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The biological activity of structurally defined inositol glycans

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Abstract

Background—The inositol glycans (IGs) are glycolipid-derived carbohydrates produced by insulin-sensitive cells in response to insulin treatment. IGs exhibit an array of insulin-like activities including stimulation of lipogenesis, glucose transport and glycogen synthesis, suggesting that they may be involved in insulin signal transduction. However, because the natural IGs are structurally heterogeneous and difficult to purify to homogeneity, an understanding of the relationship between structure and biological activity has relied principally on synthetic IGs of defined structure.

Discussion—This article briefly describes what is known about the role of IGs in signal transduction and reviews the specific biological activities of the structurally defined IGs synthesized and tested to date.

Conclusion—A pharmacophore for IG activity begins to emerge from the reviewed data and the structural elements necessary for activity are summarized.

Historical perspective

The **inositol glycans** (IGs) are a class of naturally occurring, phosphorylated, inositol-containing pseudosaccharides first identified in 1986 from bovine liver treated with insulin [1]. The finding that isolated natural IGs, released in response to insulin, are competent in stimulating insulin-sensitive cells in the absence of insulin led to the hypothesis that IGs are the long-elusive second messengers for insulin action, as implied by earlier experiments [2]. However, it was quickly realized that the IG preparations obtained from natural sources were mixtures of closely related carbohydrates, and this microheterogeneity severely complicated the elucidation of the role of IGs in insulin **signal transduction**.

Using a combination of metabolic labeling and degradation studies, the basic structural features of the natural IGs were identified. All of the IGs contain a terminal inositol, but both chiro [3] and myo-inositol [1] isomers were found. Similarly, the second sugar is invariably an unacylated aminosugar, but both galactosamine [3] and glucosamine [1] were identified. The remaining structure of the glycan was less well established, but the presence of a number of mannose residues and one or more phosphate residues was reported. Given this information and the approximate molecular weight of 1400 Da [1], it became clear that the IGs are very closely related to the glycosylphosphatidylinositol (GPI) membrane anchors that many cells use to tether cell surface proteins to the plasma membrane and some of whose structures have been unequivocally identified. The close structural relationship between IGs and GPIs was confirmed by the ability of phosphatidylinositol-specific phospholipase C (PI-PLC) to release

IGs from the cell surface [1] as well as by the observation that the trypanosome-derived variant surface glycoprotein GPI anchor, cleaved from the lipid with PI-PLC and from the protein with pronase, has significant insulin-like activity in rat hepatocytes and adipocytes [4,5].

The similarity between the partially known structures of the IGs and the known structures of the GPIs suggested a way of unraveling the relationship between IG structure and biological activity. The established GPI structures could be used as a template for the design and chemical synthesis of structurally defined IG analogues that could then be subjected to biological evaluation. The chemical synthesis of a bioactive IG analogue was first accomplished in 1992, when a simple pseudodisaccharide – comprising the glucosamine and myo-inositol terminus of the GPI anchor structure, but terminated by a cyclic phosphate as would be produced from PI-PLC hydrolysis of a glycolipid – was prepared and found to be able to mimic one of the actions of insulin: stimulation of **lipogenesis** in rat adipocytes [6]. This led to a burst of synthetic activity and over the next 16 years, various research groups synthesized a large number of IG analogues and evaluated them for biological activity. This has provided an extensive collection of data from which a pharmacophore for insulin-like activity is emerging.

This article reviews the published information on the relationship between IG structure and biological activity. The review covers only the compounds for which a fully defined structure and biological activity have been reported. Therefore, neither synthetic IGs that have not been tested in any bioassays nor natural IGs whose structures are not completely elucidated are included here. Earlier publications have reviewed the role of the natural IGs in signal transduction [7-13] and the chemical synthesis of the IGs [14], but this is the first comprehensive survey of the biological activity of structurally defined IGs.

Overview of insulin signaling

Type 2 **diabetes** mellitus is characterized by insulin resistance in the target tissues, effectively a failure in insulin signal transduction. Since the IGs are able to stimulate many insulin-like effects, but do not act via the insulin receptor (IR) they (or appropriate analogues) may be useful for the treatment of Type 2 diabetes. Any attempt to understand the role of IGs in producing insulin-like effects first requires a basic understanding of the molecular machinery of insulin signaling. A lot of information on the subject of insulin signal transduction has been published and several aspects have been reviewed elsewhere [15-18]. Insulin has two basic effects, mitogenesis and stimulation of **anabolic** metabolism. In this section we provide a brief and somewhat simplified overview of what is currently known about the stimulation of anabolism, including glucose transport, glycogen synthesis and lipogenesis. Each step in the process, described below, corresponds to a number in Figure 1:

(1) Insulin (Ins) delivers its signal by binding to the IR, a cell-surface receptor tyrosine kinase. A complex consisting of the IR and insulin is formed, leading to conformational changes of the IR. (2) The two intracellular domains of the IR, possessing kinase activity, phosphorylate each other. This leads to increased kinase activity as well as affinity toward other IR substrates. (3) Adaptor proteins (IRS-1, IRS-2) bind to the phosphorylated receptor and then (4) their tyrosine residues are phosphorylated by the IR kinase activity. IRS proteins are localized to the plasma membrane during this event, perhaps through the interaction with phosphatidylinositol-4,5-diphosphate [19]. (5) The p85 regulatory domain of phosphatidylinositol 3 kinase (PI3K) docks with the phosphorylated sites of IRS1/2. (6) This leads to phosphorylation of the lipid phosphatidylinositol-4,5-diphosphate and generation of phosphatidylinositol-3,4,5-triphosphate (PIP3). An elevated concentration of PIP3 leads to a cascade of protein phosphorylations. (7) First, binding of phosphoinositide-dependent kinase 1 (PDK1) to PIP3 occurs. (8) Then PDK1 phosphorylates and activates protein kinase B (PKB), also referred to as Akt. In fact, two kinases are required for Akt activation and the nature of

the second one was unknown for a long time; however, the putative kinase was designated PDK2. Recently, the mTOR (mammalian target of rapamycin)–RICTOR (rapamycin-insensitive companion of mTOR) protein complex has been suggested as PDK2 [20]. Akt is believed to be responsible for the regulation of various metabolic pathways via phosphorylations. For example, **(9)** it catalyzes phosphorylation of glycogen synthase kinase (GSK)-3 that **(10)** leads to deactivation of GSK-3 and as a result **(11)** prevents the inactivation of glycogen synthase (GS) by phosphorylation, and hence increases glycogen synthesis [21]. Moreover, PIP3 activates protein phosphatase 1 (PP1) localized on glycogen particles by an unknown mechanism [18], and **(13)** PP1 dephosphorylates inactive GS leading to **(12)** active GS and, therefore, to a further increase in glycogen synthesis.

Likewise, **(14)** deactivation of GSK-3 leads to a decreased level of phosphorylation of ATP citrate lyase (ACL) at one site. **(15)** Phosphorylation at a different site on ACL by PKB **(16)** increases the activity of that enzyme [22]. ACL catalyzes conversion of citrate to acetyl-CoA in cytosol where it can be utilized in lipid biosynthesis. Citrate is actively transported from the mitochondria, where it is prepared from pyruvate (in two steps). Pyruvate in turn is a product of glycolysis. This path, therefore represents lipid biosynthesis from glucose.

In addition to activation of glucose and lipid metabolism, insulin greatly stimulates glucose uptake: the GLUT-4 glucose transporter is translocated from intracellular vesicles to the cell membrane. There are at least two signaling pathways that produce this result, but neither has been fully elucidated. In the first, PKB stimulates GLUT-4 exocytosis either **(17)** directly or **(18)** via activation of atypical protein kinases C: PKC- ζ and γ (designated as aPKC in Figure 1). Several possible molecular regulators downstream of PKB and aPKC are under consideration [23]. The second pathway of GLUT-4 translocation starts right downstream of the IR [24]. Another IR substrate referred to as Cbl (existing in complex with Cbl-associated protein [CAP]) is **(21)** phosphorylated by IR kinase, however **(19)** binding to IR and **(20)** phosphorylation of adaptor protein APS is required before Cbl-CAP association with IR and APS and subsequent phosphorylation. Phosphorylated Cbl requires **(22)** adaptor protein CrkII together with guanine nucleotide exchange factor C3G. C3G **(23)** activates the TC10 protein, which belongs to the Rho family of proteins. TC10 **(24)** activates GLUT-4 vesicle translocation via an unknown mechanism, however it is hypothesized that this happens via actin cytoskeleton regulation – a function attributed to many of the Rho proteins. It is also believed that the two GLUT-4 translocation mechanisms described regulate different states of GLUT-4 transport and are not independent from each other.

Insulin mediates two more metabolic events through the inhibition of protein kinase A (PKA). The processes begin when **(26)** phosphodiesterase 3B (PDE3B) is activated at endoplasmic reticulum/Golgi [25]. The exact mechanism of the activation is not known, however **(25)** PI3K might be indirectly involved (perhaps through PKB) [26,27]. Activated PDE3B **(27)** hydrolyzes cAMP. Low cAMP level **(28)** reduces the activity of PKA since cAMP activates PKA by binding to its regulatory domain. As a result **(29)** PKA does not phosphorylate hormone sensitive lipase (HSL) and HSL is not converted into active form. Lipolysis is, therefore, decreased.

Similarly, **(30)** inactive PKA does not phosphorylate fructose 2,6-bisphosphatase (Fru-2,6P2ase), thus failing to convert it into active form. Inactive Fru-2,6P2ase **(31)** cannot catalyze conversion of fructose-2,6-biphosphate (Fru-2,6P2) into fructose-6-phosphate (Fru-6P). Hence, Fru-2,6P2 accumulates and **(32)** inhibits fructose 1,6-bisphosphatase (Fru-1,6P2ase) – an enzyme that is involved in gluconeogenesis [28].

Another observation should be noted. At least some of the IRs are found in caveolae – small cave-like structures on the cell membrane. They represent a class of so-called lipid rafts or

detergent/carbonate-insoluble glycolipid-enriched (DIG) microdomains. Like lipid rafts, the caveolae are rich in glycosphingolipids and cholesterol. Some proteins may contribute to DIG compositions; for example the IR is found to be associated with the protein caveolin (not shown in Figure 1), which is abundant in caveolae. If the caveolin-1 gene is disrupted, cells become insulin resistant.

Other molecular bases of insulin resistance have been found. It was shown that mutation of several proteins involved in insulin signal transduction, particularly in IRS-2 and the β isoform of PKB, cause the onset of diabetes or impair glucose metabolism and (or) insulin sensitivity [29]. An excess of adipose tissue may also cause diabetes and there is a correlation between Type 2 diabetes and obesity. So-called visceral adipocytes that are found in these patients are relatively resistant to antilipolytic insulin signaling and as a result they secrete a lot of nonesterified fatty acids (NEFA) into the bloodstream. NEFAs can block insulin signaling in other cells via several possible mechanisms or they can impair glucose metabolism in liver [16].

Mechanism of action of inositol glycans

How do IGs stimulate insulin-like effects in cells? Do IGs crosstalk with the insulin-signaling pathway? Are extracellularly-produced IGs transported into cells? The answer to this last question appears to be yes, at least in hepatocytes. The existence of an IG transporter is implied by the observation that a metabolically radiolabeled IG is transported into rat hepatocytes through an energy-requiring process [30], but whether this is due to a specific IG transporter or the normal detoxifying function of hepatocytes is unknown. However, both natural and synthetic IGs are able to modulate purified pyruvate dehydrogenase phosphatase [31,32] and pyruvate dehydrogenase kinase (PDK) [32] activities in cell free assays, suggesting not only an intracellular role for IGs, but also an intramitochondrial role.

Interestingly, IGs also appear to be able to stimulate cells by interacting with the exterior surface of the membranes. For example, a fluorescently-labeled synthetic IG analogue was found to stimulate lipogenesis in rat adipocytes despite the fact that it was not able to enter the cell [33]. Müller has suggested that the dynamics of plasma membrane microdomains may be a part of the insulin-signaling pathway and has collected considerable evidence suggesting that IGs act via this mechanism [34,35]. Müller's theory is summarized in Figure 2 and the numbered description below.

There is evidence that DIG microdomains are heterogeneous: there are cholesterol-rich (hcDIGs) and cholesterol-depleted micro-domains (lcDIGs). Major fractions of certain GPI anchored proteins exist at hcDIG. Other lipid-modified signaling proteins (fatty acid-anchored or prenylated) may exist preferentially in different DIG areas depending on the structure of their lipid anchor. Another targeting signal for proteins that defines their location at plasma membrane microdomains is association with caveolin or other hcDIG proteins. Moreover, association of caveolin with a signaling protein (e.g., the kinase pp59Lyn) seems to be a way of regulating the latter's activity. It was found that pp59Lyn in its inactive state resides in hcDIG bound to the caveolin scaffolding domain (CSD) of caveolin through caveolin binding domains (CBDs). One explanation for the loss of activity due to caveolin binding is that the interaction with caveolin may cause some conformation change in pp59Lyn, leading to its inactivation. On the other hand, this binding may keep the kinase away from its substrates. However, it was shown that CSDP directly interacts with CBD, blocking pp59Lyn activity, and therefore supporting the first hypothesis.

Receptors for the GPI epitope of natural GPI-anchored proteins (as well as probably for free GPIs) exist in hcDIG microdomains. It was shown that GPI-anchored proteins are freely distributed with some preference to lcDIG in membrane models. Accordingly, it is the binding

to the receptor that favors accumulation at hcDIG. The nature of the receptor is not well established other than that it is a protein with a molecular weight of 115 kDa.

Müller's hypothesis, illustrated in Figure 2, is that (1) insulin stimulates a glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) that (2) lipolytically cleaves GPI-anchored proteins (and, perhaps free GPIs) on the outer leaflet of the cell membrane. (3) The lipolytically cleaved protein-IG conjugate (and IGs) promotes GPI-anchored protein(s) to (4) dissociate from the receptor; GPI-anchored proteins are found to translocate to lcDIG microdomains after insulin treatment. (5) Redistribution of acylated non-receptor tyrosine kinases (NRTKs) such as pp59Lyn on the inner leaflet of the plasma membrane between hcDIGs and lcDIGs is also observed. (6) Kinase pp59Lyn activates another kinase pp125Fak that is able to (7) recruit IRS proteins for (8) phosphorylation by pp59Lyn at the sites that are recognized by PI(3)K. This event, which was discussed above, turns on the phosphorylation cascade and is a point of convergence between the IG mechanism of action and the insulin-signaling pathway described above.

There are several explanations for the fact that GPI-anchored proteins on the exterior leaflet and acylated proteins on the cytoplasmic side move at the same time, namely because there is communication between the leaflets within the microdomain [34]. The preference of GPI-anchored proteins for lcDIG could be considered a driving force. The lipid part of a migrating GPI may bring some other components of hcDIG (perhaps, cholesterol) with it, thus changing hcDIG composition on the cytoplasmic side. This in turn may somehow cause a protein-protein interaction-mediated signal to caveolin leading to its conformational change and to a decreased affinity toward pp59Lyn. Another possibility is that it is a change in the lipid composition of the inner leaflet that must occur after lipid rearrangement on the outer leaflet that drives pp59Lyn away.

Description of biological assays performed on structurally defined inositol glycans

Almost two dozen distinct assays or measurements have been used to probe how IG structure affects the molecule's ability to interact with cells. To help the reader connect the mechanisms of action described above with Tables 1-7 correlating IG structure to specific activity in published assays, we have compiled a list of the assays with a brief description of each. The assays are listed in alphabetical order and, where appropriate, the step in Figure 1 or Figure 2 that is being probed is noted. Finally, the specific structurally defined IG(s) tested in the assay are listed.

- cAMP-dependent protein kinase activity [36,37] (Figure 1, step 28, PKA): IG-41, 47. Rat adipocytes are prepared by collagenase digestion of epididymal fat pads. Approximately 20 μ l of adipocyte suspension is mixed with 80 μ l of solution containing 20 mM 3-(N-morpholino)propanesulfonic acid (pH 7.2) 4-mM DTT, 12.5-mM MgCl₂, 0.2-mM PMSF, 1 mM isobutylmethylxanthine, 50- μ M [³²P]-ATP (2.5 μ Ci) with or without 1- μ M cAMP. The resulting mixture is incubated at 30 °C for 10 min followed by the addition of 1 ml of ice-cold solution containing 20% trichloroacetic acid, 10 mM ATP, 5 mM NaPPi and 100 mM NaF. The resulting precipitates are separated by centrifugation and dissolved in 100 μ l of 1 M NaOH. The precipitate is restored by the addition of 1 ml of a solution containing 20% trichloroacetic acid, 10-mM ATP and 5-mM NaPPi. The precipitate is filtered over a Whatman GF/C glass fiber filter, washed several times with washing solution (20% trichloroacetic acid, and 5-mM NaPPi) dried under vacuum and then counted for radioactivity. Activity of PKA is assayed by the incorporation of ³²P from [³²P]-ATP into histone H1.

- cAMP phosphodiesterase activity (Figure 1, step 27, PDE3B) [1]: IG-2, -4, -9 and -10. The activity of cAMP phosphodiesterase, obtained from rat adipocytes, is measured at 30 °C by the method of Thompson and Appleman [38]. Membranes are incubated with Tris-HCl at pH 7.4, MgCl₂, dithiothreitol and [2,8-³²H]-cAMP. The activity of the enzyme is measured by the conversion of [³H]-cAMP to [³H]-AMP and normalized to the protein concentration.
- Cell proliferation and Fos oncoprotein expression in chicken embryo otic vesicles [39]: IG-2. fresh otic vesicles obtained from stage 18 chicken embryos [40] and the desired analyte: IGF-I, synthetic IG or no addition (control) are incubated at 37 °C in a water-saturated atmosphere containing 5% CO₂ for 24 h. The vesicles are incubated in the absence of serum for 24 h. This makes them inactive or dormant. Cell proliferation is then estimated by scintillation counting of the incorporated [³H]-thymidine into trichloroacetic acid precipitated explants [40]. The extent of incorporation of Fos protein in these explants is measured using Western blotting analysis.
- Fructose-1,6-bisphosphatase activity (Figure 1, step 32, Fru-1,6P2ase) [4]: IG-42. Fructose-1,6-bisphosphatase catalyzes the hydrolysis of fructose-1,6-bisphosphate to Fru-6P. IGs inhibit the activity of fructose-1,6-bisphosphatase. The assay for the activity of this enzyme is carried out with the cytosolic fractions of rat livers [41]. These fractions are incubated in 50 mM TRIS-HCl (pH 7.4) containing 5 mM MgCl₂, 0.1 mM EDTA, 150 mM KCl, 0.1 mM NADP⁺, 0.8 unit of phosphoglucose isomerase, and 0.8 unit of glucose-6-phosphate dehydrogenase, at 25 °C for 10 min. To assay the activity of the enzyme, 1 mM fructose-1,6-bisphosphate is added to the sample of incubated cytosol and the resulting solution is analyzed spectrophotometrically for approximately 12 min. Increase in the absorbance (340 nm) indicates the rate of conversion of NADP⁺ to NADPH, which in turn indicates the activity of fructose-1,6-bisphosphatase.
- Gluconeogenesis (Figure 1) [4]: IG-2, -4, -9, -10 and -42. Gluconeogenesis is determined by the conversion of [¹⁴C]-pyruvate into [¹⁴C]-glucose. The assay is carried out on male Sprague–Dawley rat hepatocytes isolated by collagenase digestion [42]. The hepatocytes are suspended in Hanks' buffer containing 2% bovine serum albumin with the desired IG. The solution is incubated in a shaking water bath at 37 °C for 1h. [¹⁴C]-pyruvate (4 µCi/ml) is added and the incubation is continued for 40 min. The solution is centrifuged (12,000g for 5 min) to remove the hepatocytes and the supernatant is deproteinized by the addition of ZnSO₄ and Ba(OH)₂. The solution is centrifuged again for 5 min and the precipitate is removed. The supernatant is eluted through a trilayered column consisting of 1 ml of Dowex-1 anion exchange resin (100 mesh) 1 ml of Amberlite XAD-2, and 1 ml of Dowex-1 anion exchange resin (100 mesh). [¹⁴C]-glucose production is quantified by scintillation counting.
- Glycogen synthesis in insulin-sensitive hepatoma H4IIE cells (Figure 1) [43]: IG-3. To assay the incorporation of [¹⁴C]-glucose into [¹⁴C]-glycogen, H4IIE hepatoma cells are grown in HAM's F-12/DMEM (1:1) with 10% fetal bovine serum containing 5-mM glucose. Before the assay, the cells are washed twice with PBS and incubated with [¹⁴C] glucose (0.2 µCi) along with unlabeled glucose for 60 min at 37 °C. After the incubation the cells are washed twice with PBS and incubated with 30% KOH for 60 min at 37 °C. The solution is heated to 100 °C and glycogen is precipitated using ethanol. Radioactivity of the resulting glycogen is measured using a scintillation counter.
- Glucose concentration; decrease in hyperglycemia in STZ diabetic rats [43]: IG-3. Type 2 diabetes is induced in male Sprague–Dawley rats by injecting low-dose

streptozotocin (50 mg/kg) intravenously into the tail vein. As diabetes is induced, the blood glucose level increases to approximately 250–400 mg/100 ml. These diabetic rats are anesthetized with ketamine (2-[2-chlorophenyl]-2-[methylamino] cyclohexanone). Their tails are clipped off and the blood glucose level is determined. This corresponds to the zero time blood glucose level reading. Next the synthetic IG diluted with the saline, or the saline alone (control) is injected intravenously into the tail veins and the corresponding blood glucose level is recorded using a glucometer at different time intervals. A decrease in the blood glucose level with time indicates decrease in hyperglycemia.

- Glucose-6-phosphatase activity [4]: IG-42. Glucose-6-phosphatase catalyses the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate. Microsomal fractions of rat liver are isolated [41] and suspended in 50 mM TRIS-HCl (pH 7.4) that contains Triton X-100 0.1% with some additive. The solution is incubated at 25 °C for 10 min followed by the addition of 50 mM glucose-6-phosphate solution in 0.2 M imidazole (pH 6.5). The resulting solution is further incubated at 37 °C for 20 min. The reaction is stopped by the addition of trichloroacetic acid 10%. Any precipitates are removed by centrifugation and the supernatant is assayed for the presence of inorganic phosphate [44].
- Glucose transport (Figure 1) [37,45,46]: IG-32, -47, -74 and -78. Glucose transport in adipocytes is measured as the uptake of 2-deoxy- ^3H -glucose (0.5- μCi , 100- μM final concentration). Adipocytes in KRH (1 ml, 3×10^5 cells) are incubated with different concentrations of insulin or IG in 15-ml polystyrene tubes for 20 min at 37 °C. KRH (50 μl) containing 0.5- μCi 2-deoxy- ^3H -glucose is added and the solution is incubated for another 5 min at room temperature. The reaction is stopped by the addition of cytochalasin B followed by immediate separation of 100- μl aliquots of the cells by centrifugation through dinonylphthalate oil. Radioactivity is measured to give the initial rates of glucose transport. Glucose uptake by diffusion and trapping of glucose in the intercellular spaces of the packed adipocytes is corrected by preincubating the cells with cytochalasin B before addition of the labeled glucose.
- Glucose transporter isoform 4 translocation (Figure 1, step 24, GLUT-4) [37,45]: IG-32, -47, -74 and -78. Approximately 7×10^6 adipocytes are incubated with KRH buffer containing glucose and synthetic IG. The solution is shaken for 20 min at 37 °C. The cells are washed with KRH containing 2-mM KCN and are suspended in 20-mM Tris/HCl buffer (pH 7.4) containing 250-mM sucrose, 1-mM EDTA, 0.2 mM PMSF, 100- μM benzamidine and 20 $\mu\text{g/ml}$ each of leupeptin, pepstatin, aprotinin and antipain. This solution is homogenized ten times in a Teflon-in-glass homogenizer. Plasma membranes and low-density microsomes are isolated and the GLUT4 is quantitatively immunoprecipitated using rabbit anti-GLUT4 antibodies raised against the carboxyl terminal 16 amino acids of rat GLUT4 and protein A Sepharose. The immunoprecipitated GLUT4 is separated by SDS-PAGE and its amount evaluated by immunoblotting with the same antibodies and ^{125}I protein A followed by autoradiography of the dried gel (Kodak X-Omat AR). Quantitation is performed by phosphorimaging of the dried gel.
- Glycerol-3-phosphate acyltransferase (GPAT) activity [37,45]: IG-32, -47, -74 and -78. GPAT activity is assayed as incorporation of ^3H -glycerol-3-phosphate into butanol-extractable lipids [37,47]. The crude microsomal cell extracts can be prepared as described [47]. The resulting pellet is suspended in 25-mM Tris/HCl (pH 7.4) 250-mM sucrose, 0.5-mM EDTA, 1-mM DTT, 0.1-mM PMSF at 2-mg protein/ml and stored in liquid nitrogen until determination of GPAT activity. The assay mixture containing 0.2 mM ^3H -glycerol-3-phosphate (0.5- μCi), 150- μM palmitoyl-CoA and 100-mg microsomal protein in a total volume of 0.5 ml is incubated for 3 min at 37

°C. The incubation is stopped by addition of water-saturated butanol, followed by butanol-saturated water. The butanol phase is separated and measured for radioactivity.

- Glycogen synthesis in hemidiaphragms (Figure 1) [37]: IG-32, -47, -74 and -78. Hemi-diaphragms from male Wistar rats are incubated in DMEM (15 ml/hemidiaphragm) containing 10% FCS, 5.5-mM glucose, 1% BSA, 50 U penicillin/ml, 50-μg streptomycin sulfate/ml under constant bubbling of O₂/CO₂ (95:5) for 60 min at 37 °C. For the assay, these diaphragms are incubated with insulin or the synthetic IG in DMEM (15 ml/hemidiaphragm) for 60 min at 37 °C and then washed with the buffer. Washed hemidiaphragms are incubated with [U-¹⁴C]-glucose in a solution of KRH containing 2 mM glucose for 20 min at 37 °C. After washing and homogenization of the frozen hemidiaphragms, the resulting supernatant is precipitated with ethanol. The extent of glycogen synthesis is assayed by measuring the incorporation of [U-¹⁴C]-glucose into ethanol-precipitable glycogen.
- Glycogen synthase (Figure 1, step 12, GS) [37,48]: IG-47. Rat adipocytes are incubated with insulin or IG under 5% CO₂ for 30 min at 37 °C. These cells are washed with a buffer and are frozen in liquid N₂ followed by homogenization and centrifugation. The resulting infranatant solution is used as a source of GS. This homogenate is incubated with a solution containing 33-mM Tris/HCl (pH 7.8), 0.2-mM [¹⁴C]-UDP-glucose (4 μCi) glycogen, KF and glucose-6-phosphate for 20 min at 30 °C. The reaction is terminated by adding 66% ethanol and 10 mM LiBr followed by filtration over pre-wetted Whatman GF/C glass-fiber discs. These filters are washed with 66% ethanol at 25 °C, dried and are measured for radioactivity. The GS activity is assayed by measuring the incorporation of radioactivity from [¹⁴C]-UDP-glucose into glycogen.
- Lipogenesis (Figure 1, Lipid Synthesis) [6,33,36,45,49-51]: IG-1, -2, -4, -5, -7-10, -29-33, -35-41 and -43-83. Rat adipocytes are prepared by collagenase digestion of epididymal fat pads and incubated with radioactive [3-³H/6-³H/U-¹⁴C]-glucose along with the analyte for 1.5 h at 37 °C according to the method of Rodbell [52]. The assay is stopped by addition of water and a toluene-based scintillation cocktail. The solution is vortexed and centrifuged followed by scintillation counting to measure the incorporation of radioactive glucose into the toluene-extractable lipids (triglycerides). Incorporation of ³H or ¹⁴C into lipids is expressed as a percent of the maximal insulin response (%MIR).
- Lipolysis (Figure 1) [4,37]: IG-32, -42, -47, -74 and -78. A suspension of isolated adipocytes, prepared by collagenase digestion of epididymal fat pads of male Sprague-Dawley rats, in Krebs-Ringer HEPES buffer (pH 7.4) is pre-incubated with insulin or inositol phosphate-glycan analogue at 37 °C for 30 min under an atmosphere of 5% CO₂ followed by the addition of 50 nM-1-μM isoproterenol and 1 unit of adenosine deaminase. The incubation is continued for 30-60 min and the assay is finally terminated by the addition of 50 μl of 4.6 M perchloric acid. Samples are vortexed, diluted with chloroform and centrifuged at 12,000g for 5-15 min. The aqueous layer is separated and neutralized with 5 N KOH and then assayed for glycerol content [53].
- Other biophysical/chemical studies. Conformational studies [39]: IG-2. To understand the basic structural features and dynamic properties of these compounds, several conformational studies were carried out using NMR spectroscopy, molecular mechanics and molecular dynamics calculations; optical studies [33]: IG-5. Fluorescence microscopy and fluorescence-assisted cell sorting are utilized to determine whether fluorescent IG-5 is transported to the interior of living adipocytes.

- PI3K activity (Figure 1, step 6) [54]: IG-47. IRS-1 immunoprecipitates to which phosphatidylinositol (0.2-mg/ml) is added are incubated in kinase buffer for 5 min at room temperature. [³²P]-ATP (40 μM, 0.1 μCi/μl) is added, the reaction is run for 20 min, and then terminated by the addition of 30 μl of 4 M HCl and 130 μl of chloroform/methanol (1:1, v/v). The organic phase is spotted on a silica gel TLC plate, visualized and analyzed using a bioimaging analyzer.
- Protein phosphorylation/dephosphorylation (Figure 1) [5]: IG-42. Rat adipocytes are prepared by collagenase digestion of epididymal fat pads and incubated with [³²P]-orthophosphate at 37 °C for 2 h with constant shaking. These adipocytes are further incubated with 50-nM isoproterenol (IPT) insulin, or the desired IG at 37 °C for 20 min. The assay is stopped by using poly(dimethylsiloxane) and the cells are lysed using a solution of ethanol containing sodium dodecyl sulfate 4% (SDS) 1.5-mM EDTA, 10-μg/ml leupeptin, 10-μg/ml pepstatin, and 100-μM phenylmethylsulfonylfluoride. The resulting solution is boiled in sample buffer, vortexed and centrifuged at 12,000g for 5 min. Electrophoresis (with 8% SDS-polyacrylamide gels) on the supernatant solution gives the extent of phosphorylated protein.
- *In vitro* phosphorylation/dephosphorylation is measured by incubation of frozen adipocyte cytosols with 1-μM (γ-³²P)-ATP for 10 min, followed by 100-μM unlabeled ATP and analyte for various times and SDS-PAGE, as above. Under these conditions changes in the observed phosphorylation state is caused by the direct dephosphorylation reaction.
- Pyruvate dehydrogenase kinase inhibition [32]: IG-2, -12-24, -26, -27 and -34. PDK is found to be tightly associated with the PDC complex. It is not isolated as free PDK. To determine the activity of PDK, the ATP-dependent reduction in activity of PDC is measured. First, the residual activity of nonphosphorylated (active) PDC is measured spectrophotometrically [55]. A 10-μl sample of activated pyruvate dehydrogenase complex (PDC) (10 units/ml) is incubated in 10-μl buffer solution pH 7.0 and 80 μl of inactivation mixture (containing 1.25-mM ATP) at 30 °C. 50 μl of this incubated mixture is transferred to pre-prepared Eppendorf tubes containing 10 μl of the desired IG analogue, followed by further incubation for 10 min at 30 °C. PDK activity is inferred from the rate of inactivation of PDC, measured spectrophotometrically.
- Pyruvate dehydrogenase phosphatase (PDH phosphatase) activity [36,43]: IG-3 and -41. IGs stimulate PDH phosphatase by enhancing its sensitivity to Mg²⁺ [56]. PDH phosphatase activity can be assayed in several ways: by monitoring the release of phosphate from ³²P-labeled PDC [57], by measuring the conversion of β-NAD⁺ spectrophotometrically [58], or by measuring the release of [¹⁴C]-CO₂ from pyruvic acid [56]. The assay for PDH phosphatase is based upon the initial rates of activation of inactivated, phosphorylated PDC complex. This assay is done in two phases; the first stage consists of dephosphorylation of PDC by the IG-activated phosphatase and the second stage involves the quantification of the activated PDC. The inactivated PDC complex prepared from bovine heart [59] is preincubated at 30 °C for 2–3 min with a solution containing 1-mg/ml fat-free bovine serum albumin, 10-mM MgCl₂, 0.1-mM CaCl₂, 1-mM dithiothreitol in 20-mM potassium phosphate buffer, pH 7. After 3 min 5 μl of metal, 10 μl of IG and 10 μl of PDH phosphatase are added and the solution is further incubated for 3 min. At this stage, the addition of NaF and [1-¹⁴C]-pyruvic acid inhibits the phosphatase and initiates the second stage of the assay, respectively. The second stage involves the quantitation of the PDC complex by measuring the rate of production of the reduced form by using absorbance data at

340 nm for approximately 5 min or by measuring the radioactivity of the released [^{14}C]- CO_2 with a liquid scintillation counter.

- Testosterone biosynthesis by polycystic ovary syndrome (PCOS) cells [60]: IG-3. Thecal cells (10^6 cells/ml) isolated from the ovaries of women with PCOS and non-PCOS, are incubated with serum-free culture medium and the corresponding analyte (no addition: negative control; hCG: positive control and insulin or synthetic IG) in a water-saturated atmosphere containing 5% CO_2 at 37 °C for approximately 144 h. Anti-inositolglycan antibodies corresponding to the respective IG receptors are also incubated with this solution for approximately 15 min before the addition of the analytes. Rapid freezing in an acetone-dry ice bath terminates the incubation. Testosterone content of cells and media are analyzed in duplicate by RIA (Diagnostic Systems Laboratories, Webster, TX).
- Tyrosine phosphorylation of caveolin or IRS-1 [45,48,51,54]: IG-32 and -47. After incubation with insulin or the synthetic IG, detergent insoluble complexes (caveolin) [51] or hemidiaphragms (IRS-1) [45] are collected and solubilized in 1 ml of TEST buffer followed by centrifugation (15,000 g, 10 min, 4 °C). The supernatant is precleared with protein A/G Sepharose and incubated with rabbit anticaveolin antibodies or with rabbit anti-carboxyterminal IRS-1 antibodies for 4 h at 4 °C, and then with protein A/G Sepharose for 16 h at 4 °C. The precipitates are collected by centrifugation (15,000 g, 2 min, 4 °C) and washed successively with a series of buffers. The immunoprecipitants are suspended in Laemmli sample buffer for 5 min at 95 °C followed by centrifugation (15,000 g, 2 min). The supernatant is subjected to SDS-PAGE (8 % resolving gel) followed by immunoblotting with either monoclonal antiphosphotyrosine antibodies or anti-IRS-1 antibodies using radioactive [^{125}I] protein A. The radiolabeled blots are analyzed by phosphorimaging.

Biological activity of structurally defined inositol glycans

The structures and the biological activities of all of the published IGs that have been tested to date are compiled in Figures 1 & 2 and Tables 1-7. They are organized by the number of sugar (or pseudosugar) units and span the range of monosaccharides to heptasaccharides. The tables list our compound number (designated with an IG-prefix) to allow easy correlation with the structures, the assay performed (see the preceding section for a brief description of each assay), the results of that assay, the compound number in the literature (for easy comparison when reading the primary source) and the reference.

Conclusions

The large collection of structures listed above (largely due to the prolific work of Dr Müller's team) suggests a preliminary IG pharmacophore for insulin-like action. Structural elements that appear to most often contribute to high activity are a cyclic phosphate on the inositol, though the inositol isomer is less important, a free (unacylated) amino group on the second sugar, and a distal anionic group on one or more of the mannose residues (especially the fourth residue from the inositol terminus) though the nature of the ionic group (phosphonate, phosphate, sulphate) is less important. Curiously, the anomeric configuration between the aminosugar and the inositol is relatively unimportant. The observation that a hybrid compound, IG-5, bearing a distal anionic group pendant on a noncarbohydrate scaffold attached to a disaccharide is more active than the disaccharide, IG-2, from which it was derived, suggests the possibility of noncarbohydrate or partially carbohydrate-based structures with IG receptor agonist properties. This may lead to the development of compounds with useful pharmacological properties that are also considerably simpler to synthesize than the currently most active IGs such as IG-78 or IG-39.

Future perspective

The role of the inositol glycans in insulin signal transduction has been controversial since the IGs were first identified. While there is no doubt that IGs are produced in response to insulin (and indeed to other biological signals [61-70]) and that the IGs are able to mimic the metabolic effects of insulin on insulin-sensitive cells, questions remain regarding the extent to which IGs are necessary and/or sufficient for insulin's metabolic action.

Regardless of their role in insulin signaling, the fact that the IGs elicit insulin-like effects downstream of the IR suggests their use in the treatment of diabetes and possibly other metabolic diseases. Several pharmaceutical companies with interest in developing therapies for Type 2 diabetes have dabbled in the IG area, but most have abandoned this avenue of research, presumably daunted by the lack of 'drug-like' structures among the IGs and the extremely lengthy syntheses required to prepare the most active substances. The field is still in need of considerable basic research to elucidate better the nature of the IG receptor on the cell surface, develop more efficient syntheses, establish structure–activity relationships differentiating mitogenic and metabolic activity, as well as to identify simpler and synthetically more accessible IG receptor agonists that may have greater promise as drug candidates.

The basic chemistry and biology of the IGs remains a fascinating area for study. The ubiquitous presence of GPI-anchored proteins on eukaryotic cell surfaces, the highly conserved core structure of the GPI anchors, and the strong kinship in structure between the GPI protein anchors and the hormonally released IGs all suggest a significant role for this class of compounds in cell biology, certainly in hormone signal transduction, and possibly in autocrine and paracrine signaling. It is quite probable that in the next decade we will see an expanding appreciation of the role of IGs in controlling cellular function and that this will be accompanied by a renewal of interest in the use of IGs or their analogues in the treatment of human diseases.

Executive summary

- The inositol glycans (IGs) are small oligosaccharides released from insulin-sensitive cells upon stimulation by insulin. Isolated IGs are capable of activating insulin-sensitive cells.
- The natural IGs are structurally microheterogeneous with representatives containing both myo- and chiro-inositol, both glucosamine and galactosamine, an oligomannose portion of varying length, and a variable number of phosphates and/or cyclic phosphates.
- Elucidation of a relationship between IG structure and biological activity has been difficult with natural IGs because of the microheterogeneity.
- Chemical synthesis has provided a large number of IGs with defined structures for biological evaluation. A comprehensive list of the structures and their activities is provided in this review.
- The data suggest that the most important features that an IG should possess for high insulin-like activity are a cyclic phosphate on the inositol, a free (unacylated) amino group on the second sugar and a distal anionic group.

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conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Glossary

Inositol glycan	A member of a family of inositol-containing oligosaccharides, some of which are thought to be involved in regulating various cellular processes
Signal transduction	The process of relaying an extracellular biological stimulus to the interior of a cell
Diabetes	A disease characterized by an elevated concentration of glucose in the blood
Lipogenesis	The synthesis of lipids, usually triglycerides, occurring in specialized fat cells
Anabolic stimulation	The activation of biochemical pathways leading to the synthesis of larger molecules such as proteins, lipids, and polysaccharides

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Figure 1. Insulin signal transduction

Inactive proteins are indicated by a paler color. Protein shape and color depend on the protein function: kinases (and insulin receptor substrates) are green and rectangular with rounded edges. Regulatory domains (e.g., the p85 domain of PI3 kinase) and adaptor proteins are light blue and oval. Phosphatases are dark blue and rectangular. Lyases and synthases are brown. Blue arrows denote a chemical transformation (e.g., $\text{PIP}_2 \rightarrow \text{PIP}_3$) as well as movement of proteins. Black arrows point to the protein target. A solid arrow indicates that the reaction has been established. Dashed arrows refer to pathways that are not yet well established.

Figure 2. Mechanism of inositol glycan (inositolphosphate glycan) action on cell surfaces

GPI: Glycosylphosphatidylinositol; IPG: Inositolphosphate glycan.

From [34,35].

Table 1

Monosaccharides.

Study	Comp.	Assays [*]	Activity	Lit. no. [‡]	Ref.	
Cobb JE (1991)	IG-1	Lipogenesis	No activity	2		[50]

Lit. no.: Literacy number.

^{*} Details of assays (in alphabetical order) are given in the text.

[‡] The number assigned to this compound in the cited publication.

Disaccharides

Table 2

Study	Compound	Assays [*]	Activity	Lit. no. [†]	Ref.
Plourde (1992)	IG-2	Lipogenesis	30–40% MIR at 40 μ M	1	[6]
Chakraborty (2005)			18% MIR at 10.5 μ M	IPG 1	[49]
Frick (1998)			Activity 'A' §	43	[45]
Dietrich (1999)		Cell proliferation of otic vesicles	Max. effect at 10 μ M	3	[39]
		Fos oncoprotein expression	Max. effect at 2.2 $\pm$ μ M		
		Conformational study			
Plourde (1992)		cAMP phosphodiesterase	No activity	1	[6]
		Gluconeogenesis	No activity		
McLean (2008)		PDH phosphatase	Active, but data not reported	3	[32]
		PDK inhibition			

Study	Compound	Assays*	Activity	Lit. no. [†]	Ref.
Nestler (1998)	IG-3	Thecal testosterone biosynthesis	Effective range 1.0 -100 μ M/L	INS-2	[60]
Larner (2003)		Glucose incorporation	40% decrease in hyperglycemia at 20 mg/Kg (6 mM–60 μ M)	1	[43]
McLean (2008)		PDH phosphatase	Effective at 100 μ M range only when chelated with Mn^{2+}	19	[32]
Larner (2003)		Glucose incorporation in insulin-sensitive hepatoma H4IIE cells	No activity with or without Mn^{2+} Effective from 0.1–100 μ M in presence of insulin	1	[43]
Plourde (1992)	IG-4	Lipogenesis cAMP phosphodiesterase	No activity No activity	5	[6]
Dietrich (1999)		Gluconeogenesis Conformational study	No activity	5	[39]
Turner (2005)	IG-5	Lipogenesis Optical studies	47% MIR, Kd of 12.0 μ M ($R = 0.877$) Not transported across cell membranes	1	[33]
Reddy (1993)	IG-6	No assay reported		1	[71]
Frick (1998)	IG-7	Lipogenesis	Activity $\cdot A \cdot \delta$	42	[45]
Frick (1998)	IG-8	Lipogenesis	Activity $\cdot A \cdot \delta$	44	[45]
Plourde (1992)	IG-9	Lipogenesis cAMP phosphodiesterase	No activity	13	[6]
Plourde (1992)	IG-10	Lipogenesis cAMP phosphodiesterase Gluconeogenesis	No activity	14	[6]
McLean (2008)		PDH phosphatase	No activity	1	[32]
McLean (2008)	IG-11	PDH phosphatase	No activity	2	[32]
McLean (2008)	IG-12	PDH phosphatase PDK inhibition	0.125 nmol/min at 100 μ M 0.08% inhibition of PDK at 1 mM	4	[32]
McLean (2008)	IG-13	PDH phosphatase PDK inhibition	0.8 nmol/min at 100 μ M 0.4% inhibition of PDK at 1 mM	5	[32]

Study	Compound	Assays*	Activity	Lit. no. [†]	Ref.
McLean (2008)	IG-14	PDH phosphatase PDK inhibition	2.7 nmol/min at 100 μ M 75% inhibition of PDK at 1 mM	6	[32]
McLean (2008)	IG-15	PDH phosphatase PDK inhibition	30% activation of PDP at 100 μ M 0.01% inhibition of PDK at 1 mM	7	[32]
McLean (2008)	IG-16	PDH phosphatase PDK inhibition	130% activation of PDP at 100 μ M 0.7% inhibition of PDK at 1 mM	8	[32]
McLean (2008)	IG-17	PDH phosphatase PDK inhibition	320% activation of PDP at 100 μ M 1.0% inhibition of PDK at 1 mM	9	[32]
McLean (2008)	IG-18	PDH phosphatase PDK inhibition	0.125 nmol/min at 100 μ M 0.02% inhibition of PDK at 1 mM	10	[32]
McLean (2008)	IG-19	PDH phosphatase PDK inhibition	1.0 nmol/min at 100 μ M 4.0% inhibition of PDK at 1 mM	11	[32]
McLean (2008)	IG-20	PDH phosphatase PDK inhibition	1.0 nmol/min at 100 μ M 9.5% inhibition of PDK at 1 mM	12	[32]
McLean (2008)	IG-21	PDH phosphatase PDK inhibition	No activity	13	[32]
McLean (2008)	IG-22	PDH phosphatase PDK inhibition	No activity	14	[32]
McLean (2008)	IG-23	PDH phosphatase PDK inhibition	0.25 nmol/min at 100 μ M 0.3% inhibition of PDK at 1 mM	16	[32]
McLean (2008)	IG-24	PDH phosphatase PDK inhibition	2.2 nmol/min at 100 μ M 0.4% inhibition of PDK at 1 mM	17	[32]
McLean (2008)	IG-25	PDH phosphatase PDK inhibition	Significant activity	18	[32]
McLean (2008)	IG-26	PDH phosphatase PDK inhibition	0.3 nmol/min at 100 μ M 0.07% inhibition of PDK at 1 mM	21	[32]
McLean (2008)	IG-27	PDH phosphatase PDK inhibition	0.25 nmol/min at 100 μ M 0.6% inhibition of PDK at 1 mM	22	[32]
McLean (2008)	IG-28	PDH phosphatase	Significant activity	23	[32]

IG: Inositol glycan; Lit. no.: Literacy number; MIR: Maximal insulin response; PDH: Pyruvate dehydrogenase; PDK: Phosphoinositide-dependent kinase.

[§]Note: For Muller's SAR study [45], aggregate activities were reported as follows: "A", MIR < 20%; "B", MIR 20–49%, EC20 25–200 μ M; "C", MIR 50–80%, EC50 10–100 μ M; "D", MIR > 80%, EC50 3–30 μ M.

* Details of assays (in alphabetical order) are given in the text.
[†] The number assigned to this compound in the cited publication.

Table 3

Trisaccharides.

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Jaworek (2001)	IG-29	Lipogenesis	Inactive	1	[72]
Dietrich (1999)		Conformational study		4	[39]
Jaworek (2001)	IG-30	Lipogenesis	Inactive	2	[72]
Frick (1998)	IG-31	Lipogenesis	Inactive	1	[45]
Frick (1999)			15% MIR EC ₅₀ = 25 μM	H	[101]
		Glucose transport	2% MIR, EC ₅₀ = 10 μM		
Frick (1998)	IG-32	Lipogenesis	20% MIR at 100 μM	7	[45]
Frick (1999)			25% MIR, EC ₅₀ = 15 μM	G	[101]
Frick (1998)		Glucose transport	40% MIR at 20 μM	7	[45]
Frick (1999)			9% MIR, EC ₅₀ = 15 μM	G	[101]
Frick (1998)		GPAT	25% MIR	7	[45]
		Glycogenesis	33% MIR at 20 μM		
		GLUT4 translocation	50% MIR		
		Lipolysis	42% MIR		
		Tyrosine phosphorylation of IRS-1	30% MIR at 10 μM		

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1998)	IG-33	Lipogenesis	Activity 'B'*	8	[45]
McLean (2008)	IG-34	PDH phosphatase PDK inhibition	Negligible activity for PDP activation 0.2% inhibition of PDK at 1 mM	15	[32]

Lit. no.: Literacy number; MIR: Maximal insulin response; PDH: Pyruvate dehydrogenase; PDK: Phosphoinositide-dependent kinase.

* Details of assays (in alphabetical order) are given in the text.

[‡]The number assigned to this compound in the cited publication.

[§]Note: For Muller's SAR study [45] aggregate activities were reported as follows: "A", MIR < 20%; "B", MIR 20–49%, EC₅₀ 25–200 μM; "C", MIR 50–80%, EC₅₀ 10–100 μM; "D", MIR > 80%, EC₅₀ 3–30 μM.

Table 4

Tetrasaccharides.

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Chakraborty (2006)	IG-35	Lipogenesis	Inactive	3	[73]
Chakraborty (2006)	IG-36	Lipogenesis	Inactive	4	[73]
Chakraborty (2005)	IG-37	Lipogenesis	63% MIR, EC ₅₀ = 10.6 μM	3α	[49]
Chakraborty (2005)	IG-38	Lipogenesis	51% MIR, EC ₅₀ = 9.8 μM	3β	[49]
Chakraborty (2005)	IG-39	Lipogenesis	78% MIR, EC ₅₀ = 1.1 μM	4α	[49]
Chakraborty (2005)	IG-40	Lipogenesis	47% MIR, EC ₅₀ = 9.5 μM	4β	[49]

Lit. no.: Literacy number; MIR: Maximal insulin response.

* Details of assays (in alphabetical order) are given in the text.

[‡] The number assigned to this compound in the cited publication.

Table 5

Pentasaccharides.

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Martin-Lomas (2000)	IG-41	Lipogenesis PDH phosphatase cAMP-dependent protein kinase	Inactive	1a	[36]
Misek (1992)	IG-42	Lipolysis	70% inhibition at 132 μM, IC ₅₀ = 60 μM	VSG-IPG	[4]
		Glucose-6-phosphatase activity	90% inhibition at 160 μM, IC ₅₀ = 109 μM		
		Fructose-1,6-bisphosphatase activity	100% inhibition at 226 μM, IC ₅₀ = 136 μM.		
		Gluconeogenesis	67% inhibition at 102 μM, IC ₅₀ = 84 μM		

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Misek (1992)		Protein phosphorylation/dephosphorylation	Blocked IPT-dependent phosphorylation at 200 µM ~ 90% dephosphorylation at 150 µM, IC ₅₀ = 75 µM	VSG-IPG	[4]
Frick (1998)	IG-43	Lipogenesis	Activity ^{A,§}	20	[45]
Frick (1998)	IG-44	Lipogenesis	Activity ^{C,§}	29	[45]
Frick (1998)	IG-45	Lipogenesis	Activity ^{B,§}	23	[45]
Frick (1998)	IG-46	Lipogenesis	Activity ^{C,§}	13	[45]
Frick (1999)			44% MIR, EC ₅₀ = 40 µM	Q	[101]
		Glucose transport	18% MIR, EC ₅₀ = 30 µM		

IPG: Inositol phosphate-glycan; Lit. no.: Literacy number, MIR: Maximal insulin response.

* Details of assays (in alphabetical order) are given in the text.

[‡]The number assigned to this compound in the cited publication.

[§]Note: for Muller's SAR study [45] aggregate activities were reported as follows: "A", MIR < 20%; "B", MIR 20–49%, EC₂₀ 25–200 µM; "C", MIR 50–80%, EC₅₀ 10–100 µM; "D", MIR > 80%, EC₅₀ 3–30 µM.

Table 6

Hexasaccharides.

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Muller (1997)	IG-47	Lipogenesis	15.5-fold stimulation, EC ₅₀ = 1.0 μM	PIG-P	[37]

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Muller (1998)			65–75% MIR, EC ₅₀ = 2–5 µM	PIG-P	[51]
Muller (2002)			22-fold stimulation at 10 µM	YCN-PIG	[48]
Muller (1997)		GPAT	75–85% MIR, EC ₅₀ = 3–5 µM	PIG-P	[37]
Muller (1998)			85% MIR at 5 µM	PIG-P	[51]
Muller (1997)		Glucose transport	15-fold increase, EC ₅₀ = 1.0–1.5 µM	PIG-P	[37]
Kessler (1998)			sixfold stimulation at 10 µM	PIG-P	[54]
Muller (1997)		GLUT4 translocation	85% MIR, EC ₅₀ = 2–3 µM	PIG-P	[37]
Muller (1997)		Glycogenesis	75–90% MIR, EC ₅₀ = 8–10 µM	PIG-P	[37]
Muller (2002)			eightfold stimulation at 10 µM	YCN-PIG	[48]
Muller (1997)		GS stimulation	2.8-fold stimulation, EC ₅₀ = 8.0 µM	PIG-P	[37]
Muller (2002)			fourfold stimulation at 10 µM	YCN-PIG	[48]
Muller (2002)		Glycerol-3-phosphate acyltransferase activity	sixfold stimulation at 10 µM	YCN-PIG	[48]
Muller (1997)		cAMP-dependent protein kinase activity	70% MIR, EC ₅₀ = 0.3–0.5 µM	PIG-P	[37]
Muller (1997)		Lipolysis	70% inhibition, EC ₅₀ = 0.5–0.8 µM	PIG-P	[37]
Muller (2002)			95% inhibition at 10 µM	YCN-PIG	[48]
Kessler (1998)		PI-3 kinase activity	2.3-fold stimulation,	76% MIR PIG-P	[54]
Muller (2002)		Radiolabeled study with hcDIGs of adipocyte plasma membranes	Specific interaction with hcDIGs, K _d 30–100 nM, B _{max} = 5–10 pmol/mg hcDIGs	YCN-PIG	[74]
Muller (1998)		Tyrosine phosphorylation of caveolin	80% MIR at 10 µM, EC ₅₀ = 1–2 µM	PIG-P	[51]
Kessler (1998)		Tyrosine	2.3-fold stimulation, 44% MIR	PIG-P	[54]
Muller (2002)		phosphorylation of IRS-1	24-fold stimulation at 5 nM	YCN-PIG	[48]
Frick (1998)	IG-48	Lipogenesis	Activity ·A [§]	2	[45]
Frick (1999)			10% MIR, EC ₅₀ = 20 µM	O	[101]
		Glucose transport	5% MIR, EC ₅₀ = 50 µM		
Frick (1998)	IG-49	Lipogenesis	Activity ·A [§]	3	[45]

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1998)	IG-50	Lipogenesis	Activity 'A' §	4	[45]
Frick (1999)			18% MIR, EC ₅₀ = 20 µM Glucose transport	1 14% MIR, EC ₅₀ = 50 µM	[101]
Frick (1998)	IG-51	Lipogenesis	Activity 'B' §	5	[45]
Frick (1998)	IG-52	Lipogenesis	Activity 'B' §	6	[45]
Frick (1998)	IG-53	Lipogenesis	Activity 'B' §	9	[45]
Frick (1998)	IG-54	Lipogenesis	Activity 'B' §	10	[45]
Frick (1999)		Glucose transport	33% MIR, EC ₅₀ = 20 µM 18% MIR, EC ₅₀ = 18 µM	D	[101]
Frick (1998)	IG-55	Lipogenesis	Activity 'B' §	11	[45]
Frick (1998)	IG-56	Lipogenesis	Activity 'C' §	14	[45]
Frick (1999)		Glucose transport	48% MIR, EC ₅₀ = 15 µM 19% MIR, EC ₅₀ = 10 µM	R	[101]
Frick (1998)	IG-57	Lipogenesis	Activity 'C' §	15	[45]
Frick (1999)			50% MIR, EC ₅₀ = 15 µM Glucose transport	S 34% MIR, EC ₅₀ = 20 µM	[101]
Frick (1998)	IG-58	Lipogenesis	Activity 'C' §	16	[45]
Frick (1999)			72% MIR, EC ₅₀ = 12 µM Glucose transport	T 30% MIR, EC ₅₀ = 10 µM	[101]
Frick (1998)	IG-59	Lipogenesis	Activity 'D' §	17	[45]
Frick (1999)			80% MIR, EC ₅₀ = 15 µM Glucose transport	U 34% MIR, EC ₅₀ = 15 µM	[101]
Frick (1998)	IG-60	Lipogenesis	Activity 'D' §	18	[45]

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1999)			83% MIR, EC ₅₀ = 15 µM Glucose transport	V	[101]
Frick (1998)	IG-61	Lipogenesis	Activity 'D' §	19	[45]
Frick (1999)			86% MIR, EC ₅₀ = 10 µM Glucose transport	W	[101]
				42% MIR, EC ₅₀ = 10 µM	
Frick (1998)	IG-62	Lipogenesis	Activity 'B' §	21	[45]
Frick (1998)	IG-63	Lipogenesis	Activity 'B' §	22	[45]
Frick (1998)	IG-64	Lipogenesis	Activity 'B' §	25	[45]
Frick (1998)	IG-65	Lipogenesis	Activity 'B' §	26	[45]
Frick (1998)	IG-66	Lipogenesis	Activity 'B' §	27	[45]
Frick (1998)	IG-67	Lipogenesis	Activity 'C' §	30	[45]
Frick (1999)			48% MIR, EC ₅₀ = 60 µM Glucose transport	E	[101]
				34% MIR, EC ₅₀ = 30 µM	
Frick (1998)	IG-68	Lipogenesis	Activity 'C' §	31	[45]
Frick (1999)			53% MIR, EC ₅₀ = 15 µM Glucose transport	F	[101]
			26% MIR, EC ₅₀ = 25 µM		
Frick (1998)	IG-69	Lipogenesis	Activity 'C' §	32	[45]
Frick (1998)	IG-70	Lipogenesis	Activity 'C' §	33	[45]
Frick (1999)			59% MIR, EC ₅₀ = 7 µM Glucose transport	A	[101]
			28% MIR, EC ₅₀ = 8 µM		
Frick (1998)	IG-71	Lipogenesis	Activity 'C' §	34	[45]
Frick (1999)			63% MIR, EC ₅₀ = 8 µM Glucose transport	J	[101]
			21% MIR, EC ₅₀ = 3 µM		

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1999)	IG-72	Lipogenesis	Activity 'C' [§]	35	[45]
Frick (1999)			64% MIR, EC ₅₀ = 7 µM	C	[101]
			Glucose transport	29% MIR, EC ₅₀ = 10 µM	
Frick (1998)	IG-73	Lipogenesis	Activity 'C' [§]	36	[45]
Frick (1999)			72% MIR, EC ₅₀ = 8 µM	K	[101]
			23% MIR, EC ₅₀ = 15 µM		
Frick (1998)	IG-74	Lipogenesis	80% MIR at 20 µM, IC ₅₀ = 2.2 ± 0.2 µM	37	[45]
Frick (1999)			77% MIR, EC ₅₀ = 8.2 µM	B	[101]
Frick (1998)		GPAT	90% MIR, IC ₅₀ = 8.0 ± 1.1 µM	37	[45]
			Glucose transport 90–95% MIR at 20 µM, IC ₅₀ = 8.3 ± 1.5 µM		
Frick (1999)			41% MIR, EC ₅₀ = 8.1 µM	B	[101]
Frick (1998)		GLUT4 translocation	50% MIR	37	[45]
			Glycogenesis 65% MIR at 20 µM, IC ₅₀ = 7.1 ± 2.0 µM		
		Lipolysis	95% MIR, IC ₅₀ = 2.5 ± 0.5 µM		
		Tyrosine phosphorylation of IRS-1	65% MIR at 10 µM		
Frick (1998)	IG-75	Lipogenesis	Activity 'D' [§]	38	[45]
Frick (1999)			90% MIR, EC ₅₀ = 7 µM	M	[101]
			39% MIR, EC ₅₀ = 6 µM		
Frick (1998)	IG-76	Lipogenesis	Activity 'D' [§]	39	[45]
Frick (1999)			84% MIR, EC ₅₀ = 7 µM	L	[101]
			36% MIR, EC ₅₀ = 8 µM		
Frick (1998)	IG-77	Lipogenesis	Activity 'D' [§]	40	[45]
Frick (1998)	IG-78	Lipogenesis	90% MIR at 20 µM, IC ₅₀ = 2.5 ± 0.9 µM	41	[45]

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1999)			96% MIR, EC ₅₀ = 3 μM	N	[101]
Frick (1998)		GPAT	90% MIR, IC ₅₀ = 3.5 ± 0.8 μM	41	[45]
Frick (1999)		Glucose transport	90–95% MIR at 20 μM, IC ₅₀ = 3.5 ± 0.6 μM		
Frick (1998)			52% MIR, EC ₅₀ = 5 μM	N	[101]
			GLUT4 translocation 50% MIR	41	[45]
			Glycogenesis 70% MIR at 20 μM, IC ₅₀ = 3.3 ± 0.5 μM		
		Lipolysis	95% MIR, IC ₅₀ = 1.6 ± 0.5 μM		
Frick (1998)	IG-79	Tyrosine phosphorylation of IRS-1 Lipogenesis	95% MIR at 10 μM Activity 'C'·§	45	[45]
		Tyrosine phosphorylation of IRS-1	55% MIR at 10 μM		
Frick (1998)	IG-80	Lipogenesis	Activity 'C'·§	46	[45]

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[§] Note: for Muller's SAR study [45] aggregate activities were reported as follows: "A", MIR < 20%; "B", MIR 20–49%, EC₅₀ 25–200 μM; "C", MIR 50–80%, EC₅₀ 10–100 μM; "D", MIR > 80%, EC₅₀ 3–30 μM.

Table 7

Heptasaccharides (cont.).

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1998)	IG-82	Lipogenesis	Activity 'B' [§]	24	[45]

Study	Compound	Assays*	Activity	Lit. no. [‡]	Ref.
Frick (1998)	IG-83	Lipogenesis	Activity 'B' [§]	28	[45]

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[§] Note: for Muller's SAR study [45] aggregate activities were reported as follows: "A", MIR < 20%; "B", MIR 20–49%, EC₅₀ 25–200 μM; "C", MIR 50–80%, EC₅₀ 10–100 μM; "D", MIR > 80%, EC₅₀ 3–30 μM.