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Metformin (Glucophage) inhibits tyrosine phosphatase activity to stimulate the insulin receptor tyrosine kinase

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Abstract

Metformin is a commonly used anti-diabetic but whether its mechanism involves action on the insulin receptor or on downstream events is still controversial. With a time course that was slow compared with insulin action, metformin increased tyrosine phosphorylation of the regulatory domain of the insulin receptor (specifically, tyrosine residues 1150 and 1151). In a direct action, therapeutic levels of metformin stimulated the tyrosine kinase activity of the soluble intracellular portion of the beta subunit of the human insulin receptor toward a substrate derived from the insulin receptor regulatory domain. However, metformin did not alter the order of substrate phosphorylation by the insulin receptor kinase. Using a *Xenopus* oocyte preparation, we simultaneously recorded tyrosine kinase and phosphatase activities that regulate the insulin receptor by measuring the tyrosine phosphorylation and dephosphorylation of peptides derived from the regulatory domain of the human insulin receptor. In an indirect stimulation of the insulin receptor, metformin inhibited endogenous tyrosine phosphatases and purified human protein tyrosine phosphatase 1B that dephosphorylate and inhibit the insulin receptor kinase. Thus, there was evidence that metformin acted directly upon the insulin receptor and indirectly through inhibition of tyrosine phosphatases.

Keywords: Protein tyrosine phosphatase; Signal transduction; Diabetes; Biguanide; Phosphotyrosine

1. Introduction

Without causing hyperinsulinaemia, weight gain or hypoglycaemia, metformin (*N*,*N*-dimethylbiguanide or *N*,*N*-dimethylimidodicarbonimidic diamide; with proprietary inclusions, sold under name of ''Glucophage'') reduces macroangiopathy, hyperglycemia, and gluconeogenesis and improves lipid profile [1,2]. However, the exact mechanism(s) of action of metformin is still debated.

1.1. Metformin stimulation of the insulin receptor tyrosine kinase

Since therapeutic levels of metformin elevated the level of phosphotyrosine on the insulin receptor and IRS-1 in *Xenopus* oocytes or a plasma membrane-cortex (PMC) preparation obtained from the oocyte, we have suggested that metformin acts through stimulation of the insulin receptor tyrosine kinase activity [3]. This mechanism of action is very different from a recent suggestion that the drug acts through inhibition of mitochondrial function and stimulation of AMP-dependent protein kinase [4–10].

In support of a mechanism involving stimulation of insulin receptor kinase activity, metformin has also been found to stimulate insulin receptor tyrosine phosphorylation in rat tissue (muscle from diabetic rats [11], human vascular smooth muscle cells [12], human hepatoma

Abbreviations: ELISA, enzyme linked-immunosorbent assay; IPβIRK, the kinase activity of the recombinant, soluble intracellular portion (residues 941–1343) of the beta subunit of the human insulin receptor; IRS-1, insulin receptor substrate 1; *p*NPP, *para*-nitrophenol phosphate; PI3-kinase, phosphatidylinositol 3′-kinase; PMC, plasma membrane-cortex; hPTP-1B, human protein tyrosine phosphatase 1B

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HepG2 cells [13], primary human hepatocytes and hepatoma Huh7 cells [14], and human erythrocytes [15]). In another study, chronic insulin treatment of C2C12 skeletal muscle cells drastically reduced insulin receptor tyrosine phosphorylation, PI3-kinase activity and glucose uptake, however metformin treatment was able to restore all three measures of insulin action [16]. Metformin was also able to restore insulin-induced receptor tyrosine phosphorylation and PI3-kinase activation in cholesterol hemisuccinate-treated HepG2 cells [13]. Tyrosine kinase blockers (Tyrphostin B46 or AG 555, and AG 1024) inhibited metformin stimulation of glycogen synthase activity in oocytes [17], and 2-deoxyglucose uptake in human hepatoma cells [14]. In addition, diabetic patients with mutant insulin receptors (due to a deletion of exon 17 and subsequent loss of a portion of the tyrosine kinase domain of the insulin receptor) are unresponsive to metformin [18].

Metformin inhibition of hepatic glucose production [14] may be central in the ability of metformin to lower blood glucose. Since activation of the insulin receptor reduces hepatic glucose production, the fact that metformin increases insulin receptor tyrosine phosphorylation and tyrosine kinase activity in primary human hepatocytes and human hepatoma cells [14] is support for the belief that metformin acts at least in part through activation of the insulin receptor.

1.2. Regulation of the insulin receptor

As human protein tyrosine phosphatase 1B (hPTP-1B) lowers insulin receptor tyrosine phosphorylation and inhibits insulin action in *Xenopus* oocytes [19,20], we examined the effect of metformin on hPTP-1B activity. hPTP-1B preferentially removes phosphate from tyrosine residues 1150 and 1151 of the regulatory domain of the insulin receptor [21,22] to attenuate insulin signaling [23–34].

We have developed a new assay system that simultaneously records phosphorylation and dephosphorylation of specific regulatory tyrosine residues within the regulatory domain of the insulin receptor. When the insulin receptor regulatory domain (amino acids 1142–1153) is phosphorylated on tyrosines 1146, 1150 and 1151, the insulin receptor kinase activity is fully active and insulin signaling is maximized [35]. Since the PMC preparation retains coupling between the insulin receptor and immediate downstream signaling enzymes [3,36] and reduces ''background'' signals (e.g., phosphorylation or dephosphorylation events occurring in the cytoplasm, organelles, nuclei or nonmembrane events in whole cells), this membrane preparation provides significant improvement over other assay systems. With this new assay system, we find that metformin indirectly stimulates the insulin receptor through a decrease in inhibitory tyrosine phosphatase activity.

2. Materials and methods

2.1. A model system: the Xenopus oocyte

Since, at therapeutic levels, metformin or other biguanide derivatives enhanced and mimicked insulin action in the *Xenopus* oocyte [3,4,17,37,38], the oocyte has also been used as a model system in the study of metformin and insulin [3,20,23,39–55].

Xenopus laevis (Xenopus Express) stage VI oocytes were obtained by manual dissection from ovaries of primed animals. Priming was achieved with injection of 50 IU of pregnant mare's serum gonadotropin (Sigma Chemical Co.) into the animals 3–10 days before use. This maximizes and synchronizes the response of oocytes to insulin [56]. Cells were kept in modified 0-R2 solution (83 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.9).

2.2. ELISA for phosphorylation of insulin receptor phosphotyrosines 1150 and 1151

To record metformin action on the level of tyrosine phosphorylation of the insulin receptor regulatory domain in whole cells, metformin hydrochloride (MERCK-Santé) was added (10 μg/ml) to groups of 30 *Xenopus* oocytes. Since metformin uses water as a carrier, water was added to all control groups in all experiments. For the analysis by ELISA (KHR9131; BioSource International), cells (∼100 μl total volume in 1.7 ml Eppendorf v vials) were homogenized in 300 μl of a mixture of protease inhibitors ([final]: 3.75 mM 4-(2-aminoethyl)-bezenesulfonylfluoride; HCl, 3 μM aprotinin, 188 μM bestatin, 56 μM E-64, 75 μM leupeptin, and 38 μM pepstatin A; cat. no. 539134; Calbiochem), tyrosine phosphatase inhibitors (dilute 1:100 mixture of sodium orthovanadate, molybdate, tartrate and imidazole in extraction buffer; P5726; Sigma) and PMSF ([final]: 1 mM; prepared fresh daily). To remove storage yolk proteins, 600 μl of freon (1,1,2-trichlorotrifluoroethane; Sigma) was added and the samples centrifuged (15,000 × *g*, 10 min; 4 °C). The supernatant (∼200 μl; ∼6 mg protein) was incubated in wells precoated with a monoclonal (capture) antibody to the beta subunit of the insulin receptor. After incubation (overnight, 4 °C), the cell homogenate solution was removed, the wells washed (four times with working wash buffer included in the kit) and rabbit (detection) antibody to phosphorylated tyrosines 1150/1151 of the insulin receptor regulatory domain was added. After 1 h (room temperature), the wells were washed again and anti-rabbit IgG conjugated to horse radish peroxidase was added. Following incubation (30 min, room temperature) and washing, stabilized chromagen (tetramethylbenzidine) was added and the 96-well plate was incubated (30 min, room temperature) in the dark. Subsequent to addition of a stop solution, a plate reader (Multiskan Ascent 354, Thermo Labsystems) was used to record the absorbance at 450 nm. The assay was

linear over the range of cells (8–30 oocytes) and over the time points utilized.

2.3. Plasma membrane-cortices (PMCs)

PMCs were isolated from *Xenopus* oocytes as previously described by Stith et al. [3]. Briefly, in a solution of 10 mM NaCl, 10 mM HEPES, pH 7.2, oocytes were broken open with the points of microdissection tweezers and the nucleus and most of the cytoplasm (~99.5% of cellular protein) was washed away. Each PMC is about 10–50 nm thick and contains ~5 μg of protein. In this preparation, insulin can stimulate the insulin receptor tyrosine kinase activity and enzymes down-stream of the insulin receptor [3].

2.4. Phosphatase activity in PMC preparations measured with non-insulin receptor peptides

To measure endogenous serine-threonine phosphatase activity, a phosphopeptide (K-R-pT-I-R-R; 250 μM; Upstate Biotechnology Inc.) was added to 15 PMC preparations. After 15 min (15 °C) in the presence or absence of metformin (0.1–100 μg/ml), samples were briefly centrifuged (few seconds, 15,000 × *g*; Beckman Microfuge) and 25 μl was removed from the supernatant and free phosphate was measured by the malachite green assay [57].

To measure the effect of metformin on tyrosine phosphatase activity, the phosphopeptide was changed to: T-S-T-E-P-Q-pY-Q-P-G-E-N-L (17–126; Upstate Biotechnology). The phosphopeptide, which is a sequence from the src pp60 and includes tyrosine 527, is a substrate for hPTP-1B and other tyrosine phosphatases [57]. Phosphopeptide (200 μM final; 20 μl of 1 mM stock in distilled H_2O) was added to 15 PMCs (in 80 μl of 10 mM NaCl, 10 mM HEPES, pH 7.2) and incubated for 15 min at 15 °C. After a flash spin in a centrifuge (Beckman Microfuge E), 20 μl of supernatant was analyzed for free phosphate with malachite green [57]. After this initial period to measure control tyrosine phosphatase activity, PMCs were resuspended and the control groups (see "C" in Fig. 2) received 2 μl of H_2O. Some PMC groups ("M + W," Fig. 2) were briefly incubated with 150 mM NaCl, 10 mM HEPES, pH 7.2 to wash away peripheral membrane proteins (followed by reconstitution in low tonicity control buffer). Metformin (2 μl if 40 μg/ml for a final concentration of 10 μg/ml) was added to salt washed groups ("M + W", Fig. 2) and to PMCs that had not been salt washed ("M," Fig. 2). After a second 15-min incubation period, the PMCs were again gently pelleted and the supernatant analyzed for free phosphate with malachite green. Less than ~30% of substrate was broken down under the conditions used.

2.5. Human protein tyrosine phosphatase 1B in vitro activity measurements

In initial experiments, recombinant hPTP-1B (14–358; Upstate Biotechnology) activity was recorded with the use of *para*-nitrophenol phosphate (*p*NPP) as a substrate. After some groups were preincubated in metformin (10 μg/ml) for 20 min, hPTP-1B (750 ng in 25 mM HEPES, 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM ethylene-diamine-tetraacetic acid, pH 7.2, 100 μg/ml bovine serum albumin, and, to start the reaction, 5 mM *p*NPP) activity was measured over 10 min (37 °C). Reactions were stopped with 100 μl of 2 M K_2CO_3. After raising the volume to 1 ml with water, absorbance was recorded (as is well-known, after hPTP-1B removal of the phosphate from *p*NPP, the absorbance at 405 nm increases).

Subsequent experiments measured hPTP-1B (173 ng; 49 units of activity where 1 unit is 1 nmol of phosphate of *p*NPP released per min at 37 °C) activity in the presence of 20 PMCs. The reaction was begun by addition of 5 mM *p*NPP, and after 15 min (37.5 °C), the sample was flash spun (pressing the spin button of a Beckman microfuge) to pellet the PMCs and the supernatant was then analyzed for pNP production.

2.6. Use of peptides derived from the regulatory region of the human insulin receptor

To measure tyrosine kinase or phosphatase activity directed against the regulatory domain, we used commercially-available peptides derived from the human insulin receptor regulatory domain (Biomol). The amino acid sequence of the peptides was: T-R-D-I-Y^{1146}-E-T-D-Y^{1150}-Y^{1151}-R-K. The peptides contained no phosphate (referred to as "IR 0"), one phosphate ("IR 5" has a phosphate on tyrosine 1146; "IR 9" has one phosphate on tyrosine 1150; "IR 10" has one phosphate on tyrosine 1151), two phosphates ("IR 5, 9"; "IR 9, 10"; and "IR 5, 10"), or three phosphates ("IR 5, 9, 10"; with phosphates on tyrosines 1146, 1150, and 1151).

To separate the unphosphorylated, mono-, bis-, and trisphosphorylated forms of the insulin receptor peptides, we utilized an HPXL 2 pump HPLC system with UV detector set to 267 nm (Varian Instruments). The flow rate through the LC-318 (Supelco) column was 1 ml/min. Over a 40-min period, the percent acetonitrile was in increased from 5 to 19.1% (0.05% trifluoroacetic acid was in all solutions). Over the next 5 min, the percentage was increased to 25% and then decreased to 5% over the subsequent 5 min (total run time of 50 min).

To determine the amount of various peptides produced through the action of kinases and phosphatases, the peak of absorbance for the various peptides was electronically integrated. The peak area was then converted to micromoles or micrograms through the use of standard lines.

Before or after treatment with metformin, various insulin receptor regulatory domain peptides were added to the PMCs with or without the human insulin receptor kinase IPβIRK or hPTP-1B. The action of tyrosine kinases or phosphatases on specific tyrosine residues was compared

by the relative amounts of phosphorylated or dephosphorylated peptides produced.

2.7. Measurement of tyrosine kinase activity with insulin receptor regulatory domain peptides

We determined which tyrosine is preferentially phosphorylated by the human insulin receptor and tested the influence of metformin on the human insulin receptor tyrosine kinase activity. For these studies, recombinant intracellular portion of the human insulin receptor (IPβIRK) (Stratagene or Biomol Research Laboratories) was used to phosphorylate the unphosphorylated insulin receptor peptide (IR 0). IPβIRK exhibits the major functional properties of the intact insulin receptor [58,59] and consists of the juxtamembrane, tyrosine kinase, regulatory and C terminal domains of the human insulin receptor.

In the presence or absence of metformin (10 μg/ml), IR 0 (32 μg or 1.6 units of 1 nmol/min of phosphorylation of poly(glu:Tyr)$_{4:1}$) was added to the reaction mixture (final volume of 100 μl:1 μg IPβIRK in 5 mM $MnCl_2$, 5 mM $MgCl_2$, 250 μM ATP, 1 mM DTT, 50 mM Tris, pH 7.4). After 20 min (37 °C), 100 μl of 10% TCA was added and the mixture was centrifuged (5 min; Beckman Microfuge E). A portion (150 μl) of the supernatant was injected into the HPLC and the phosphorylated and dephosphorylated insulin receptor peptides were separated. After integration of UV absorption peaks, the amount of peptides were calculated from standard lines.

2.8. Simultaneous measurement of tyrosine kinase and phosphatase activities directed against the insulin receptor regulatory domain peptide IR 9

Since the PMC preparation contains both tyrosine phosphatase and kinase activities, the effect of metformin on these activities was determined simultaneously. After metformin treatment (10 μg/ml) of some groups, phosphopeptide IR 9 (one phosphate located on tyrosine 1150 of the insulin receptor regulatory domain; 16 μg of IR 9 in 16 μl) was added to 15 PMCs in 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 1% BSA, 50 mM Tris (pH 7.4) (total final volume of 100 μl). After 40 min at room temperature, 100 μl of 10% TCA was added to stop the reaction. After centrifugation (5 min in Beckman Microfuge E), 150 μl of the total 200 μl was removed and injected into the HPLC and the amount of insulin receptor peptides without phosphate or with two phosphates was recorded.

3. Results

Since metformin increases the level of phosphotyrosine on the insulin receptor, we [3] and others (see Section 1) have suggested that this action is central to metformin's ability to potentiate insulin action. To confirm and extend our prior work with whole cells (involving nonspecific phosphotyrosine antibodies), we quantified the level of phosphorylation of the specific regulatory sites on the insulin receptor (tyrosine residues 1150 and 1151). Both insulin and metformin increased the tyrosine phosphorylation of the regulatory domain to about the same level but metformin required a much longer incubation period (90 min) than insulin (20–30 min) (Fig. 1). The basal level of phosphorylated insulin receptor was 33.4 ± 2.8 pg per oocyte ($n = 8$) whereas insulin or metformin addition increased this number to about 47 pg per oocyte.

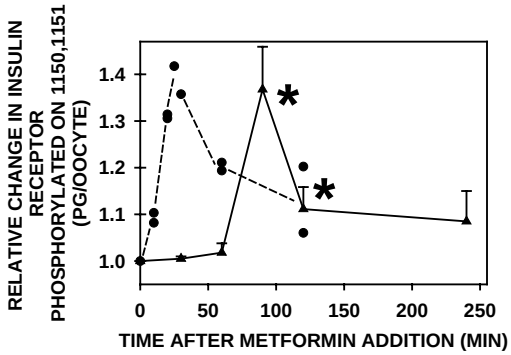

Fig. 1. Using a phosphospecific antibody, metformin increased phosphorylation of insulin receptor regulatory sites. Addition of either 2 μM insulin (dashed line) or 10 μg/ml metformin (solid line) to *Xenopus* oocytes increases the level of phosphorylation of sites 1150 and 1151 on the insulin receptor. An ELISA was used to quantify the level of phosphorylation. Individual determinations are graphed for insulin and there were four to eight determinations for metformin. Due to variance between basal levels, data were expressed relative to the control value for each experiment. Asterisks with the metformin data denote significance at $P < 0.05$.

Since tyrosine phosphatases can lower phosphorylation of the tyrosine residues found in the regulatory domain of the insulin receptor to inhibit the receptor tyrosine kinase activity, we examined the effect of metformin on tyrosine phosphatase activity. That is, we examined whether metformin could inhibit tyrosine phosphatases found in the PMC preparation or hPTP-1B.

With a substrate utilized by hPTP-1B and many other tyrosine phosphatases [57], metformin inhibited endogenous tyrosine phosphatase activity in the PMC preparation (Fig. 2; compare control release of phosphate, control group "C," versus metformin treated group "M"). Due to prior washing of the PMC preparation with a low tonicity medium, the tyrosine phosphatase activity measured would be bound to the plasma membrane or cortex.

Various therapeutic levels (1–10 μg/ml, which is equivalent to 8–80 μM) of metformin were able to inhibit the tyrosine phosphatase activity however 0.01 μg/ml was not effective. Monomethylbiguanide (10 μg/ml), a very close derivative of metformin that is ineffective in fighting diabetes and unable to stimulate isolated IPβIRK [3], was unable to inhibit tyrosine phosphatase activity (data not shown).

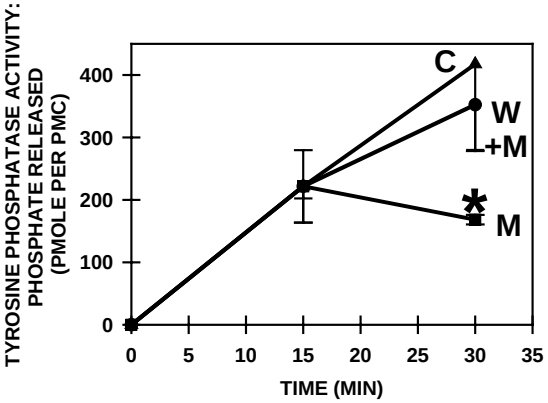

Fig. 2. Metformin inhibited tyrosine phosphatase activity in PMCs. The release of phosphate from a tyrosine on a phosphopeptide in the presence of 15 PMCs (that contain "endogenous" tyrosine phosphatase activity) is linear over 45 min ("C"). The phosphopeptide, T-S-T-E-P-Q-pY-Q-P-G-E-N-L, is a substrate for many tyrosine phosphatases [60]. Addition of 10 μg/ml metformin to some groups ("M") at 15 min inhibited tyrosine phosphatase activity in the PMC preparation. Metformin did not inhibit tyrosine phosphatase activity if the PMC was first washed with 150 mM NaCl first (W + M). For each group, $N = 9$ and an asterisk represents $P < 0.05$.

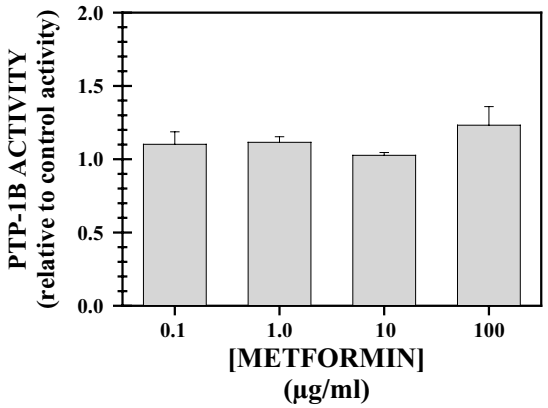

Fig. 3. Metformin did not directly inhibit hPTP-1B in vitro. Metformin was unable to inhibit the in vitro activity of human recombinant hPTP-1B. Using standard in vitro assay conditions (see Section 2), *p*NPP was used as a substrate for the tyrosine phosphatase. There were 18 determinations per column. To compare results across experiments that utilized different amounts of hPTP-1B activity, for each experiment, the activity in the presence of metformin was divided by the activity in the absence of metformin.

In addition, when PMCs were washed with high salt to remove peripheral proteins, metformin was unable to inhibit the phosphatase activity ("W + M," Fig. 2). This suggested that metformin inhibited tyrosine phosphatases through an intermediate that was located at or near the plasma membrane and was removed by salt washing. In addition, it shows that metformin does not simply interfere with the tyrosine phosphatase assay or malachite green determination of phosphate. Since tyrosine phosphatase activity in the washed PMCs did not significantly decrease from control values (PMCs not salt washed, "C"), tyrosine phosphatase activity must be bound to the plasma membrane (or structures associated with the plasma membrane) rather tightly. Consistent with this finding is that human tyrosine phosphatases that act upon the insulin receptor are not removed from membranes by washing with 0.6 M KCl [60,61].

To see if metformin inhibited other types of phosphatases, we examined serine-threonine phosphatase activity. With a commonly-used peptide substrate (K-R-pT-I-R-R) and oocyte homogenates or the PMC preparation, metformin (0.1–200 ug/ml) had no effect on activity (data not shown).

3.1. Metformin inhibition of recombinant hPTP-1B

Since metformin inhibited tyrosine phosphatase activity in the PMC preparation, and since hPTP-1B is believed to be a major inhibitor of the human insulin receptor, we determined whether the drug could directly inhibit hPTP-1B in vitro. After utilizing various incubation times and concentrations of metformin with purified recombinant hPTP-1B, metformin was unable to directly inhibit hPTP-1B (Fig. 3).

If metformin acts through an intermediate located at or near the plasma membrane, addition of PMCs would rescue the inhibition of hPTP-1B by metformin. Indeed this is the case as, in the presence of PMCs, therapeutic levels of metformin (greater than 1 μg/ml) inhibited hPTP-1B by 57% (Fig. 4).

3.2. The action of kinases and phosophatases on the insulin receptor regulatory domain

Our model suggests that metformin stimulates insulin signaling by increasing phosphorylation of specific tyrosines in the insulin receptor regulatory domain. Thus, we

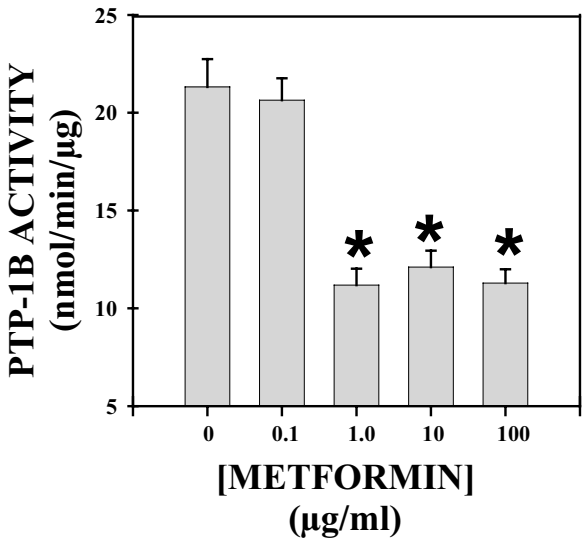

Fig. 4. PMCs rescued metformin inhibition of hPTP-1B. The effect of metformin on hPTP-1B was assayed in the presence of 15 PMCs. Phosphatase activity was measured with *p*NPP. For each concentration, $N = 4$ and an asterisk represents $P < 0.05$.

Table 1
HPLC separation of peptides derived from the insulin receptor regulatory domain

Phosphorylated tyrosine (amino acid number in the human insulin receptor)	Abbreviated name	HPLC elution time (min)
None	IR 0	36
Monophosphorylated		
1146	IR 5	32.7
1150	IR 9	34.6
1151	IR 10	33.7
Bisphosphorylated		
1146, 1150	IR 5, 9	26.8
1146, 1151	IR 5, 10	28.3
1150, 1151	IR 9, 10	27.3
Trisphosphorylated		
1146, 1150, 1151	IR 5, 9, 10	19

switched from artificial substrates to a more appropriate substrate derived from the human insulin receptor regulatory domain. The human insulin receptor is stimulated by phosphorylation of the three tyrosines (residues 1146, 1150 and 1151) located in a regulatory region of the intracellular portion of the insulin receptor [35].

Peptides derived from the insulin receptor regulatory domain (amino acids 1142–1153 of the human insulin receptor: T-R-D-I-Y^{1146}-E-T-D-Y^{1150}-Y^{1151}-R-K) can have up to three phosphotyrosines; "IR 0," "IR 5," "IR 9," or "IR 10" (no phosphate, phosphate on tyrosine 1146, 1150 or 1151, respectively). When two phosphates are present, there are three possibilities: "IR 5, 9"; "IR 9,10"; and "IR 5, 10." With three phosphates, the peptide is referred to as "IR 5, 9, 10." The time of elution for each peptide is found in Table 1 and a typical HPLC run showing separation of different phosphopeptides is shown in Fig. 5.

With addition of the IR 5, 9, 10 peptide, the activity of a tyrosine phosphatase can be followed. With the use of IR 0, tyrosine kinase activity directed toward the insulin regulatory domain can be recorded by measuring the rate at which phosphate is added to the three tyrosine residues of the insulin receptor regulatory domain peptide. With the use of IR 9 (one phosphate, on tyrosine 1150), we can simultaneously quantify tyrosine phosphatase and kinase action on the insulin receptor domain. PMCs, which contain both can be used as a source phosphatases and kinases, or purified enzymes such as IPβIRK or hPTP-1B can be added to the insulin receptor peptides. The use of the PMC preparation offers the additional advantage that endogenous kinases and phosphatases measured are those found at or near the plasma membrane (lowering background noise from those phosphorylation events that occur deep within the cytoplasm or nucleus).

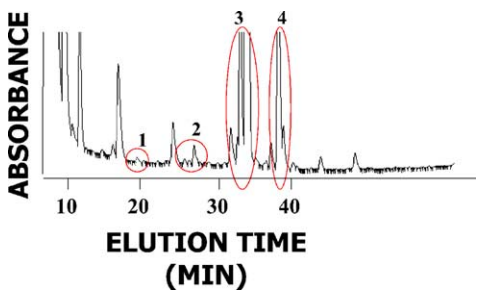

Fig. 5. HPLC separation of human insulin receptor regulatory domain peptides. IPβIRK was incubated with the unphosphorylated, human insulin receptor regulatory domain peptide (IR 0) (32 μg) for 40 min. After stopping the reaction with TCA, the phosphopeptides were separated on an HPLC. Peak 1 is the trisphosphorylated regulatory domain peptide (IR 5, 9, 10); peak 2 is a bisphosphorylated regulatory domain peptide; peak 3 is a monophosphorylated regulatory domain peptide, and peak 4 is the unphosphorylated regulatory domain peptide (IR 0).

3.3. The effect of metformin on IPβIRK activity directed toward the regulatory domain from the human insulin receptor

IR 0 (the insulin receptor regulatory domain peptide without any phosphate) was added to IPβIRK and by recording the mass of insulin receptor peptides with different levels of tyrosine phosphorylation, the order of phosphorylation preferred by human IPβIRK can be determined. After determining optimal conditions, we found that IPβIRK showed an approximately 60-fold preference toward phosphorylation of tyrosine 1150 (making the peptide referred to as "IR 9") over tyrosines 1151 and 1146 (Fig. 6). As noted in Fig. 6, metformin did not alter the substrate specificity of IPβIRK.

In the presence of metformin (Fig. 6), there appeared to be a small increase in the tyrosine kinase activity of IPβIRK that is directed toward the insulin receptor regulatory domain. In a prior publication, we described a small in vitro, direct stimulation of IPβIRK by metformin (no PMCs or cell extract were present) with an artificial tyrosine kinase substrate (RR-SRC, derived from src pp60 protein and modified with the addition of two arginine

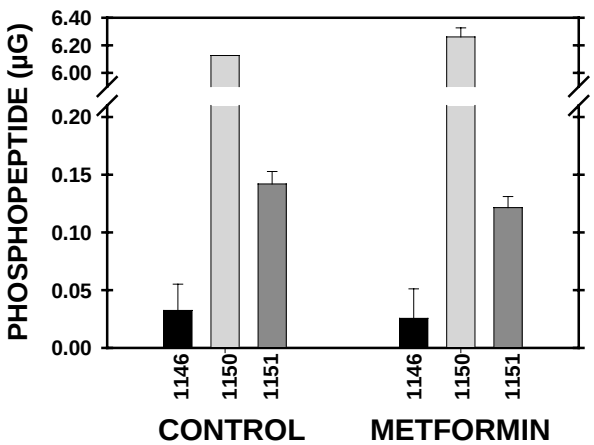

Fig. 6. Recombinant intracellular portion of the human insulin receptor (IPβIRK) preferentially phosphorylated tyrosine 1150 of the regulatory domain. Metformin had no significant effect on the relative order of preference of IPβIRK for the phosphorylation of the insulin receptor regulatory domain. $N = 3$ for each column.

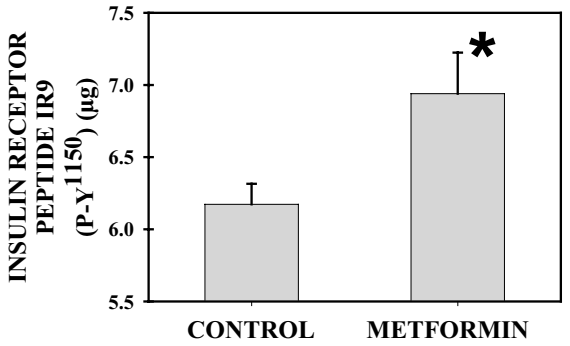

Fig. 7. Metformin stimulated IPβIRK in vitro activity directed toward tyrosine 1150. With or without metformin, IPβIRK was incubated with unphosphorylated insulin receptor regulatory domain peptide IR 0. The appearance of IR 9 phosphopeptide (one phosphate is present, located on tyrosine 1150) was recorded as a measure of human insulin receptor kinase activity directed toward the regulatory domain of the insulin receptor. The asterisk represents $P < 0.05$ and there were four determinations per column.

residues) [37]. With further experiments, metformin was able to weakly (13%) stimulate the ability of IPβIRK to phosphorylate the preferred site on the insulin receptor regulatory domain: tyrosine 1150 (Fig. 7).

However, no tyrosine phosphatases were present in these in vitro assays. In a whole cell, it is possible that the small in vitro stimulation of the insulin receptor kinase would be negated by the presence of tyrosine phosphatases. Thus, to more closely replicate cellular conditions (i.e., both tyrosine kinase and phosphatase activities present), we utilized the insulin receptor peptide IR 9 (the peptide with one phosphate on the site that is preferred by the insulin receptor kinase, tyrosine 1150) and the PMC preparation. More specifically, tyrosine phosphatase (IR 9 being metabolized to IR 0) and tyrosine kinase (IR 9 metabolized to bisphosphorylated insulin receptor peptides) activities were measured.

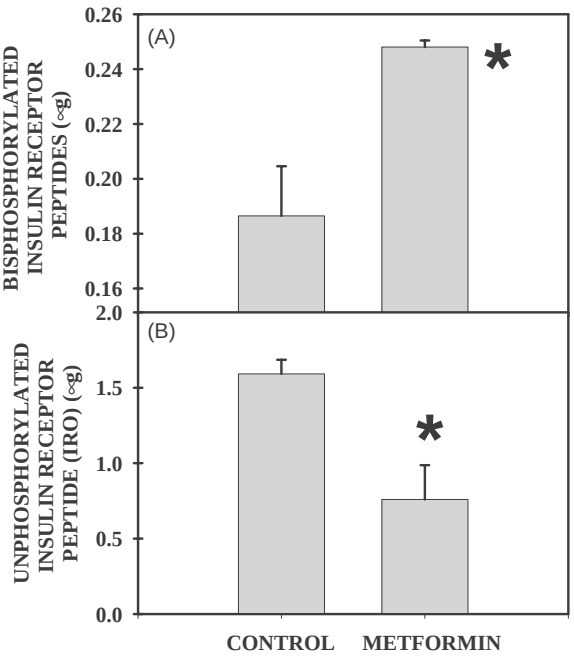

Fig. 8. Metformin stimulated tyrosine kinase (A) and inhibited tyrosine phosphatase (B) activities directed toward the human insulin receptor regulatory domain. PMCs (15) were incubated 40 min with 200 μM IR 9 phosphopeptide (one phosphate on the insulin receptor regulatory domain peptide tyrosine 1150). In (A) which reflects tyrosine kinase activity directed toward the insulin receptor regulatory domain, peak areas of all bisphosphorylated peptides were measured and combined. In (B) which reflects tyrosine phosphatase activity directed toward phosphotyrosine 1150 of the insulin regulatory domain, the unphosphorylated peptide (IR 0) was measured. There were three determinations per column and an asterisk represents $P < 0.05$.

Metformin, in the presence of both tyrosine kinases and phosphatases from the PMC preparation, stimulated the rate of insulin receptor peptide phosphorylation by 31% (Fig. 8A) and inhibited an endogenous tyrosine phosphatase that acts upon the insulin receptor regulatory domain by 55% (Fig. 8B).

4. Discussion

Metformin addition to whole *Xenopus* oocytes stimulated phosphorylation of the regulatory domain 1150/1151 tyrosine residues in the insulin receptor regulatory domain when metformin was added to whole *Xenopus* oocytes (Fig. 1). The prior studies of metformin action on insulin receptor phosphorylation (e.g., [3,14]) were obtained with a less specific anti-phosphotyrosine antibody and Western blotting. It was always possible that the general phosphotyrosine antibody used in these prior studies recorded unimportant phosphorylation on tyrosines not located in the regulatory domain of the insulin receptor, that contaminating ∼94 kDa phosphoproteins were present in the Western blot, or that the antibody was not specific for tyrosine phosphorylation.

The increase in the levels of tyrosine phosphorylation on the insulin receptor in whole cells is consistent with the finding that metformin stimulated the activity of endogenous tyrosine kinase activity, located at or near the plasma membrane (i.e., in the PMC preparation), that was directed toward the human insulin receptor regulatory domain (Fig. 8A).

In whole oocytes, metformin took much longer to increase phosphorylation of regulatory tyrosine residues 1150 and 1151 than insulin (90 min versus 20–30 min). In prior results with a nonspecific anti-phosphotyrosine antibody and Western blotting, metformin required ∼60 min to increase tyrosine phosphorylation in a 94 kDa band [3], whereas insulin required 10 min to double the level of phosphorylation and a maximum was achieved at ∼15–30 min after insulin addition (insulin experiments conducted at the same time as metformin; unpublished).

A relatively longer incubation time metformin action (versus insulin action) was also found in hepatocytes: metformin required 15 min to increase insulin receptor phosphotyrosine versus only 1 min for insulin [14].

In contrast to the 90-min incubation time with whole cells, and similar to its action on hepatocytes [14], metformin

action on PMCs required only 15 min [3]. Based on the relative incubation times between the PMC and whole cells, we have suggested that metformin must enter the cell to act [3]. There are other data consistent with an intracellular site of action for metformin: since the15-min incubation time with hepatocytes is much shorter than 60–90 min required for metformin action on the oocyte, cellular metformin concentrations may build up faster in the hepatocytes (which is ~1 million times smaller than the *Xenopus* oocyte). Further evidence that metformin must enter the cell is that when metformin was covalently linked to beads (thus preventing the entry of metformin through the membrane), the drug was unable to stimulate insulin action [60].

We now find (Fig. 1) that metformin was able to stimulate levels of tyrosine phosphorylation of the regulatory domain of the insulin receptor that were equivalent to the level stimulated by insulin. Prior results with *Xenopus* oocytes and human hepatocytes found that metformin addition produced lower levels of insulin receptor tyrosine phosphorylation than insulin addition [3,14]. Perhaps the use of a nonspecific anti-phosphotyrosine antibody in these prior studies resulted in a perceived lower level of metformin stimulation.

4.1. Direct action of metformin on the insulin receptor tyrosine kinase

We have described both a direct and indirect stimulation of the insulin receptor. There is a small 15–25% stimulation of recombinant, intracellular portion of the beta subunit of the insulin receptor (IPβIRK) when metformin is added in vitro [37]. An artificial tyrosine kinase substrate, RR-SRC, was used in this prior work. We go beyond simple confirmation of this prior result to show that metformin stimulated human IPβIRK activity directed toward the human insulin receptor regulatory domain tyrosine 1150 by ~13% (Fig. 7) and that metformin did not alter substrate specificity of the insulin receptor kinase (Fig. 6).

In contrast, addition of 1 μg/ml metformin to whole insulin receptor partially purified (by antibody or WGA) from human hepatocytes stimulated tyrosine kinase activity by ~125% [14]. Although the effect of metformin on the recombinant beta subunit IPβIRK is relatively weak, these results provide further evidence that metformin can directly stimulate the insulin receptor kinase (e.g., metformin binds to the intracellular portion of the human insulin receptor).

4.2. A second action: the indirect action of metformin on the insulin receptor through inhibition of tyrosine phosphatase activity

Metformin (a) inhibited, through an intermediate, endogenous tyrosine phosphatase activity, located at or near the plasma membrane, that is directed toward a commonly-used tyrosine phosphatase substrate (Fig. 2), (b) inhibited, through an intermediate, hPTP-1B by 57% (Fig. 4), (c) inhibited an endogenous tyrosine phosphatase activity (located at or near the plasma membrane) directed toward the human insulin receptor regulatory domain by 55% (Fig. 8B), and (d) did not affect serine-threonine activity.

Some of these experiments recording tyrosine phosphatase activity involved the use of PMCs and since PMCs have been washed with low salt buffers to remove most cytoplasmic proteins, one wonders whether important enzyme activity was removed. It is important to note that over 90% of protein tyrosine phosphatase activity (including hPTP-1B) is associated with membrane fractions [25] and hPTP-1B may be held at the membrane due to its interaction with docking proteins such as p130 Cas [62].

Metformin inhibition of endogenous tyrosine phosphatase activity, measured with release of free phosphate from RR-SRC (Fig. 2), demonstrated over 100% inhibition (the slope of the line showing release of phosphate was actually negative). This unusual level of inhibition may in part be due to incorporation of free phosphate since the PMC preparation contains many relatively intact cellular components located near the plasma membrane.

However, as noted above, two other phosphatase assays also showed that metformin inhibited tyrosine phosphatase activity by about 56%. One assay utilized measurement of pNP production by hPTP-1B (Fig. 4) and the second utilized measurement of insulin receptor regulatory domain peptide by endogenous tyrosine phosphatases located at or near the plasma membrane (Fig. 8B).

There are two lines of evidence for the presence of an intermediate between metformin and the inhibited tyrosine phosphatase: one, the salt washing of the PMCs (which removes proteins loosely bound to the membrane preparation) inhibits metformin action, and, two, metformin inhibits hPTP-1B only in the presence of the PMCs. Additional studies will attempt to identify the intermediate(s). Initial studies with PMCs failed to detect an increase in ^{32}P in hPTP-1B in the presence of metformin.

4.3. Multiple actions of metformin

Since metformin has many diverse effects (in addition to stimulating insulin receptor kinase activity, the drug decreases diabetes-related myocardial infarction and stroke, adipose tissue lipolysis, blood free fatty acids, hepatic glycogenolysis, gluconeogesis by glucagon, weight gain and increases muscle glucose uptake, PI3-kinase and glycogen synthesis), the drug may act through multiple mechanisms. For example, due to the finding that hPTP-1B deficient mice are very resistant to obesity [32,33], the ability of metformin to inhibit hPTP-1B

may be related to the finding that patients using metformin do not show a large gain in weight [63]. Metformin inhibition of hPTP-1B is consistent with the fact that metformin often lowers blood lipid levels and inhibition of hPTP-1B induces similar benefits [64].

4.4. Other mechanisms of metformin action

Metformin also inhibits the respiratory chain complex 1 of mitochondria [4,5,8] that could lead to the simulation of the AMP-activated protein kinase activity noted with metformin [6,7,9,10]. In contrast to relatively rapid two- to fourfold stimulation of insulin receptor tyrosine phosphorylation by low levels of metformin (in *Xenopus*: 60 min, 8–80 μM metformin [3]; in human HepG2 cells: 120 min, 100 μM metformin [13]) or a rapid twofold stimulation of PI3-kinase (10 min, 100 μM metformin [13]), or a rapid twofold stimulation of glycogen synthase (60 min; 1–10 μM [13,17,38]), similar concentrations of metformin required 4–18 h to inhibit mitochondrial respiratory complex 1 and stimulate AMP-activated protein kinase [4,10]. Gunton et al. [14] suggest that metformin causes a rapid stimulation of the insulin receptor in human hepatocytes to increase phosphorylated IRS-2 (not IRS-1) which would stimulate Glut1 translocation. This rapid metformin action on the insulin receptor would be contrasted with more long term metformin effects that would include transcriptional/translational regulation of enzymes such as glucokinase, pyruvate kinase, or glucose-6-phosphate. The relative contribution to metformin's ability to enhance insulin signaling through rapid and long term effects is still not clear.

In summary, we provide evidence that metformin can directly and indirectly stimulate the insulin receptor tyrosine kinase. The indirect path involves metformin inhibition of tyrosine phosphatase(s)—such as hPTP-1B—that would in turn inhibit the insulin receptor.

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