by maximal S597 resembled that of the lower insulin concentration with the absence of a peak at 5 min seen with high insulin (Fig. 1B). Similar results were also obtained with other insulin peptide mimetics (data not shown).

The difference in receptor phosphorylation by insulin and S597 could be due to S597 phosphorylating a lower number of insulin receptors than insulin, or S597 phosphorylating the same number of receptors but to a lower degree. To elucidate which one was the case, we stimulated cells and performed immunoprecipitations with anti-phosphotyrosine antibody followed by Western blotting with insulin receptor antibody, thus only bringing down the activated receptors and giving a measure of how many receptors each ligand affects. Fig. 1C shows that 1 or 10 nM insulin and 10 or 100 nM S597 all lead to phosphorylation of the same number of insulin receptors. Fig. 1D shows the corresponding amount of tyrosine phosphorylation of the insulin receptors without immunoprecipitation. This means that the same number of insulin receptors were phosphorylated by S597 but to a lower degree.

Differential Activation of the Insulin Receptor—Next we analyzed the tyrosine phosphorylation of IRS proteins (the 180-kDa band on a Western blot detecting tyrosine-phosphorylated IRS proteins) as well as activation of effectors ERK1/2 and PKB by using antibodies specific for their active forms. IRS was tyrosine-phosphorylated by maximal S597 up to 43% of maximum insulin levels at 10 min, with roughly similar kinetics (Fig. 2, A and B) and to a level similar to that induced by 1 nM insulin. ERK1 and ERK2 were only very weakly activated, to 27 and 18% of maximum insulin levels respectively, but with a flat kinetic profile compared with insulin where both 1 and 10 nM have a peak at 10 min, and to a
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much lower level than even 1 nM insulin (Fig. 2, C and D). In striking contrast, PKB shows nearly full activation, 94% compared with insulin, although the dose response curve is shifted to the right, and similar kinetics (Fig. 2, E and F). Interestingly, PKB shows maximal activation already at 1 nM insulin where the insulin receptor is only partially phosphorylated (29% of maximum). These results clearly show that S597 activates the insulin signaling cascade differently than insulin, with only half the receptor phosphorylation, full PKB stimulation but nearly no ERK1 and ERK2 stimulation.

To further elucidate the phosphorylation of the proximal substrates of the insulin receptor we analyzed cells stimulated with either 10 nM insulin or 100 nM S597 for IRS1 and IRS2 phosphorylation, by immunoprecipitation of the two proteins, and Shc phosphorylation by antibodies specific for Shc phosphorylated on the tyrosine that regulates the activity of Shc. As can be seen in Fig. 3, IRS1 and IRS2 were phosphorylated to 59 and 41% of insulin levels, respectively, when analyzed specifically by immunoprecipitation, consistent with the results from the less specific method measuring both proteins presented above. In contrast, Shc was not activated, with specific phosphorylation of 5.5 or 0% at 10 and 20 min, respectively. So IRS1 and IRS2 followed the receptor phosphorylation pattern, whereas there was no phosphorylation of Shc by S597.

Previously it was shown that S597 binds specifically and with a similar affinity as insulin to the insulin receptor. However, to control that the obtained effect of S597 is indeed through the insulin receptor we also performed experiments with untransfected L6 cells that predominantly express IGF-I receptors and very few insulin receptors. S597 had no effect on phosphorylation of the IGF-1 receptor, IRS, ERK1/2, or PKB in these cells (supplemental Fig. B) consistent with that the effect on signaling we observe in the L6 hIR is because of activation of the insulin receptor.

Internalization—There is a clear link between receptor internalization and which signaling pathways there gets initiated, as for instance full activation of Shc but not PI3-kinase seems to be dependent on insulin receptor internalization (for review, see Refs. 11, 12). Therefore we investigated whether S597 was internalized to a similar degree as insulin. When labeled ligand is bound at 4 °C, and the cells are then shifted to 37 °C the internalization of the ligand-receptor complex can be followed. The surface-bound ligand and the internalized ligand can be separated by washing off surface-bound ligand with acidic buffer. These experiments shows that insulin is internalized within 20 min and almost no ligand is associated with the cells after 60 min, meaning it has been internalized and released (Fig. 4). S597 binding is more stable at acid pH, and therefore not all surface S597 came off in the acidic wash. At time 0 where all the ligand is on the outside of the cell ~80% was removable, and this picture was unchanged with nearly all the 125I-labeled S597
associated with the cells staying on for the 2 h the cells are followed, and ~80% being removable by acidic wash. This method therefore suggests that S597 stays surface bound, and is associated with the cells for much longer than insulin. We also used trypsinization in the absence of EDTA, which does not release the cells but only removes extracellular proteins. With this procedure the pattern for insulin and S597 internalization looked similar to the one obtained with the acid wash experiment, most of the S597 was removable with trypsin for up to 90 min, meaning that it is surface bound for this time (data not shown). This demonstrates that the two ligands have very different sites of residence in the period where the signaling pathways have been analyzed.

Glycogen Synthesis and Cellular Proliferation—We also analyzed some of the endpoints, known to be stimulated by insulin via the signaling pathways analyzed so far, namely glycogen synthesis and cell proliferation. Fig. 5A shows that glycogen synthesis is stimulated almost to the same level by insulin and S597 (72%), although with a lower potency (Table 1). Again there appears to be receptor spareness in the activation of glycogen synthesis compared with stimulation of insulin receptor tyrosine phosphorylation, with maximal signal already at 1 nM insulin, as was the case for PKB. In sharp contrast, S597 was much less potent at stimulating cell proliferation/growth compared with insulin as seen in Fig. 5B, confirming our recent results.4 The stimulation of cell growth was measured by [6-3H]thymidine incorporation assays where DNA synthesis was quantified as [6-3H]thymidine incorporation into DNA and used as a measurement for cell proliferation. At the highest ligand concentration, 100 μM, the effect of insulin was ~3 times bigger than of S597. To further elucidate the effect of S597 we also performed assays where 10 nM insulin was incubated in the presence of increasing amounts of S597 and vice versa. These assays demonstrate that S597 is a partial agonist, and antagonizes the effect of insulin down to the effect of S597 alone. To verify that the effect on cell proliferation is through the insulin receptor the effect of insulin and S597 was also studied on the untransfected L6 cells with only few insulin receptors. S597 had no effect on cell proliferation in these cells (supplemental Fig. C) supporting that the weak mitogenic signal by S597 is mediated through the insulin receptor. The data from the experiments are summarized in Table 1.

DISCUSSION

Insulin exerts a wide range of metabolic and mitogenic responses by binding to its tyrosine kinase receptor and phosphorylating tyrosines on several intracellular protein substrates. In this study we have stimulated L6 hIR myoblasts expressing the insulin receptor A-isoform with an optimized insulin mimetic peptide, S597, and investigated the activation of the insulin receptor, the post-receptor signaling mechanisms (IRS1/2, ERK1/2, Shc, and PKB) as well as the biological properties of S597 i.e. stimulation of glycogen synthesis and cell proliferation.

The binding kinetics and gene expression profile of S597 have recently been studied in detail.5,4 Furthermore we have earlier published on other peptides that bind to the insulin receptor with regard to affinity for the receptor and ability to stimulate lipogenesis in mouse adipocytes (8, 9), but intracellular signaling was not investigated.

In this study we have found striking differences in insulin receptor activation and downstream signaling pathways initiated by S597 and insulin. We have tested various other insulin mimetic peptides that show similar results (data not shown) supporting that the insulin receptor is activated in a different way by the peptides. The main findings of this study are summarized in Fig. 6 and Table 1.

S597 phosphorylates the same number of insulin receptors as insulin but to a lower degree. This suggests that even though S597 is dissociating much more slowly than insulin it is not able to activate the insulin receptor to the same degree as insulin. This is in sharp contrast with studies of insulin analogues where prolonged residence time on the receptor was shown to have an enhanced mitogenic/metabolic potency ratio, seemingly through sustained Shc activation (13). As the duration of the phosphorylation is similar to insulin stimulation it seems that it is not the duration that is responsible for the differences in biological properties of S597 and insulin. The receptor intracellular β-subunit domain contains six phosphorylatable tyrosines, including three in the regulatory loop of the kinase (Tyr1146, Tyr1150, and Tyr1151); whose phosphorylation is required for amplification of the kinase activity (3). At present there is no clear evidence for specific roles of individual phosphorylation sites in divergent metabolic and mitogenic signaling pathways (14). However a study where the IR was mutated at Tyr1146 to phenylalanine led to; a massive reduction in IR autophosphorylation, lack of insulin stimulated internalization and DNA synthesis but equally induction of insulin-stimulated

![Figure 5](https://example.com/figure5.png)

**TABLE 1**

| Assay                  | Insulin EC<sub>50</sub> (nM) | S597 EC<sub>50</sub> (nM) | % Maximal activation |
|------------------------|-------------------------------|---------------------------|----------------------|
| Binding                | 2.1                           | 2.5                       | ~                   |
| IR tyrosine phosphorylation | 2.6                         | 2.4                       | 58                   |
| IRS1 tyrosine phosphorylation | 1.2                         | 4.1                       | 43                   |
| ERK1 phosphorylation   | 0.55                          | 3.5                       | 18                   |
| PKB phosphorylation    | 0.11                          | 3.9                       | 94                   |
| Glycogen synthesis     | 0.11                          | 3.2                       | 71                   |
| Cell growth (WST-1)    | 1.2                           | 3.0                       | 23                   |

**FIGURE 5.** Glycogen synthesis and cell proliferation. A, ability of S597 (■) and insulin (●) to stimulate glycogen synthesis measured by incorporation of radiolabeled glucose into L6 hIR cells. B, ability of S597 (■), insulin (●), or 10 nM insulin plus increasing amounts of S597 (○) to stimulate cell proliferation measured by [6-3H]thymidine incorporation after incubation of cells with increasing amounts of ligands for 19 h and additional 2 h of incubation with [6-3H]thymidine.
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![Diagram](image)

**FIGURE 6. Summary of the effects of insulin and S597 on signaling and gene expression.**

A. Insulin binding to the insulin receptor results in receptor phosphorylation, internalization of the complex and phosphorylation of IRS1, IRS2, and Shc. This initiates the two major pathways: the PI 3-kinase pathway (including PKB) and MAPK (including ERK) leading to expression of several genes (many interact with ERK) as well as cell proliferation and glycogen synthesis. B. Binding of S597 phosphorylates the insulin receptor although to a lesser extent. No phosphorylation of Shc occurs, and IRS1 and IRS2 are phosphorylated less than by insulin. This leads to activation of the PI 3-kinase pathway as seen by full activation of PKB, whereas the MAPK pathway is only very weakly activated (almost no phosphorylated ERK is detected). This leads to full activation of glycogen synthesis but less induction of gene expression and cell proliferation.

Glycogen synthesis as wild-type IR (15). This is very similar to the effect of S597 making it plausible that S597 is less efficient in phosphorylating (specifically) Tyr1146, which could be the primary basis for the differences in cellular responses between S597 and insulin. Further work will focus on mapping the pattern of phosphorylated residues induced by insulin and S597.

Activation of the insulin receptor leads to phosphorylation of several intracellular protein substrates. These include IRS1, IRS2, and Shc. This initiates the two major signaling cascades, the MAPK pathway that includes activation of ERK1 and ERK2 leading to gene transcription, protein translation and cell growth and the PI 3-kinase pathway that includes activation of PKB leading to activation of glycogen synthase and other enzymes/proteins necessary for the acute metabolic effects of insulin (3). S597 is able to phosphorylate 41–59% of IRS1 and IRS2 compared with insulin, fully activate PKB, while hardly affecting Shc and ERK. This demonstrates that insulin and S597 initiate different signaling responses through the same receptor isoform making it apparent that activation of the insulin receptor is not only dependent on the concentration of a ligand but also on the nature of the ligand. Fig. 6 summarizes the similarities and differences of insulin and S597 signaling, internalization and biological responses found in this and earlier studies.

We have shown that S597 interacts with the insulin receptor in a different way than insulin suggesting that it does not bind to the exact same spots as insulin. It is likely that the different interaction is the reason for the reduction of the receptor phosphorylation resulting in differences in the downstream signaling. That a reduction in insulin receptor phosphorylation results in differences in downstream signaling has also been shown by Thirone et al. (16) who found that a reduction of insulin receptor phosphorylation induced by chronic *in vivo* growth hormone treatment or acute epinephrine infusion is accompanied by a reduction in the level of IRS1 but not Shc phosphorylation in rat hindlimb muscle, suggesting that in contrast to IRS1, Shc activation is not dependent on full insulin receptor phosphorylation. In our case, Shc phosphorylation was in contrast abolished.

Which pathways get activated also depends on whether the insulin receptor is located on the cell surface or in the endosomal compartment (reviewed in Refs. 11, 12). In this study we find that S597 and insulin have different sites of residence in the period where the signaling pathways have been analyzed, with S597 staying on the cell surface for much longer than insulin. We believe that this is an important reason for the differences seen with S597 and insulin stimulation.
Cellular growth is also stimulated very little by surface-activated insulin receptors and therefore seems to depend on signals, such as Shc and ERK1/2 that are activated intracellularly. Our results are consistent with the general assumption that the Shc pathway is a major player in inducing the mitogenic response to insulin which is also illustrated in Fig. 6 (7).

While our report was readied for submission, a study by Uhles et al. (21) was published, which strongly supports our proposal above that spatial segregation results in differential signaling, in that case via the B-isoform of the receptor. The authors showed that in beta cells, insulin activates the glucokinase gene from the plasma membrane (and via a PI3K/PKB pathway), while c-fos gene is activated from early endosomes, and via an Shc/ERK pathway. This dichotomy is consistent with our observations via the A-isoform on a more classical insulin target cell.

Contradictory results have been obtained when inhibiting the insulin receptor internalization with regard to the effect on DNA synthesis; Hamer et al. (18) found that inhibiting internalization leads to decreased DNA synthesis which is consistent with our findings, whereas Ceresa et al. (19) could not detect any difference. Further studies are needed to explore exactly how and how much the different pathways contribute to the different biological responses.

As the lack of activation of the MAPK pathway could explain some of the differences in gene expression induced by insulin and S597 found in a previous study we took a closer look at the differentially regulated genes. In this study we reported 131 transcripts (72 genes and 59 ESTs) differentially regulated by insulin and S597. We imported these genes into the Ingenuity Pathway Analysis software and found that 13 of the differentially regulated genes either directly or indirectly interact with ERK, as shown in Fig. 7. The genes are; early growth response 1 (EGR-1), chemokine ligand 2 (CCL13), colony stimulating factor 1 (CSF1), interleukin 6 (IL6), plasminogen activator, urokinase receptor (PLAUR), heparin-binding EGF-like growth factor (HBEGF), vascular endothelial growth factor (VEGF), v-myc avian myelocytomatosis viral oncogene homolog (MYC), v-myelocytomatosis viral-related oncogene (MYCN), v-jun sarcoma virus 17 oncogene homolog (JUN), Jun-B oncogene (JUNB), heme oxygenase 1 (HMOX1), fos-like antigen 1 (FOSL1). Many of the genes are involved in growth and proliferation and are all except MYCN more up-regulated by insulin than by S597 compared with untreated. This finding that many of the genes that were more up-regulated by insulin than by S597 interact with ERK supports the idea that the main difference between S597 and insulin is that the mitogenic signaling pathway is much less activated by S597 than insulin. The difference in signaling initiated by insulin and S597 appears to explain the differences between insulin and S597 induced gene expression. Furthermore a study by Harada et al. (22) demonstrates that insulin receptor phosphorylation and Shc phosphorylation is necessary for insulin-induced EGR-1 expression, whereas IRS1 phosphorylation is not necessary or sufficient for the expression. The reduced insulin receptor phosphorylation and low Shc phosphorylation when stimulating with S597 compared with insulin is therefore most likely the mechanism behind the lower expression of EGR-1 found with S597 compared with insulin-stimulated samples. Insulin-inducible DNA synthesis and repair after injury are processes critically dependent upon the activation of EGR-1 (in aortic endothelial cells) (23). This also supports that the lesser effect on DNA synthesis by S597 is caused by lack of activation of the MAPK pathway.

The finding that two ligands activating the same insulin receptor isoform can lead to different biological effects has also been shown in studies investigating the effect of insulin and IGF-2 on the insulin receptor A-isoform (24–27). These found that insulin and IGF-2 bind with a similar affinity to the receptor, whereas insulin led primarily to metabolic effects (measured by glucose uptake), IGF-2 led primarily to mitogenic effects (measured by [3H]thymidine assays). These differences in the biological effect were associated with differential recruitment and activation of intracellular substrates as well as selective changes in gene expression.

Thus, our work using newly designed, synthetic non-analogue ligand mimetics, strengthens the idea that two ligands upon binding to the same insulin receptor isoform, and presumably to binding sites in close proximity, can preferentially activate different signaling pathways such as the IRS/Pi3/Akt or the Shc/ERK pathway, and therefore have a different potency in eliciting different biological effects. Our work also established for the first time that it is possible to design insulin receptor mimetics with equipotent metabolic effects but low mitogenicity. High mitogenicity has been a problem with some insulin analogues (28).

CONCLUSION

The work presented here shows that the binding of an insulin mimetic peptide to the insulin receptor results in a different
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signaling response than insulin binding. The work suggests that both the magnitude (autophosphorylation of the IR) of the signal as well as location (cell surface versus endosomal compartment) contributes to the different biological responses. Furthermore the differences in signaling response appear to explain both the differences in gene expression, cell proliferation and glycogen synthesis induced by insulin and S597. These results raise the possibility of designing non-internalizing ligands for the insulin receptor that could selectively induce metabolic but not mitogenic pathways.

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