Differential Pathway Coupling of the Activated Insulin Receptor Drives Signaling Selectivity by XMetA, an Allosteric Partial Agonist Antibody

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ABSTRACT

The monoclonal antibody XMetA is an allosteric partial agonist of the insulin receptor (IR), which activates the metabolic Akt kinase signaling pathway while having little or no effect on the mitogenic extracellular signal-regulated kinase (ERK) signaling pathway. To investigate the nature of this selective signaling, we have conducted a detailed investigation of XMetA to evaluate specific phosphorylation and activation of IR, Akt, and ERK in Chinese hamster ovary cell lines expressing either the short or long isoform of the human IR. Insulin activated both pathways, but the phosphorylation of Akt was more sensitive to the hormone than the phosphorylation of ERK. Maximally effective concentrations of XMetA elicited phosphorylation patterns similar to 40–100 pM insulin, which were sufficient for robust Akt phosphorylation, but had little effect on ERK phosphorylation. These data indicate that the preferential signaling of XMetA is due to an innate difference in pathway sensitivity of Akt versus ERK responses to IR activation and partial agonism by XMetA, rather than a separate pathway-biased mechanism. The metabolic selectivity of partial IR agonists like XMetA, if recapitulated in vivo, may be a desirable feature of therapeutic agents designed to regulate blood glucose levels while minimizing undesirable outcomes of excessive IR mitogenic activation.

Introduction

Most patients with type 2 diabetes mellitus (T2DM) are both hyperglycemic and resistant to both endogenous and exogenous insulin (Reaven, 1988; Deftos, 2009). In many patients, fasting hyperglycemia can be corrected only by providing exogenous insulin, often in the form of long-acting insulin or a long-acting insulin analog (Pollock et al., 2011; Hilgenfeld et al., 2014). Insulin treatment, while effective, has potential risks, including weight gain, episodic hypoglycemia, and activation of potential risks of insulin highlight challenges in the management of T2DM patients with insulin, and the need for new agents that maximize the beneficial metabolic effects of insulin while minimizing the negative effects of the hormone.

When insulin binds to the insulin receptor (IR), it triggers a conformational change that allows for the autoprophosphorylation of tyrosines on the receptor’s intracellular β-subunit (Roth and Cassell, 1983; Shia and Pilch, 1983; Kahn, 1985; De Meyts, 2008). These phosphotyrosines serve kinase regulatory functions and become binding substrates for SH2 domain–containing adapter proteins, such as the IR substrate (IRS) proteins, Shc, and GRB2 (Taniyuki et al., 2006). The canonical metabolic pathway activation by insulin involves IR autoprophosphorylation and IRS protein binding and phosphorylation, followed by the association and activation of phosphatidylinositol-4,5-bisphosphate 3-kinase, which leads to the activation of phosphoinositide-dependent kinase 1. Then, phosphoinositide-dependent kinase 1 activates Akt by phosphorylating it at Thr308 (Taniyuki et al., 2006; Tan et al., 2012). Akt kinase is a key enzyme in metabolic insulin

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ABBREVIATIONS: BSA, bovine serum albumin; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; IR, insulin receptor; IRS, insulin receptor substrate; KinExA, kinetic exclusion assay; T2DM, type 2 diabetes mellitus.
action (Minea et al., 2005; Dong et al., 2008). This insulin-mediated signaling cascade acts in various cell types to orchestrate a shift from the use of stored energy in the body (glycogen, fat, and amino acids) to the storage and utilization of abundant prandial glucose and amino acids.

In addition to its metabolic effects, insulin can also trigger the activation of mitogenic pathways (Vigneri et al., 2009). Phosphorylated IR and IRS-1, bound by the Shc protein, serve as effective adaptors for the GRB2-SOS complex, thus activating RAS and the mitogen-activated protein kinase cascade (Hansen et al., 1996; Ceresa and Pessin, 1998). The mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK1/2, also referred to as p44/p42-activated protein kinase), is a key regulator of the mitogenic response to insulin (Johnson and Lapadat, 2002).

IR monoclonal antibodies may represent a novel class of long-acting therapeutics for regulating glucose metabolism in T2DM (Ussar et al., 2011). Monoclonal antibodies to the IR also have the potential to elicit metabolic effects while minimizing mitogenic responses. We have recently reported the development of XMeta, a fully human monoclonal antibody to the IR (Bhaskar et al., 2012). XMeta is an allosteric partial agonist of the IR that does not influence either insulin’s binding to the receptor’s orthosteric site or insulin’s ability to activate downstream signaling. In cell culture, XMeta stimulates key metabolic functions of insulin signaling, including glucose transport. XMeta is also active on the mouse and monkey IR. In rodent models of diabetes (Bhaskar et al., 2012, 2013), and in spontaneously diabetic primates (Zhao et al., 2014), XMeta decreases fasting blood glucose levels. Moreover, XMeta did not cause hypoglycemia in these diabetic animals (Bhaskar et al., 2012, 2013; Issafras et al., 2014).

Unlike insulin, which can stimulate both metabolic and mitogenic pathways via Akt and ERK, respectively, we observed that XMeta stimulated the Akt metabolic pathway, but generated little or no activation of the mitogenic ERK pathway. Moreover, unlike insulin, XMeta did not induce proliferation of tumor cells (Bhaskar et al., 2012, 2013). This observation suggested that XMeta activated downstream signaling pathways of the IR in a manner that differed from that of insulin. Therefore, to fully interpret this selective signaling, in the present study we analyzed the high-affinity insulin binding site and minimized the influence of the negative cooperativity effect (De Meyts, 2008; Whitten et al., 2009). To analyze the high-affinity insulin binding site and minimize the influence of the negative cooperativity effect (De Meyts, 2008), we employed equilibrium assays under conditions in which there was either no insulin present or a saturating insulin concentration (175 nM) present. XMeta (50 pM) was incubated with increasing concentrations of CHO cells (maximum 4 × 10^7 cells/ml) for 18 hours on a rotator at 5°C in phosphate-buffered saline with 0.25% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich). Cells were pelleted by centrifugation and the amount of free XMeta remaining in the supernatant solution was measured by immunofluorescence using a kinetic exclusion assay (KinExA) instrument (Sapidyne Instruments, Boise, ID). Antibody concentration data were fit using KinExA software (Xie et al., 2005; Rathanawasmi et al., 2008) to yield an estimate of the equilibrium dissociation (K_D) values. Preliminary data indicated that XMeta binds divalently to the IR dimer, establishing a 1:1 stoichiometry between the IgG molecule and receptor dimer (data not shown).

**Insulin Binding Assessed by KinExA.** To measure the effect of XMeta on the binding affinity of insulin for both isoforms of the human IR, we employed equilibrium assays under conditions in which there was either no XMeta present or where a saturating XMeta concentration (33 nM) was present. Then, 50 nM human insulin (Sigma-Aldrich) and either XMeta or an anti-keyhole limpet hemocyanin IgG2 isotype control antibody (33 nM) was incubated for 18 hours on a rotator at 5°C in phosphate-buffered saline with 0.25% BSA and 0.1% sodium azide with increasing concentrations of either CHO-hIR-A or CHO-hIR-B cells. Cells were pelleted by centrifugation and the amount of free insulin in the solution was measured by immunofluorescence using a KinExA instrument. Briefly, polymethylmethacrylate beads were coated with 65 μg/ml D6C4 anti-insulin monoclonal Ab (Fitzgerald Industries, Acton, MA), and the captured insulin was detected with 0.15 μg/ml biotin-labeled D3E7 anti-insulin monoclonal antibody (Fitzgerald Industries) mixed with 1 μg/ml streptavidin-phycoerythrin. Insulin binding data were fit using KinExA software as described previously to determine the insulin binding affinity (K_D) (Xie et al., 2005; Rathanawasmi et al., 2008). This methodology, employed to measure insulin affinity, was designed to analyze the high-affinity insulin binding site and minimize the influence of the negative cooperativity effect (De Meyts, 2008; Whitten et al., 2009).

**The Effect of XMeta on Insulin Signaling in Cultured Cells.** For studies evaluating the activation of insulin signaling events, CHO-K1 cells expressing either the human IR-A or human IR-B isoform were first incubated in Dulbecco’s modified Eagle’s medium (25 mM glucose) with 0.2% BSA for 5 hours to reduce background signals, and then incubated with increasing concentrations of either insulin or XMeta for 10 minutes. In preliminary studies, this time was found to yield a robust response to insulin. Both longer and/or shorter incubations were used in the time course assays, as specified in the text. Cells were pelleted by centrifugation at 4°C, the supernatant was decanted, and the cells

**Materials and Methods**

**Establishment of Cell Lines.** The Chinese hamster ovary (CHO)-K1 cells employed in the current studies contained less than 5000 hamster IRs and less than 5000 IGF-1Rs (Bhaskar et al., 2012). These CHO-K1 cells were transfected with a stable plasmid containing a neomycin-selective marker and either the long form (IR-B) or the short form (IR-A) of the human IR cDNA (Yamaguchi et al., 1991; Bhaskar et al., 2012). The CHO-hIR-A and CHO-hIR-B cells were cloned by limited dilution, screened by flow cytometry for high expression, and cultured in EX-CELL-302 media (Sigma-Aldrich, St. Louis, MO). Both IR-transfected cell lines had approximately 250,000 receptor dimers per cell. The 3T3R-IR-A cells were obtained from the University of California at San Francisco (San Francisco, CA) and carry a deletion of the IGF-1 receptor and express approximately 400,000 IR-A receptor dimers per cell.

**XMeta Binding Assessed by Kinetic Exclusion Assay.** To measure the effect of insulin on the binding affinity of XMeta for both isoforms of the human IR, we employed equilibrium assays under conditions in which there was either no insulin present or a saturating insulin concentration (175 nM) present. XMeta (50 pM) was incubated with increasing concentrations of CHO cells (maximum 4 × 10^7 cells/ml) for 18 hours on a rotator at 5°C in phosphate-buffered saline with 0.25% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich). Cells were pelleted by centrifugation and the amount of free XMeta remaining in the supernatant solution was measured by immunofluorescence using a kinetic exclusion assay (KinExA) instrument (Sapidyne Instruments, Boise, ID). Antibody concentration data were fit using KinExA software (Xie et al., 2005; Rathanawasmi et al., 2008) to yield an estimate of the equilibrium dissociation (K_D) values. Preliminary data indicated that XMeta binds divalently to the IR dimer, establishing a 1:1 stoichiometry between the IgG molecule and receptor dimer (data not shown).

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resuspended in cold Tris lysis buffer [150 mM NaCl, 20 mM Trizma-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, phosphatase inhibitors 2 and 3 (100 µM/10 µl), and 2 mM PMSF, all from Sigma-Aldrich], and cOmplete Protease Inhibitor tablets (Hoffman-La Roche, Basel, Switzerland). Samples were then analyzed by Western blot using antibodies recognizing total Akt, Akt phosphorylated at Thr308, total Erk1/2, Erk1/2 phosphorylated at Thr202/Tyr204, Thr185/Tyr187, IR phosphorylated at Tyr1328, and β-actin (Cell Signaling Technology, Danvers, MA). The total IR β-subunit antibody and the antibody to IR phosphorylated at Tyr1162/1163 were from EMD Millipore (Billerica, MA), and the antibody to IR phosphorylated at Tyr972 was from Invitrogen (Carlsbad, CA). Samples were reduced and run on a 10% 2-[bis(tris(hydroxymethyl)methyl)amino]-2-(hydroxymethyl)propane-1,3-diol gel using 4-morpholinepropanesulfonic acid–SDS running buffer, and then transferred to polyvinylidene difluoride membranes. Blots were blocked with Tris-buffered saline with 0.1% Tween 20 and 5% BSA, and then probed with primary antibodies at 1:5000 dilution. Detection was performed with anti-rabbit IgG(H + L)-HRP (Jackson Immunoresearch, West Grove, PA), imaged using SuperSignal West Dura Chemiluminescent Substrate (Pierce/Thermo Fisher, Waltham, MA) on a ChemiDoc MP charge-coupled device imager (Bio-Rad, Hercules, CA), quantitated in the Image Lab software (Bio-Rad) and normalized to percentage of maximal insulin response (25 nM insulin) for each marker. Data were fit using Prism software (sigmoidal dose response, variable slope 4 parameter fit) (GraphPad Software, La Jolla, CA) to generate EC50 values. Lysates were analyzed for IRS-1 phosphorylation by plate-based immunoassay using a kit from Meso Scale Discovery (Rockville, MD). Analysis of signaling in 2T3R-IR-A cells was carried as described previously with the modification that no centrifugation was required because the cells were adherent and the supernatant was aspirated. Unless stated otherwise, all figures represent results from at least three independent experiments, each with either duplicate or triplicate determinations. Values are reported as the mean ± S.E.M.

**Evaluation of Agonist Bias Using the Black-Leff Operational Model.** Signaling response data were evaluated using the Black-Leff operational model to calculate log(t/Ko) values (Kenakin et al., 2012). The log(t/Ko) values were calculated using the operational model in Prism (GraphPad Software) and the data are presented as ± S.E.M.

**Results**

**Binding of XMetA to Cells Expressing Either Isoform of the Human IR (CHO-hIR-A or CHO-hIR-B).** Studies were first carried out in cultured CHO-K1 cells expressing either the hIR-A or hIR-B isoform to determine the binding affinity (Kd) of XMetA to each isoform (Fig. 1). XMetA bound to the hIR-A isoform with an affinity of 55 ± 16 pM (Fig. 1A) and bound to the hIR-B isoform with a nearly identical affinity of 50 ± 11 pM (Fig. 1B). The affinity of XMetA to both IR isoforms was independent of the presence of insulin. This analysis indicated that the presence of the region encoded by exon 11 of the IR did not influence XMetA binding.

**Binding of Insulin to Cells Expressing Different Forms of the Human IR (CHO-hIR-A and CHO-hIR-B).** Studies were carried out in cultured CHO-K1 cells to determine the affinity of insulin to both isoforms of the human IR (Fig. 2). Insulin bound to cells expressing the hIR-A isoform with an affinity of 156 ± 14 pM in the presence of control antibody, and 216 ± 100 pM in the presence of XMetA (Fig. 2A). Insulin bound to cells expressing the hIR-B isoform with an affinity of 221 ± 28 pM in the presence of control antibody and 277 ± 112 pM in the presence of XMetA (Fig. 2B). The affinity of insulin to both IR isoforms was independent of XMetA (P = 0.45). In the absence of XMetA, the affinity of insulin was slightly higher for the hIR-A form (P = 0.024) than for the hIR-B form. This slightly higher affinity for the hIR-A form is in agreement with previously published results using other techniques (Mosthaf et al., 1990; Yamaguchi et al., 1991, 1993; Sciaccia et al., 2010; Knudsen et al., 2011).

**Effect of Insulin and XMetA on Autophosphorylation of the IR Kinase Regulatory Domain of the Two hIR Isoforms.** Autophosphorylation of IR beta subunit tyrosines 1150/1151 of the hIR-A isoform and the beta subunit tyrosines 1162/1163 of the hIR-B isoform are necessary to allow activation of IR tyrosine kinase activity (Hubbard, 2013). In the hIR-A isoform, insulin stimulated this phosphorylation half-maximally (EC50) at 486 ± 238 pM (Fig. 3A). The effect of insulin on the hIR-B isoform phosphorylation occurred at slightly lower concentrations, stimulating this function half-maximally at 118 ± 32 pM (Fig. 3B). The maximal effect of XMetA on IR autophosphorylation of both receptor isoforms was markedly lower than that of insulin, achieving only 20–30% of the maximal effect of insulin. XMetA stimulated hIR-A isoform phosphorylation half-maximally at 445 ± 77 pM (Fig. 3A), and hIR-B isoform phosphorylation half-maximally at 1430 ± 42 pM (Fig. 3B).

**Comparison of the Effects of Insulin and XMetA on Other Tyrosine Phosphorylation Sites on the IR and IRS-1.** Because the IR has additional phosphorylation sites, we evaluated whether the lower maximal autophosphorylation by XMetA was unique to the kinase regulator loop tyrosines (vide supra). Thus, we also evaluated two other tyrosine autophosphorylation sites representing the juxtamembrane domain (Tyr960 for hIR-A and Tyr972 for hIR-B) and the C-terminal region (Tyr1316 for hIR-A and Tyr1328 for hIR-B) (Fig. 3). Insulin stimulated phosphorylation of the juxtamembrane

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**Fig. 1.** Effect of insulin on XMetA binding to CHO-K1 cells expressing either human IR-A or IR-B. Varying concentrations of CHO-K1 cells in suspension, expressing either IR-A (A) or IR-B (B), were incubated in the presence (filled squares) and absence (open circles) of 175 nM insulin plus 50 pM XMetA antibody at 4°C for 18 hours. After removal of the cells by centrifugation, the free antibody in the supernatant at equilibrium was analyzed by immunofluorescence-based KinExA, and then analyzed with KinExA software to determine binding affinity. Data are the mean ± S.E.M. from triplicate experiments.
tyrosine of the IR-A isoform with an EC\textsubscript{50} of 690 ± 141 pM and the IR-B isoform with an EC\textsubscript{50} of 394 ± 88 pM. Insulin stimulated the phosphorylation of the C-terminal domain with an EC\textsubscript{50} of 1.55 ± 0.17 nM for the IR-A isoform and 1.54 ± 0.09 nM for the IR-B isoform. Thus, while the IR-B isoform kinase regulatory loop tyrosines were more sensitive to insulin-stimulated autophosphorylation than the IR-A isoform, the juxtamembrane and C-terminal tyrosines had similar insulin-stimulated dose responses in both isoforms.

In the CHO-hIR-A cells, XMetA induced the autophosphorylation of all these tyrosines to a level of approximately 20% that of insulin with similar dose responses (Fig. 3A). In the CHO-hIR-B cells, XMetA induced autophosphorylation for tyrosines 972 that was also 20% that of insulin, but the tyrosine near the C terminus (Tyr1328) had a slightly higher level of activation to approximately 40% that of insulin (Fig. 3B). XMetA stimulated phosphorylation of the juxtamembrane tyrosine of the IR-A isoform with an EC\textsubscript{50} of 807 ± 360 pM and the IR-B isoform with an EC\textsubscript{50} of 1.96 ± 0.46 nM. XMetA stimulated the phosphorylation of the C-terminal domain with an EC\textsubscript{50} of 1.32 ± 0.41 nM for the IR-A isoform and 4.52 ± 0.15 nM for the IR-B isoform. Thus, XMetA was a modestly more potent activator of IR-A autophosphorylation than IR-B in terms of dose response; however, the maximal levels of autophosphorylation induced by XMetA were very similar between the two isoforms.

After autophosphorylation, the IR phosphorylates tyrosines on IRS proteins. Thus, the effect of insulin and XMetA was also studied on IRS-1 phosphorylation (Fig. 3C). XMetA at a maximal concentration stimulated IRS-1 phosphorylation at a level approximately 20% that of a maximally effective concentration of insulin.

**Effect of Insulin on Activation of Akt and ERK in CHO-IR Cells.** We next studied the effect of insulin on phosphorylation of Akt at Thr\textsuperscript{308}, the site required for its kinase activation and activation of IR-mediated metabolic signaling (Taniguchi et al., 2006). In the cells expressing the IR-A isoform (Fig. 4A), 10 minutes of incubation with insulin stimulated Akt phosphorylation half-maximally at 123 ± 36 pM. In the cells expressing the IR-B isoform (Fig. 4A), insulin stimulated Akt phosphorylation half-maximally at 42 ± 10 pM (Fig. 4B).

We next studied the effect of insulin on the phosphorylation of ERK1/2 at Thr\textsuperscript{202}/Tyr\textsuperscript{204} and Thr\textsuperscript{185}/Tyr\textsuperscript{187}, the sites required for its kinase activation and activation of IR-mediated mitogenic signaling. To stimulate ERK1/2 activation, much higher concentrations of insulin (10- to 20-fold) were required when compared with insulin stimulation of Akt: in cells expressing the IR-A isoform (Fig. 4A) insulin stimulated ERK phosphorylation half-maximally at 1450 ± 274 pM, and in cells expressing the IR-B isoform insulin stimulated ERK phosphorylation half-maximally at 837 ± 274 pM (Fig. 4B).

**Fig. 2.** Effect of XMetA on insulin binding to CHO-K1 cells expressing either human IR-A or IR-B. Varying concentrations of CHO-K1 cells, expressing IR-A (A) or the IR-B (B), were incubated in the presence of either 33 nM XMetA (filled squares) or control antibody of the same isotype (open circles) plus 50 pM insulin at 4°C for 18 hours. After removal of the cells by centrifugation, the free insulin in the supernatant at equilibrium was analyzed by immunofluorescence-based KinExA, and then analyzed with KinExA software to determine the affinity. Data are the mean ± S.E.M. from triplicate experiments.

**Fig. 3.** Effect of insulin and XMetA on IR autophosphorylation in CHO-hIR-A and hIR-B cells. IR autophosphorylation of the juxtamembrane, kinase regulatory loop, and C-terminal tyrosines was evaluated in the CHO-IR-A cells (A) at Tyr\textsuperscript{960} (squares), Tyr\textsuperscript{1150/1151} (circles), and Tyr\textsuperscript{1316} (triangles) by either insulin (solid symbols and solid curves) or XMetA (open symbols and dashed curves). The analogous tyrosines were also evaluated for autophosphorylation in CHO-IR-B cells (B) at Tyr\textsuperscript{972} (squares), Tyr\textsuperscript{1162/1163} (circles), and Tyr\textsuperscript{1328} (triangles). CHO cells expressing IR-A or IR-B isoforms were cultured in growth factor–deficient media for 5 hours, stimulated with various concentrations of either XMetA or insulin for 10 minutes at 37°C, pelleted by centrifugation at 4°C, and then lysed. Lysates were analyzed by Western blot using specific IR phosphotyrosine antibodies and imaged on a charge-coupled device imager. Densitometry data were normalized to percentage of maximal insulin response for each phosphotyrosine and shown as the mean ± S.E.M. from triplicate experiments. (C) Lysates were analyzed for IRS-1 phosphorylation using a plate-based immunoassay (Meso Scale Discovery). Four stimulations per column are shown from a single experiment, with the mean ± S.D.
Effect of XMetA on Activation of Akt and ERK in CHO-IR Cells. Next, we studied the effect of XMetA on the phosphorylation of Akt at Thr^308. Maximal phosphorylation of this enzyme was approximately 60% that of insulin for both IR isoforms. In cells expressing the hIR-A isoform (Fig. 4C), XMetA stimulation of Akt phosphorylation half-maximally at 356 ± 60 pM, and in cells expressing the hIR-B isoform, XMetA stimulated Akt phosphorylation half-maximally at 968 ± 42 pM (Fig. 4D). Thus, when Akt is measured, CHO-hIR-B cells are more sensitive to insulin and less sensitive to XMetA than the CHO-hIR-A cells.

The maximal effect of XMetA on the phosphorylation of ERK1/2 was only 14% that of insulin in cells expressing the hIR-A isoform (Fig. 4C) and less than 4% that of insulin in cells expressing the hIR-B isoform (Fig. 4D). XMetA stimulated ERK phosphorylation in cells expressing the hIR-B isoform half-maximally at 1000 ± 190 pM and stimulated ERK phosphorylation in cells expressing the hIR-B isoform at 1430 ± 270 pM.

Effect of Duration of Incubation on IR Autophosphorylation, Akt Phosphorylation, and ERK Phosphorylation. The effect of duration of incubation was studied on the aforementioned functions for both insulin and XMetA. Incubation times were 2, 5, 10, and 20 minutes (Fig. 5). The concentrations tested at these times were a maximally effective insulin concentration of 100 nM, a maximally effective insulin concentration of 25 nM, and a lower insulin concentration of 100 pM. The lower concentration of insulin caused a level of pIR and pAkt activation similar to that of 100 nM XMetA. This analysis indicated that the differences between XMetA and insulin on the aforementioned parameters were not the result of kinetic differences.

Comparison of the Effects of Insulin and XMetA on 3T3R-IR Cells. To explore whether the differential in activation of Akt versus ERK by both insulin and XMetA was specific for CHO cells, Akt and ERK activation was investigated in a second cell line, mouse fibroblasts expressing hIR-A. In this cell line, insulin stimulated Akt activation half-maximally at 81 ± 30 pM (Fig. 6A). XMetA stimulated Akt activation to a level approximately 80% that of insulin (Fig. 6B). The one-half-maximal XMetA concentration was 1960 ± 244 pM.

As with CHO cells expressing the human IR, much higher concentrations of insulin were required to stimulate ERK1/2 activation in the 3T3R-IR cells; the half-maximal insulin concentration was 473 ± 116 pM (Fig. 6A). For XMetA, the maximal pERK1/2 response was approximately 5% that of insulin (Fig. 6B). These studies indicated that the effect of insulin on ERK activation also required higher concentrations of insulin when compared with Akt activation in a second cell line, and that XMetA had much greater effects on Akt activation than on ERK activation. Thus, the differential effects of XMetA were not specific to CHO cells.

Evaluation of Agonist Bias Using the Black-Leff Operational Model. Signaling response data were evaluated using the Black-Leff operational model to calculate log(Δt/K_A) values. The log(Δt/K_A) value is a transduction coefficient that can be used as a measure of ligand potency in terms of dose response (Kenakin et al., 2012). This transduction coefficient incorporates both an affinity factor (K_A) and an efficacy factor (Δt) to estimate the relative potency of an agonist, and can be calculated for each activation pathway or function and used to quantify signaling bias of agonists relative to a reference agonist. The log(Δt/K_A) values were calculated for insulin or XMetA stimulation of Akt and ERK1/2 phosphorylation (Fig. 7, A and B). The Δlog(Δt/K_A) values (Fig. 7, C and D) show the difference in log(Δt/K_A) values of XMetA from insulin for either the Akt or the ERK pathway. The insulin-subtracted Δlog(Δt/K_A) of the two pathways’ stimulation by XMetA have overlapping errors and are not significantly different, demonstrating a lack of agonist bias. To further illustrate this relationship, bias plots were created (Fig. 7, E and F). These plots compare the relative activation
of the Akt and ERK pathways at the same concentration of agonist. The XMetA response on the bias plot closely follows that of insulin for both the hIR-A and hIR-B experiments. This demonstrates that the innate differential sensitivities of the ERK and AKT pathways to IR stimulation are sufficient to describe the pathway activation characteristics observed for XMetA, as opposed to XMetA having a unique differential potency for AKT relative to ERK compared with insulin.

Discussion

The monoclonal antibody, XMetA, was isolated from a human antibody phage display library (Schwimmer et al., 2013), and has been shown to bind to both the hIR-A and hIR-B isoforms of the receptor. XMetA is an allosteric partial agonist that binds to and activates the IR, and activates the Akt, but not the ERK pathway. Therefore, XMetA behaves as a selective IR modulator (Vigneri et al., 2012). The current studies investigated in detail the mechanism(s) whereby XMetA selectively activates the Akt pathway. We also determined whether these signaling properties of XMetA are the same for both the A and B isoforms of the human IR.

In the case of insulin, a differential dose response for Akt and ERK activation via the IR has been previously observed in several studies (Jensen et al., 2007; Malaguarnera et al., 2012), but the effects of an IR partial agonist, such as XMetA, on this
differential dose response have not been explored. We measured signaling functions downstream from the IR: the phosphorylation of Akt, a major regulator of the metabolic effects of insulin, and ERK1/2, a major regulator of the mitogenic effects of insulin. Interestingly, the insulin dose response for Akt phosphorylation and activation occurred at significantly lower hormone concentrations than that of ERK1/2 phosphorylation. This differential sensitivity of Akt versus ERK1/2 was seen with both IR isoforms and in several cell types. Thus, these data indicate that the Akt pathway inherently has greater signal amplification (Tan et al., 2012) than the ERK1/2 pathway following IR activation (Kahn, 1978; Ish-Shalom et al., 1997). Previously, we compared the effect of insulin and XMetA on glucose uptake and cell proliferation (Bhaskar et al., 2012). Glucose uptake was enhanced at subnanomolar concentrations by both insulin and XMetA. In contrast, cell proliferation was only enhanced by insulin at nanomolar concentrations and XMetA had no effect on this parameter. These data are compatible with the observations (1) that insulin and XMetA both activate the IR/Akt metabolic pathway; and (2) that insulin at relatively higher concentrations activates the IR/ERK mitogenic pathway, whereas XMetA stimulation of this pathway is negligible.

XMetA, as a partial IR agonist, exploits this differential pathway sensitivity in both isoforms of the IR to elicit an apparent metabolic signaling bias. Because XMetA stimulates sufficient IR autophosphorylation to activate Akt, the antibody mimics the metabolic effects of low concentration insulin. Because more IR activation is required to activate the ERK pathway than the Akt pathway, a compound that is capable of only partial IR activation would have little or no ability to activate the less efficiently coupled ERK pathway. Thus, a major reason why XMetA does not activate ERK is that, as a partial IR agonist, the antibody does not stimulate sufficient IR autophosphorylation to activate the ERK pathway. When the insulin dose-response curves for these various markers of insulin activation, as well as the maximal level of stimulation by XMetA, are plotted together for comparison (Fig. 8), it can be demonstrated that XMetA, at its maximum effective dose, behaves much like a physiologic concentration of insulin (40–100 pM) in CHO-hIR cells. Therefore, these data suggest that the observed metabolic selectivity by XMetA is a direct consequence of its partial agonism combined with the natural differential signaling pathway sensitivities of Akt versus ERK when stimulated by IR activation. This conclusion is supported by the biased agonist analysis (Fig. 7) as applied from the Black-Leff operational model (Kenakin et al., 2012).

Another potential mechanism for differential post-IR signaling by XMetA was alternate phosphorylation of $b$-subunit tyrosines. The IR has seven identified tyrosines that are autophosphorylated by the IR kinase domain (Wilden et al., 1990), and these tyrosines are divided into three main regions: (1) the juxtamembrane region (965 and 972); (2) the kinase regulatory domain (1158, 1162, and 1163); and (3) the C-terminal region (1328 and 1334) (Hubbard, 2013). There are no reports of IR tyrosines that specifically regulate mitogenic activation.
because all tyrosine depletions decrease the metabolic response to insulin (Ellis et al., 1986; Maegawa et al., 1988; McClain et al., 1988; White et al., 1988). We observed that for both IR-A and IR-B the kinase regulatory domain was most sensitive to insulin, and the C-terminal region was least sensitive to insulin. Mutation and deletion studies regarding the C-terminal phosphotyrosines’ contributions to insulin signaling support the notion that the C-terminal tyrosines have a moderate role in activating metabolic insulin signaling by acting as a binding substrate to the Akt-activating PDK-1 enzyme (Fiory et al., 2005), but have little if any effect on mitogenic insulin signaling (Maegawa et al., 1988; McClain et al., 1988; Takata et al., 1991).

In the CHO-hIR-A cells, the dose response of all three sites to XMetA was similar; XMetA was able to only partially stimulate receptor autophosphorylation to a level about 20% that of insulin. In the CHO-hIR-B cells, but not the CHO-hIR-A cells, the tyrosine near the C terminus (1328) had a slightly higher level of activation to about 40% that of insulin. This difference is modest and would not be sufficient to describe the Akt selective tyrosine near the C terminus (1328) had a slightly higher level of activation to about 40% that of insulin. This difference is modest and would not be sufficient to describe the Akt selective tyrosine phosphorylation profile.

When compared with insulin, several studies have reported that certain insulin analogs enhance activation of the mitogenic pathway. These studies are difficult to interpret in terms of IR signaling because different mutants and analogs often have enhanced activities against the IGF-1R receptor, which contributes to the mitogenic response (Hansen et al., 2011). Sciacca et al. (2010) evaluated insulin analogs in cells that had no IGF-1R expression and demonstrated that some analogs maintained a preference for the mitogenic pathway activation when compared with insulin. The mechanisms of this preference are not clear, but analogs with a slow receptor dissociation rate demonstrated more mitogenic signaling (Hansen et al., 1996). The different affinities and binding properties of the insulin analogs may be able to effect IR trafficking and internalization, which can influence levels of ERK activation (Ceresa et al., 1998; Morcavallo et al., 2012). However, unlike XMetA, these insulin analogs are all orthosteric binders and, importantly, act as full agonists of the IR, with maximal levels of receptor activation similar to that of insulin. While some analogs have enhanced mitogenic activity compared with insulin, none have been reported to have enhanced metabolic activity. Thus, XMetA is a unique molecule in predominately stimulating metabolic activity via the IR.

A molecule such as XMetA that has enhanced metabolic relative to mitogenic activity may be clinically useful. It has been reported that in most insulin-resistant T2DM subjects, the metabolic pathway of the IR is selectively more resistant to insulin when compared with the mitogenic pathway (Jiang et al., 1999; Cusi et al., 2000). Therefore, a molecule that selectively stimulates the metabolic pathway might provide clinical benefits, while avoiding untoward effects of high tonic or episodic insulin exposure.

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Conducted experiments: Bedinger.
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Pathway Coupling Drives Selectivity of IR Agonist


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