D-Chiro-Inositol Glycans in Insulin Signaling and Insulin Resistance

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Classical actions of insulin involve increased glucose uptake from the bloodstream and its metabolism in peripheral tissues, the most important and relevant effects for human health. However, nonoxidative and oxidative glucose disposal by activation of glycogen synthase (GS) and mitochondrial pyruvate dehydrogenase (PDH) remain incompletely explained by current models for insulin action. Since the discovery of insulin receptor Tyr kinase activity about 25 years ago, the dominant paradigm for intracellular signaling by insulin invokes protein phosphorylation downstream of the receptor and its primary Tyr phosphorylated substrates—the insulin receptor substrate family of proteins. This scheme accounts for most, but not all, intracellular actions of insulin. Essentially forgotten is the previous literature and continuing work on second messengers generated in cells in response to insulin. Treatment and even prevention of diabetes and metabolic syndrome will benefit from a more complete elucidation of cellular-signaling events activated by insulin, to include the actions of second messengers such as glycan molecules that contain D-chiro-inositol (DCI). The metabolism of DCI is associated with insulin sensitivity and resistance, supporting the concept that second messengers have a role in responses to and resistance to insulin.

DUALITY OF INSULIN EFFECTS ON GLUCOSE UPTAKE AND GLYCOGEN SYNTHESIS

Insulin stimulates both glucose transport and glycogen synthesis; however, these actions sometimes occur in a disconnected manner. Current models for the mechanism of action for insulin, for which the dominant paradigm involves the activity of the insulin receptor Tyr kinase and its primary Tyr phosphorylated substrates—the insulin receptor substrate (IRS) family of proteins (1), are inadequate to account for these historical observations. Under certain conditions, control of glucose transport by insulin is observed in the absence of an effect on glycogen synthesis, whereas under other conditions, control of glycogen synthesis by insulin is observed in the absence of

an effect on glucose transport. For example, application of insulin during perfusion of the rat heart stimulated glucose transport, but did not activate glycogen synthase (GS) (2). On the other hand, when the rat diaphragm was treated with N-ethylmaleimide, to test the effect of this sulfhydryl reagent on metabolism, no effect of insulin was observed on glucose transport, but insulin-activated GS and glycogen synthesis (3). Thus, insulin signaling proceeded along one pathway while another pathway was unaffected, suggesting the possibility that no single pathway accounts for events downstream of the IR, but parallel signaling connects the IR to activation of glucose transport and glucose metabolism. These considerations led us to the concept that a cytoplasmic second messenger was

generated in parallel with the phosphorylation events initiated by the receptor Tyr kinase (4). We have emphasized the hypothesis that the phosphorylation network and the second messenger pathway operate in parallel and together are required to fully account for insulin effects on metabolic disposal of intracellular glucose (4).

EVIDENCE FOR INSULIN SECOND MESSENGERS

The initial evidence to support the existence of a second messenger for insulin followed classical methods used to discover cAMP. Rats were injected with insulin and killed, and then muscle and/or liver were used to prepare heat-inactivated, deproteinized extracts. The extracts from insulin-stimulated rat tissues had one or more substances that inhibited protein kinase A (PKA) and activated GS phosphatase, compared with extracts from control rats. Insulin administration maintained PKA in muscle as an inactive holoenzyme, presumably desensitized to cAMP by the soluble second

messengers that were recovered in tissue extracts (5). Independent experimental evidence for this concept came from the work of Popp *et al.* (6), who demonstrated that a low molecular weight factor was produced when insulin was incubated with adipocyte cell membranes. This factor activated PDH phosphatase (PDHP) when it was added to mitochondria. In collaborative experiments, which were reported together in *Science* in 1979 (5,7), we demonstrated that our putative insulin second messenger from muscle also activated mitochondrial PDHP.
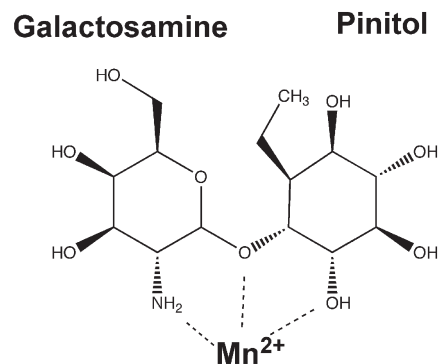
At the time of the initial description 30 years ago (5,7), no exact chemical composition or structure of the second messengers was determined. There also was no identification of an intracellular receptor protein for the messenger that would transduce the signals (like PKA for cAMP). Even worse, doubts were cast over the claims of an insulin second messenger by follow-up reports that the second messenger (also called a mediator) was generated by proteases, and that peptides derived from serum albumin had insulin second messenger activity (8,9). The concept of insulin second messengers was largely dismissed by the mainstream of investigators, and the results considered as artifacts by many inside this field of research. Isolation and study of insulin second messengers was thought to be a fool's errand. Indeed, such investigation proved to be a lonely and unappreciated pursuit of many years, but ultimately it was not fruitless (10,11). The work was continued by Joe Larner, who first identified the presence of an amino sugar (12); Alan Saltiel, who first identified the presence of an inositol (13); and Leonard Jarett, who first found a cell membrane–generated transferable factor that activated mitochondrial PDH (6). Misek and Saltiel importantly demonstrated early on that an inositol glycan generated from the Trypanosoma brucei membrane glycoprotein by pronase digestion had insulin mimetic actions, inhibiting lipolysis, glucose-6 phosphatase and fructose-1,6-diphosphatase (14).

IDENTIFICATION OF INOSITOL GLYCANS AS SECOND MESSENGERS

Biochemical purifications led to isolation of two factors and allowed initial chemical analyses. Assays for PKA inhibition and PDHP activation were used to identify separate fractions from rat liver. The first glycan, eluted from a mixed bed ion exchange resin at pH 2.0 with HCl, activated PDHP. This glycan contained galactosamine plus D-chiro-inositol (DCI) (and was later shown to be pinitol, the 3-O-methyl ether of DCI) (15). The second glycan, eluted at pH 1.3 with HCl, inhibited PKA and contained myo-inositol, glucosamine, galactose and ethanolamine (16). Both glycans were insulin mimetic when administered *in vivo* (17). Injected intravenously, they dose-dependently reduced hyperglycemia in streptozotocin (STZ) diabetic rats. Injected intraperitoneally, the glycans stimulated labeled glucose incorporation into glycogen in rat diaphragm muscles. These results stimulated further efforts.

STRUCTURE DETERMINATION OF THE FIRST CHIRO-INOSITOL GLYCAN

Results of investigations with a scaled-up preparation of the glycan from beef liver were disappointing. Then, it was realized that most cattle are fasted prior to being slaughtered for commercial meat production, so the livers fetched from the abattoir had been exposed to low levels of insulin and contained almost no glycogen and only traces of second messenger. Changing to livers from fed cattle greatly increased yields of second messenger, and extensive purification by column and thin-layer chromatography allowed for recovery of enough pure material, called INS-2 (insulin second messenger) (Figure 1), for chemical analysis, which identified 1:1 molar ratios of pinitol (3-O-methyl-D-chiro-inositol) and galactosamine, chelated to ionic manganese (10). Because pinitol is a polyol and not a proper monosaccharide, the compound is called a pseudo-disaccharide. Nuclear magnetic resonance imaging revealed a β-1,4 linkage between the rings. The structure was ultimately con-

Galactosamine   **Pinitol**

Figure 1. INS-2 structure showing Mn^{2+} as the chelating metal.

firmed by complete chemical synthesis (10) and the synthetic messenger was used to study its bioactivity. The unique nature of the pinitol β-1,4 galactosamine structure contrasts with the more common myo-inositol α-1,6 glucosamine structure determined in other myo-inositol glycans (18).

Bovine liver has four glycosylphosphatidylinositol (GPI) lipid species with 1:1 molar ratios of chiro-inositol and galactosamine (19). These GPI lipids also contain galactose, mannose and organic phosphate and are the potential precursors for generation of INS-2. GPI-linked proteins such as alkaline phosphatase are a second potential precursor (20). We propose that chiro-inositol glycans such as INS-2 are an important part of the cellular responses to insulin (3,4,6). Like the well-established release of the soluble inositol second messenger inositol 1,4,5-triphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2), we propose that the DCI glycans that act as second messengers for insulin are products of hydrolysis of membrane phospholipids and/or GPI linked proteins.

INS-2 ACTIONS *IN VIVO* AND *IN VITRO*

The *in vivo* biological and *in vitro* biochemical responses to INS-2 support the proposal that this is a second messenger for insulin. *In vivo* intravenous injection of INS-2 produces a dose-dependent reduction in blood sugar in STZ diabetic rats (10). Independently, Nestler *et al.* (21) confirmed the insulin-mimetic action

of INS-2 on testosterone biosynthesis in human ovarian thecal cells. Both insulin (20 µg/mL) and INS-2 (100 µmol/L) maximally stimulated (almost nine-fold) testosterone biosynthesis. INS-2 dose-dependently stimulated testosterone biosynthesis between 1 and 100 µmol/L. A polyclonal antibody raised against INS-2 completely blocked both insulin and INS-2 actions. A control nonimmune antibody was ineffective. The fact that the antibody blocked insulin action as well as that of INS-2 suggests that INS-2 or a similar inositol-glycan was involved in this action of insulin.

When added to hepatoma cells, INS-2 potentiates insulin stimulation of glycogen synthesis from glucose (10). *In vitro*, INS-2 allosterically activates protein phosphatase 2Cα (PP2Cα) (11). PP2C dephosphorylates and activates GS (22) as well as phosphoinositide-3 kinase (PI3K) (23). INS-2 as a manganese chelate also activates mitochondrial PDHP, the enzyme that dephosphorylates and activates mitochondrial PDH (10), thereby accounting for an otherwise unexplained mitochondrial mechanism of action of insulin.

INS-2 ALLOSTERIC MECHANISM OF ACTIVATION OF MG²⁺-DEPENDENT PROTEIN PHOSPHATASE FAMILY PROTEIN SER/THR PHOSPHATASES PP2Cα AND PDHP

Synthetic INS-2 activates two members of the Mg^{2+}-dependent protein phosphatase (MPP) family. Biochemical assays used purified recombinant PP2Cα and a phosphopeptide substrate, with the chemical p-nitrophenyl phosphate (pNPP) as a second, control substrate. INS-2 gave a dose-dependent increase in phosphatase activity with the phosphopeptide, but not with pNPP, the hydrolysis of which was unaffected by addition of INS-2 to the assay. We used Flex-X module of Sybyl software for molecular modeling and docked the INS-2 molecule into a low-energy site that was adjacent to the phosphopeptide substrate-binding site in the catalytic cleft of the enzyme (11). In this site we propose that INS-2 contacts surface loops to assist in

positioning active site residues to facilitate hydrolysis of the substrate.

As a further test of this concept a residue visualized as a contact for INS-2 was mutated, and the single residue substitution D163A resulted in complete ablation of the INS-2 activation with no change in the basal phosphatase activity with either phosphopeptide or pNPP (11). Furthermore, a pseudodisaccharide lacking the amine moiety from the galactosamine in INS-2 was synthesized and it showed a complete lack of activation of both PP2Cα and PDHP (24). This finding is in agreement with the modeling and shows a critical function for the amino group of the glycan. The results support a model for INS-2 acting as an allosteric activator of PP2Cα. This model would position PP2Cα as the intracellular receptor of the INS-2 second messenger, like PKA for cAMP or calmodulin for Ca^{2+}.

In contrast to PP2Cα, which is activated by unchelated INS-2, PDHP is not activated by unchelated INS-2. The Mn^{2+} chelate is required for bioactivity with PDHP (10), which suggests differential glycan availability to the bimetallic center in the two enzymes. The lack of activation of PDHP by unchelated INS-2 has been independently observed and noted (25). Unchelated INS-2 is biologically active in H4IIE hepatoma cells and in STZ diabetic rats (10) to sensitize insulin stimulation of glycogen synthesis (10), as well as in STZ diabetic rats to reduce hyperglycemia (11).

ALLOSTERIC SENSITIZATION OF PDHP MG²⁺ DEPENDENCE BY INSULIN AND CHIRO-INOSITOL GLYCAN

Another classical action of insulin that is not accounted for by present signaling schemes is the activation of mitochondrial PDHP to promote the oxidative disposal of glucose via pyruvate. This PDHP was found to be an Mg^{2+}-dependent phosphatase, and sequence and structure comparisons have shown that PDHP and PP2Cα are related and members of the same family of enzymes (previously called type 2C, now called MPP). The concentration of Mg^{2+} required for maxi-

mal activity in biochemical assays is beyond the physiological concentration range. The interpretation has been that the enzymes probably have endogenous cofactors that improve their avidity for the two divalent metals (Mg^{2+} or Mn^{2+} support activity) that were visualized at the active site in the 3-dimensional structure. Fatty acids such as oleic acid are reported to activate PP2Cα as possible endogenous ligands. Years ago, Denton and colleagues (26) showed that insulin treatment of adipocytes shifted the Mg^{2+} dose dependence of mitochondrial PDHP to the left, sensitizing the enzyme (26). The same effect is seen with a chiro-inositol glycan from rat liver, which reduces the concentration of Mg^{2+} needed for full activation of PDHP (27), a finding that is also compatible with a possible role for an inositol glycan in insulin action. Computer docking of INS-2 to the PDHP near the active site supports an allosteric mechanism for increasing activity at physiological concentrations of Mg^{2+}. Thus, the chiro-inositol glycans fulfill a second messenger action by activation of cytosolic and mitochondrial protein Ser/Thr phosphatases of the MPP family.

AN INSULIN-SIGNALING MODEL THAT INCLUDES SECOND MESSENGERS

Our proposed model to incorporate how insulin signaling activates GS and PDH via the inositol glycan INS-2 is shown in Figure 2. Binding of insulin activates the IR Tyr kinase that autophos-
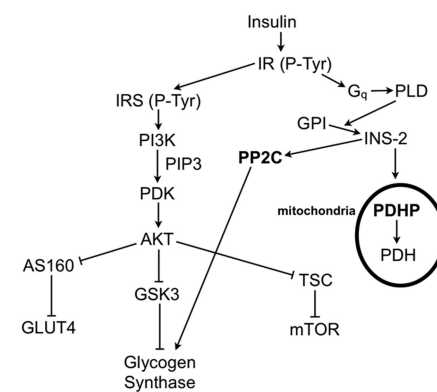

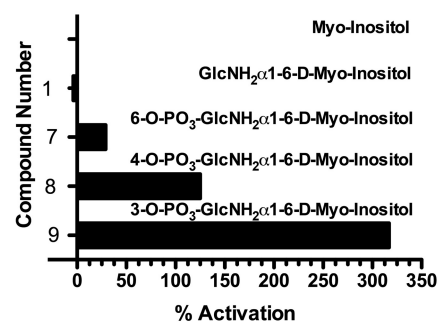**Figure 2.** Pathways of insulin signaling.

phorylates, recruits the IRS proteins, and Tyr-phosphorylates them to serve as scaffolds. A principal IR/IRS target is PI3K, which generates PIP3 to activate the PDK kinase to phosphorylate and activate Akt kinase. Phosphorylation of Akt has proven to be a reliable reporter of insulin signaling. IR activation is also coupled to the heterotrimeric G protein G_q (28) and activation of a GPI-phospholipase, possibly GPI phospholipase D (29). These responses to insulin are less well recognized. The action of the phospholipase releases INS-2 from a GPI lipid precursor that may be in the inner and/or outer leaflets of the plasma membrane. INS-2 can be directly released into the cytoplasm as a second messenger. An alternative is that INS-2 is released outside the cell and transported back into the cell of origin, or into neighboring cells, via an ATP-dependent inositol glycan transporter (30). At least one specific transporter for chiro-inositol has been identified (31). Inside the cell INS-2 binds and allosterically activates PP2Cα, which directly dephosphorylates and activates GS and indirectly activates GS via PI3K-PDK-Akt–GS kinase-3 (GSK3). Olefsky and colleagues reported that PP2Cα dephosphorylates Ser-608 of PI3Kα on its regulatory p85 subunit, resulting in activation of the catalytic subunit of PI3Kα (23). The PIP3/PDK/Akt/GSK3 pathway is proposed to activate GS via inactivation of GSK3 by Akt phosphorylation of Ser-9 (32). In addition, INS-2 is transported into mitochondria to activate PDHP, which dephosphorylates and activates PDH. Other signaling events downstream of activated Akt are depicted (Figure 2). Akt phosphorylates and inactivates different GTPase-activating proteins (AS160 and TSC [tuberous sclerosis complex]) (33–36) to allow activation of the monomeric G proteins Rab and Rheb, respectively, leading to membrane fusion of GLUT4 vesicles and activation of mammalian target of rapamycin (mTOR) kinase. Not shown but possibly important, PP2Cα also dephosphorylates Thr172 in AMP-activated protein kinase (AMPK), thereby inactivating

AMPK (37,38). Dephosphorylation and inactivation of AMPK by insulin has been observed in hepatocytes (39), the heart (40) and the hypothalamus (41). This overall model provides a conceptual framework for the origin, production and action of INS-2 as a second messenger that works in concert with the better-accepted pathways of insulin signaling. Perhaps most important, the model specifies members of the MPP family as the intracellular targets for activation by glycan second messengers.

INOSITOL-GLYCAN STRUCTURE-ACTIVITY RELATIONSHIPS—FUTURE DRUG DESIGN

With the aim of producing an orally bioavailable INS-2 derivative, we synthesized the carbon bridge analog C-INS-2 and the deaminated analog C-INS-2-OH. C-INS-2 as the Mn^{2+} chelate selectively activated PDHP but did not activate PP2Cα, whereas C-INS-2-OH was without effect on both enzymes (24). *In silico* studies of the inositol-glycans into the X-ray crystal structures of PDHP and PP2Cα are consistent with the bioactivity studies. Results with these two INS-2 analogues make two points. First, an amino group is essential for bioactivity, because of its ability to chelate metal (for example, Mn^{2+}, Mg^{2+}) in the INS-2 itself or its ability to form H-bonds in the allosteric site of the phosphatase. This finding reinforces results of earlier studies with inactivation of inositol glycans by nitrous acid deamination (13). Second, selective activation of PDHP and not PP2Cα by C-INS-2 may be a useful therapeutic property in designing a drug capable of reducing carbon overload in diabetes by favoring oxidation over glycogen storage.

d'Alarcao and collaborators demonstrated that the synthetic inositol-glycan pseudodisaccharide glucosamine α(1,6)-myo-inositol containing an inositol cyclic phosphate is insulin mimetic in stimulating lipogenesis from labeled glucose. The cyclic phosphate was shown to be essential for bioactivity (42). León *et al.* (43) demonstrated that the synthetic pseudo-

Figure 3. Structure-activity relationship of phosphate analogues on PDP activity. Percent activation of PDP by 100 μmol/L analogue (40).

disaccharide also mimicked IGF-1 action, stimulating mitogenesis and jun and fos expression in growing chicken inner-ear explants. Chakraborty and d'Alarcao (44) recently synthesized and tested two pseudotetrasaccharides of myo-inositol, glucosamine, two mannoses with inositol cyclic phosphate. The terminal mannose was decorated with sulfate in positions two and six. Both pseudotetrasaccharides were insulin mimetic in stimulating lipid synthesis from glucose in adipocytes, but the more active compound decorated in the two position of the terminal mannose was 80% as active compared with insulin, whereas the six-position compound was 60% as active as insulin. The pseudodisaccharide was 20% as active as insulin. This work demonstrates that both size and ionic charge location are important for bioactivity of glycans.

McLean *et al.* (25) synthesized 23 inositol-glycan pseudodisaccharides containing myo-inositol and/or DCI and glucosamine and/or galactosamine, assaying their activity *in vitro*. Active compounds had reciprocal actions to stimulate PDHP and to inhibit PDH kinase. The presence of phosphate enhanced bioactivity. As shown in Figure 3, the presence of phosphate in the 6, 4 and 3 positions of the glucosamine increased bioactivity up to 300%, whereas the unphosphorylated inositol-glycan was inactive, again demonstrating the importance of charge and its location to bioactivity.
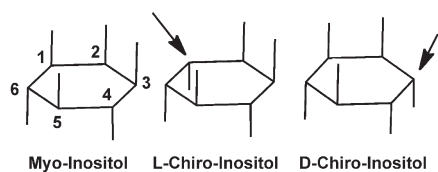
In an extensive series of studies Müller *et al.* (45) synthesized a series of 46 larger glycans of myo-inositol, glucosamine and mannose, some with attached peptidyl-ethanolamine segments based on the structure of a known yeast cAMP-binding GPI-protein Gce1p. These larger chemical species were insulin mimetic in all tests, including glucose transport, lipogenesis, GS activation, glycerol phosphate acyl transferase activation, inhibition of lipolysis, GLUT 4 translocation in adipocytes and glucose transport in isolated rat diaphragms. Of interest, the glycans stimulated tyrosine phosphorylation of IRS1 and IRS2, without tyrosine phosphorylation of the insulin receptor. Tyrosine phosphorylation of pp59[Lyn] was observed (45), potentially explaining the phosphorylation of IRS1 and IRS2. The most active compound, 41, was a pseudo-hexasaccharide with 4 mannoses with an inositol cyclic phosphate; mannoses 2 and 3 are decorated with anionic sulfate. An inactive pentasaccharide, compound 2, contained 3 mannoses, with a cyclic phosphate on the inositol but undecorated with phosphate or sulfate.

It is clear from these emerging structure-activity studies that both larger and smaller glycan species are insulin mimetic *in vitro* and *in vivo,* with the larger species generally more active. The presence of the free amino group is essential for activity, and the presence and location of anionic charge either as phosphate or sulfate enhances bioactivity.

EARLY INVESTIGATIONS OF MYO-INOSITOL AND CHIRO-INOSITOL IN RAT TISSUES AND IN URINE OF HUMANS AND MONKEYS WITH TYPE II DIABETES
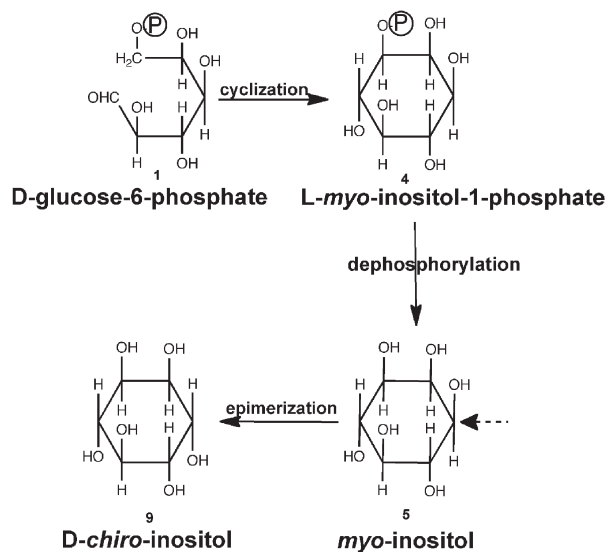
The presence of the unusual inositol, DCI, in the pH 2.0 HCl–eluted glycan fraction from liver, in contrast to myo-inositol, raised questions about DCI physiological significance. DCI had previously been found only in plants and insects. One report had identified chiro-inositol in a partially characterized mediator prepared from cultured cells but had not determined whether it was

Figure 4. Myo-inositol, L-chiro-inositol and DCI. Inversion of the C1 hydroxyl C1 produces L-chiro, and inversion of the C3 hydroxyl produces D-chiro.

the D or L species (46). We examined the distribution in rat tissues of D and L chiro-inositol and found both widespread in all tissues, with the D form generally predominant over the L (47). Of interest, in skeletal and smooth muscle and in heart, D and L were approximately equal in content (47). Inversion of 3 OH of myo-inositol converts it to DCI, whereas inversion of 1 OH of myo-inositol converts it to L-chiro-inositol (Figure 4). We originally found that the increased excretion of myo-inositol in urine of patients with type II diabetes is caused by a renal tubular competition between glucose and myo-inositol in which the hydroxyls have similar positions (48).

The conversion of glucose to myo-inositol is shown in Figure 5, together with the epimerization of myo-inositol to DCI. Glucose-6-phosphate is first cyclized to L-myo-inositol-1-phosphate, which is dephosphorylated to free myo-inositol. The carbon 1,6 ring closure of glucose-6-phosphate (49) gives positions of the OH's in glucose similar to those in myo-inositol, explaining the tubular competition. The epimerization of myo-inositol to DCI by the oxido-reductive inversion of hydroxyl 3 of myo-inositol will be further discussed.

THE INOSITOL IMBALANCE OF MYO-INOSITOL AND CHIRO-INOSITOL IN HUMAN DIABETIC TISSUES

We examined myo-inositol and chiro-inositol levels in muscle from biopsy and autopsy and hemodialysate, as well as urine of type II diabetic patients and control subjects. From autopsy specimens we prepared the pH 2.0 and pH 1.3 HCl eluates of tissues and urine containing the inositol glycan fractions. We assayed bioactivities as well as inositol contents following acid hydrolysis. In the type II diabetic autopsy muscle and urine and hemodialysate samples, chiro-inositol was decreased about 50% compared with samples from control subjects (50). In the

Figure 5. Conversion of D-glucose-6-phosphate to myo-inositol-1-phosphate by cyclization. Myo-inositol-1-phosphate is then dephosphorylated to myo-inositol, which then is converted by epimerization of the C3 hydroxyl (dashed left arrow) to DCI.

muscle biopsy specimens, no chiro-inositol was detected in the type II diabetic samples either before or after insulin administration. Myo-inositol, in contrast, was present in the type II diabetic samples in increased amounts over controls and was further increased with insulin administration (51). In the biopsies from the control subjects, both myo-inositol and chiro-inositol were present and both were increased after insulin administration (Table 1). The higher myo-inositol and the lower chiro-inositol in the type II diabetic tissue and urine compared with controls was termed an inositol imbalance. It was expressed as a ratio of myo-inositol to chiro-inositol (52). In urine, for example, ratios of myo-inositol to chiro-inositol of about two in control subjects were increased to about 20-fold in type II diabetic subjects. In type I diabetic patients and in first-degree relatives of type II diabetic patients, the same trend was observed with ratios of about 10–12 (Table 2).

In an independent study, urine ratios of myo-inositol/chiro-inositol in control subjects were ~3 compared with 10 in type I diabetic patients and ~20 in type II diabetic patients (53). In a review of the published literature, we reported increased ratios of urine myo-inositol to chiro-inositol in our data, data from a Japanese study and data from separate investigators in the US (52). This latter group had studied inositol urinary excretion in obese type II diabetic patients compared with obese control subjects and had found, in contrast to our findings, higher urine chiro-inositol in the obese type II diabetic patients compared with obese control subjects (54). However, ratios of myo-inositol to chiro-inositol calculated from their data were elevated in the type II diabetic patients as well (52,54). Why had they observed high chiro-inositol in the urine of their obese type II diabetic patients in contrast to our findings of lower chiro-inositol in our nonobese diabetic subjects? Baillargeon *et al.* (55) have now shown that with increasing obesity and insulin resistance there is increased renal

Table 1. Clamp-study biopsy of myo-inositol and chiro-inositol.[a]

Group	Time from start of clamp study to biopsy, min	Myo-inositol concentration, ng/mg net weight of tissue	Chiro-inositol concentration, ng/mg net weight of tissue
Normal subjects	0	0.28 ± 0.11	0.09 ± 0.03
	15	2.58 ± 0.41	0.52 ± 0.14
	20	0.10 ± 0.02	0.03 ± 0.02
NIDDM	0	0.57 ± 0.33	<0.01
	15	6.48 ± 3.10	<0.01
	20	1.32 ± 0.81	<0.01

[a]Plus-minus values are mean ± SEM. Four normal subjects and five patients with non–insulin-dependent diabetes mellitus (NIDDM) were studied immediately before the insulin infusion. Samples were obtained 15 min after the beginning of the insulin infusion in two normal subjects and two patients with NIDDM and 20 min after the beginning of the infusion in two normal subjects and three patients with NIDDM (51).

clearance of chiro-inositol. Thus with two sets of subjects, one set nonobese and the other obese, different urinary chiro-inositol excretion results are observed. However, in all studies of urine and tissues higher myo-inositol to chiro-inositol ratios have been observed. Similarly elevated myo-inositol/chiro-inositol ratios have been reported in the urine and tissues, for example, kidney, liver, muscle, of the GK rat, a nonobese type II diabetes model (56). Thus the myo-inositol chiro-inositol imbalance appears to be a body-wide phenomenon.

INOSITOL IMBALANCE AND INSULIN RESISTANCE

In a collaborative study with our Japanese colleagues we demonstrated that, as subjects progressed from normal glucose tolerance to impaired glucose tolerance to type II diabetes, urine chiro-inositol decreased progressively (57). These data and data from Rhesus monkeys (Table 3)

Table 2. Ratio of myo-inositol to chiro-inositol in controls, patients with type II diabetes, nondiabetic relatives, and patients with type I diabetes (52).

	Myo/chiro
Control	2.5
Type II diabetes patients	20.4
Nondiabetic relatives of type II diabetes patients	13.2
Type I diabetes patients	13.6

suggest that the chiro-inositol deficiency and imbalance with myo-inositol are related more directly to the insulin resistance *per se* rather than to the type II diabetes. Additional correlative evidence from Rhesus monkeys has been obtained (58). Insulin resistance determined by either a GS activation state or by a glucose clamp method in muscle biopsy samples was inversely correlated with urinary chiro-inositol excretion.

Even further evidence in support of this hypothesis comes from studies in two other diseases with underlying insulin resistance. Baillargeon *et al.* (59) studied women with polycystic ovary syndrome (PCOS) and Scoscia *et al.* (60) studied women with preeclampsia. Both diseases are associated with insulin resistance. In women with PCOS, Baillargeon *et al.* (55) demonstrated a deficiency of chiro-inositol in blood compared with control subjects with no difference in myo-inositol. They further found that in women with PCOS during a glucose tolerance test there was a three-fold decrease in the release of the chiro-inositol glycan compared with control subjects. Similar findings were reported by Shashkin *et al.* in type II diabetic patients compared with control subjects (61). Scoscia *et al.* studied placental membranes from women with preeclampsia, a disease with insulin resistance, compared with controls (60). With administered insulin *in vitro*, re-

Table 3. Urinary excretion of chiro-inositol and myo-inositol in monkeys (51).

Monkey No.	Diagnosis	Chiro-inositol, mol/d	Myo-inositol, mol/d
1	Normal	18.5	21.9
2	Normal	3.1	5.9
3	Normal	1.8	6.7
4	Normal	3.7	3.5
Mean ± SE		6.7 ± 2.3	9.5 ± 2.4
5	Obese	1.7	7.0
6	Obese	3.6	6.5
7	Obese	2.9	11.3
8	Obese	0.3	3.1
9	Obese	<0.1	72.2
10	Obese	<0.1	63.2
11	Obese	<0.1	4.9
Mean ± SE		1.2 ± 0.2	24 ± 4.7
12	Diabetic	1.0	169
13	Diabetic	<0.1	61.4
14	Diabetic	<0.1	312.5
15	Diabetic	<0.1	61.3
Mean ± SE		0.2 ± 0.1	151.1 ± 34.3

lease of chiro-inositol glycan from placental membranes of women with preeclampsia was reduced compared with placental membranes from control subjects. In general, decreased chiro-inositol glycan generation in insulin resistance could be explained by deficient chiro-inositol content or by a decreased release mechanism. Thus, the correlative evidence suggests that the chiro-inositol deficit and imbalance is related to insulin resistance *per se* rather than solely to diabetes.

MYO-INOSITOL TO CHIRO-INOSITOL EPIMERASE

To explain the myo-inositol to chiro-inositol imbalance associated with insulin resistance, we hypothesize a mechanism involving a faulty epimerase for conversion of myo-inositol to chiro-inositol. A myo-inositol to chiro-inositol epimerase had been reported in extracts of the cockroach (62), but not in animal tissues. Our first approach was to study the *in vivo* conversion of [3H]myo-inositol to [3H]chiro-inositol in normal rats (63). We found conversions ranging from 0.7% in heart up to 36% in urine. In the phospholipids, conversions ranged from 2.2% in liver to 60.4% in blood. Conversions to other inositol isomers, including scyllo-, muco-, neo- and epi-inositol, were less

than 0.06% in terms of myo-inositol radioactivity (63).

We compared the *in vivo* conversion of [3H]myo-inositol to [3H]chiro-inositol in GK type II diabetic rats versus control Wistar rats. Strikingly, in the insulin sensitive tissues, liver, fat and muscle conversions of 20%–30% in normals were 5% or less in the GK diabetic rats (64).

We set up assay conditions like those used in the cockroach epimerase experiments and studied the epimerase in rat liver (56). We partially purified the enzyme over two columns and then determined its pH optimum, time and concentration dependence and its absolute requirement for NADH and NADPH (56). The absolute nucleotide requirement demonstrated that the epimerase operates via an oxido-reductive mechanism of action. We then compared epimerase activities in extracts from GK type II diabetic and control Wistar rat liver, muscle, fat and kidney. GK rat tissue extracts all had lower enzyme activity compared with control Wistar extracts. Studies are now underway to further purify the rat liver epimerase to obtain sufficient amino acid sequence to clone the rat and human enzymes. The aim is to search for deficiencies in the epimerase that are associated with insulin resistance.

VEGAN DIET THERAPY FOR DIABETES AND CHIRO-INOSITOL

Interest has developed in the use of vegan diets as a therapeutic approach to treatment of type II diabetes. A number of authors including Barnard (65) and Campbell (66) have written extensively of their research on vegan diets. Striking results have been obtained in diabetic patients, with reduction of insulin dosage and weight loss.

Cordain *et al.* (67) discuss the evolution of the Western diet and its health complications. As summarized by these authors "The evolutionary collision of our ancient genome with the nutritional qualities of recently introduced foods may underlie many of the chronic diseases of Western civilization." Elements of the vegan diets identified as beneficial include nonheme iron, in contrast to heme iron, flavonoids and other antioxidants.

It is of interest that chiro-inositol is rich in plant sources, for example, soybeans, other legumes including chickpeas, fruits including oranges and arrowroot, to mention a few. In South Africa, African ginseng contains pinitol and is used to treat various diseases, including diabetes. Other plants, including bougainvillea in India, Siberian ginseng in Korea, and palo azul in Paraguay all contain pinitol and are used medicinally. Two elements of the INS-2 structure, Mn^{2+} and pinitol, were independently discovered in folk medicine as antidiabetic agents; pinitol from bougainvillea leaves in India (68), and Mn^{2+} from alfalfa (*Medicago sativa*) in South Africa (69). Thus the presence of chiro-inositol in plants may well play an additional role in the beneficial effects of vegan diets in treatment of diabetes.

CONCLUSION

We have identified DCI glycans that function as second messengers in the classical phosphorylation signaling pathways activated by insulin. These glycans activate two MPP phosphatases, which control the nonoxidative and oxidative disposal of glucose. Other groups have demonstrated insulin-mimetic effects

with synthetic myo-inositol and chiro-inositol glycans. Metabolism and uses of chiro-inositol may lead to a new insight into insulin resistance.

Unanswered Questions

Work thus far leaves many unanswered questions, some of which are listed here:

• What is the phospholipase for inositol-glycan release, from which the precursor lipids and proteins originate?

• Are glycans released intracellularly or extracellularly, or both?

• What is the glycan transporter in plasma and mitochondrial membranes?

• What is the structure of the glycan(s) in the circulation?

• What is the myo-inositol to chiro-inositol epimerase gene and are there gene mutations associated with insulin resistance?

• What is the role and function of L-chiro-inositol, especially in muscle?

CONCLUSION

In this review we have sought to bring together the evidence that inositol glycans particularly of the DCI class are insulin mimetic and thus may serve as insulin second messengers. INS-2 meets these requirements. Studies have identified the binding of INS-2 into allosteric sites in the X-ray crystal structures of PP2Cα and PDHP. Activation of these phosphatases provides critical evidence for mechanism of action of glycan messengers. The presence of a chiro-inositol glycan in blood has been shown by Nestler and colleagues (59), who demonstrated the presence of chiro-inositol in a pH 2.0 HCl–eluated fraction with PDHP bioactivity. In glucose tolerance tests on women with PCOS and controls, there was three-fold less DCI glycan released in blood in the PCOS subjects versus controls. What is still lacking is knowledge of the chemical structure of the DCI species in blood. Independent laboratories have shown that synthetic myo-inositol and chiro-inositol glycans are insulin mimetic in a wide variety of *in vitro* and cellular assays, pro-viding further evidence for their potential for drug development. An inositol imbalance of myo-inositol to myo-inositol was found in urine and tissues, which correlates with insulin resistance in diabetes. In type II diabetes, PCOS and preeclampsia, there is markedly less release of chiro-inositol glycans with insulin. A myo-inositol to chiro-inositol conversion *in vivo* was severly dimished in a GK rat model of type II diabetes. The epimerase identified in rat liver is nucleotide dependent, demonstrating a oxido-reductive mechanism. Epimerase enzyme activity was lower in insulin sensitive tissues of the GK rat compared with control Wistars. We are seeking to find the gene for this enzyme and look for mutations as a cause of disease.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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