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; DeFronzo, 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 the mitogenic insulin signaling pathway (Hemkens et al., 2009). The canonical metabolic pathway activation by insulin involves IR autophosphorylation and IRS kinase 1. Then, phosphoinositide-dependent kinase 1 activates 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 (Taniguchi et al., 2006). The canonical 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.

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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 (Miine 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 a more detailed analysis of the activation properties of XMetA on these different pathways (in comparison with insulin) has been undertaken. First, studies were performed to determine whether the effects of XMetA on the insulin signaling cascade were IR isoform specific. In humans and other mammals, the IR exists in two splice variant isoforms (Frasca et al., 1999). A long form (IR-B) contains the 12 amino acid exon 11 segment of the extracellular α-chain and is the dominant isoform in adult insulin target tissues, specifically, liver and fat (Moller et al., 1989; Mosthaf et al., 1990; Glendorf et al., 2011). The shorter isoform, IR-A, which does not contain exon 11, is the predominant isoform in fetal tissues, lymphocytes, endothelium, and many tumor cells, and is also expressed to varying degrees in insulin-sensitive tissues (Moller et al., 1989; Mosthaf et al., 1990; Sesti et al., 1994). Second, to understand XMetA signaling via the IR, a more detailed analysis of XMetA and insulin binding and their activation of Akt and ERK has been carried out. Our results provide important insights into the relative activation of the metabolic and mitogenic pathways downstream from the IR by insulin and XMetA, and may have implications for development of potential new agents for the treatment of diabetes.

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-IRs (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 limiting 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 × 10^6 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; Rathanasawami et al., 2008) (standard affinity curve fit model) 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 pM 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-phycocerythrin. Insulin binding data were fit using KinExA software as described previously to determine the insulin binding affinity (K_D) (Xie et al., 2005; Rathanasawami 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
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 (Mostafai et al., 1990; Yamaguchi et al., 1991, 1993; Sciacca 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 487 ± 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...
tyrosine of the IR-A isoform with an EC$_{50}$ of 690 ± 141 pM and the IR-B isoform with an EC$_{50}$ of 394 ± 88 pM. Insulin stimulated the phosphorylation of the C-terminal domain with an EC$_{50}$ 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 auto-phosphorylation 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$_{50}$ of 807 ± 360 pM and the IR-B isoform with an EC$_{50}$ of 1.96 ± 0.46 nM. XMetA stimulated the phosphorylation of the C-terminal domain with an EC$_{50}$ of 1.32 ± 411 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$_{308}$, 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$_{202}$/Tyr$_{204}$ and Thr$_{185}$/Tyr$_{187}$, 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).
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<sup>308</sup>. 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 stimulated Akt phosphorylation half-maximally at 356 ± 60 nM, and in cells expressing the hIR-B isoform, XMetA stimulated Akt phosphorylation half-maximally at 968 ± 42 nM (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-A 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 XMetA 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((t/K<sub>A</sub>)) values. The log((t/K<sub>A</sub>)) 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<sub>A</sub>) and an efficacy factor (t) 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((t/K<sub>A</sub>)) values were calculated for insulin or XMetA stimulation of Akt and ERK1/2 phosphorylation (Fig. 7, A and B). The ∆log((t/K<sub>A</sub>)) values (Fig. 7, C and D) show the difference in log((t/K<sub>A</sub>)) values of XMetA from insulin for either the Akt or the ERK pathway. The insulin-subtracted ∆log((t/K<sub>A</sub>)) 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

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**Fig. 5.** Effect of duration of incubation on IR autophosphorylation, Akt phosphorylation, and ERK1/2 phosphorylation. IR autophosphorylation at pTyr1162/1163 (A), phospho-Akt at Thr308 (B) and phospho-ERK1/2 at Thr202/Tyr204 (C) were evaluated in the CHO-IR-B cell at various incubation times with either insulin or XMetA. Cells were cultured in growth factor–deficient media for 5 hours, and then stimulated with either 25 nM insulin (open square), 100 nM insulin (open triangle), 100 nM XMetA (closed square), or control media (closed circle) for the indicated times at 37°C. Cells were then pelleted by centrifugation, and lysed. Lysates were analyzed by Western blots using specific antibodies and imaged on a charge-coupled device imager. Densitometry data were normalized to percentage of maximal insulin response for each function. Data from two independent experiments were analyzed.

**Fig. 6.** The effect of insulin and XMetA on phospho-Akt and phospho-ERK1/2 in 3T3R-hIR-A cells. Phospho-Akt at Thr308 (circles) and phospho-ERK1/2 at Thr202/Tyr204 (squares) were evaluated in the 3T3R-IR-A cells. Cells were cultured in monolayer with growth factor–deficient media for 5 hours, and then stimulated with various concentrations of either insulin (A) or XMetA (B) for 10 minutes at 37°C. Supernatants were aspirated and the cells were lysed. Lysates were analyzed by Western blot using specific detection antibodies and imaged on a charge-coupled device imager. Data were normalized to percentage of maximal insulin response for each phosphotyrosine and are shown as the mean ± S.E.M. from triplicate experiments.
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 δ-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.

**Fig. 7.** Analysis of agonist bias using the Black-Leff operational model. Dose/response data shown in Fig. 4 were analyzed using the Black-Leff operational model to calculate the $\log(t/K_A)$ transduction coefficient of insulin or XMetA for Akt phosphorylation (circles) and ERK phosphorylation (squares) for the CHO-IR-A (A) and CHO-IR-B (B) cells. From these the $\Delta \log(t/K_A)$ values for XMetA for both pathways was calculated in reference to insulin in CHO-IR-A (C) and CHO-IR-B (D) cells. Bias plots are shown comparing the percentage of maximal insulin stimulation of Akt and ERK phosphorylation for insulin (red circles) and XMetA (black squares) at each concentration of agonist for CHO-IR-A (E) and CHO-IR-B (F).

**Fig. 8.** Working model illustrating that XMetA behaves similarly to a low-concentration of insulin. Phospho-IR (triangle), phospho-Akt (circles), and phospho-ERK (squares) insulin dose-response curves presented in Figs. 3 and 4 have been combined. The horizontal dashed lines are the maximal activation levels achieved by XMetA (100 nM) for the respective markers. The vertical line highlights a single insulin concentration that would approximate the maximal XMetA effect across the three markers of insulin activation.
because all tyrosine deletions 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 (1326) 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 selectivity observed from XMetA because pathway selectivity occurred in a similar manner for both the hIR-A and hIR-B cells. Thus, it is unlikely that XMetA induced pathway specificity via a 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-1 receptor, which contributes to the mitogenic response (Hansen et al., 2011). Sciaccia et al. (2010) evaluated insulin analogs in cells that had no IGF-1R expression and demonstrated that some analogs maintained a preference for IR 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 shown 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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Authorship Contributions

Participated in research design: Bedinger, Corbin, Roell, Adams.
Conducted experiments: Bedinger.
Performed data analysis: Bedinger.

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References


Pathway Coupling Drives Selectivity of IR Agonist


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