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A fluorescent inositol phosphate glycan stimulates lipogenesis in rat adipocytes by extracellular activation alone

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Abstract—The chemical synthesis of 2,6-dideoxy-2-amino-6-mercaptoglucopyranosyl-(α1–6)-*myo*-inositol 1,2-cyclic phosphate and its conjugation with a lucifer yellow derivative are reported. The resulting fluorescent IPG analogue was able to stimulate lipogenesis in rat adipocytes despite the fact that it was not internalized into the cell. The results demonstrate that internalization of the IPG is not required for manifestation of its insulin-like effects.

The inositol phosphate glycans (IPGs) are small, ionic oligosaccharides that are released from the exterior surface of insulin-sensitive cells in response to insulin stimulation. IPGs isolated from the medium after insulin-induced release or generated artificially by phosphatidylinositol–specific phospholipase C (PI–PLC) cleavage of glycolipids on the cell surface are able to stimulate insulin-sensitive cells upon exogenous addition, even in the absence of insulin. These facts have led to the hypothesis that the IPGs are second messengers of insulin action.[1–4] Their extracellular release may provide a mechanism for signal amplification, since one molecule of insulin can result in the release of numerous IPG molecules and could thereby activate more than one cell.

The mechanism by which IPGs stimulate insulin-like activities in sensitive cells, including even the site(s) of IPG action in or on the cell, is still not fully understood. Alvarez et al.[5] convincingly demonstrated that a natural IPG is actively transported into the cytoplasm of hepatocytes in a process that is inhibited by cyanide and therefore is probably energy dependent. This discovery lead to the hypothesis that cell activation by IPGs occurs once the IPG is in the cytosol where it modulates the metabolic activity of the cell. This hypothesis is consistent with the observations from several laboratories that the IPGs are able to modulate the activities of several insulin-responsive enzymes, including c-AMP phosphodiesterase,[6] pyruvate dehydrogenase phosphatase,[7] protein phosphatase 2C,[8] c-AMP-dependent protein kinase,[9] and adenylate cyclase[6] in cell-free assays. On the other hand, Muller and coworkers have demonstrated that synthetic and yeast-derived IPGs are able to bind to a 115 kDa protein on the exterior surface of rat adipocytes and that this binding activates the pp59(Lyn) kinase, leading to insulin-like effects in the cell.[10] However, it has not been determined if surface binding, or transport into the cell, or both are necessary and sufficient for cell activation by IPGs.

We have been interested in studying the relationship between IPG transport and cell activation. To facilitate the study of transport by optical techniques including fluorescence microscopy and fluorescence assisted cell sorting (FACS), it was desirable to have a fluorescent IPG analogue. In this communication we describe the chemical synthesis of a small IPG tethered to a lucifer yellow fluorescent probe and report the finding that, while the conjugate is competent to stimulate lipogenesis in native rat adipocytes, it is not transported into the cell. We conclude that, despite the fact that the natural IPGs are transported into cells, this internalization is not required for activation of adipocytes.

We opted to prepare a pseudodisaccharide IPG analogue since it is the structurally simplest (and synthetically least demanding) IPG analogue known to exhibit insulin-like activity, albeit modestly.[11] To permit easy conjugation of the pseudodisaccharide with a fluorophore or other moiety, the primary hydroxyl was replaced

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with a thiol group. A thiol-modified IPG has been previously reported[12] but we pursued a different synthetic strategy with the pseudodisaccharide analogue. Lucifer yellow was chosen as the fluorescent dye for the conjugate because it is dianionic and therefore is expected to preclude nonspecific association of the conjugate with cell surfaces and passive diffusion through the cell membrane.

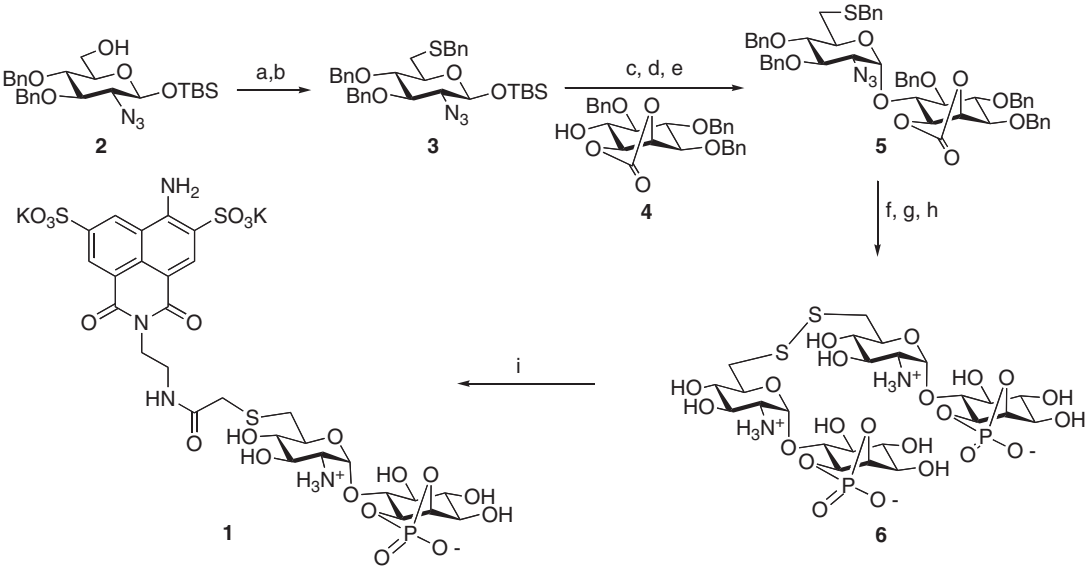
Our synthesis of fluorescent IPG **1** is shown in Scheme 1. The known,[13] selectively protected 2-azido-2-deoxyglucose **2** was tosylated at the 6-position, then treated with lithium benzylthiolate in THF to afford the protected thiol **3**. Removal of the silyl group at the anomeric position with tetrabutylammonium fluoride and acetic acid, followed by formation of the trichloroacetimidate by Schmidt's method[14] produced the glycosyl donor. Trimethylsilyl triflate-induced glycosylation of known inositol **4**[15] (diethyl ether, 4 Å molecular sieves, −42 °C) produced α-pseudodisaccharide **5**, together with the corresponding β-anomer (α:β, 2.4:1). The mixture of anomers was not separated at this stage but was treated with lithium hydroxide in THF–water to hydrolyze the cyclic carbonate producing the corresponding anomeric diols. The α-anomer was isolated chromatographically and treated with the reagent produced by CH_3OPOCl_2 in pyridine,[16] followed by sodium in liquid ammonia to reduce the azide function and remove all of the benzyl protective groups, in analogy to earlier IPG syntheses.[15] The deprotected IPG was obtained from this reaction mixture as the disulfide **6**, presumably due to air oxidation during workup, in 76% yield (two steps). Finally, coupling of **6** with the commercially available lucifer yellow iodoacetamide at pH 7.1 in the presence of tris(carboxyethyl)phosphine (TCEP) to reduce the disulfide in situ, produced fluorescent IPG **1**. The structure of the conjugate was confirmed by ^1H and ^{31}P NMR and ESI MS (negative ion mode).[17]

Conjugate **1** was evaluated for its ability to stimulate lipogenesis in intact native rat adipocytes by a modification[18] of the published method.[19] The stimulation of lipogenesis as a function of concentration for conjugate **1**, the parent pseudodisaccharide (2-deoxy-2-amino-glucopyranosyl-(α1–6)-*myo*-inositol 1,2-cyclic phosphate, **7**,[11]) and insulin are shown in Figure 1. The data were fitted to a standard receptor-binding model (Eq. 1).

$$\text{activity} = (\text{activity}_{\max}{}^{*}[\text{analyte}])/(K_d + [\text{analyte}]). \quad (1)$$

Conjugate **1** was found to stimulate lipogenesis with a maximal activity of 47% that of the maximal insulin response (MIR, defined as the stimulation by 5 nM insulin) and a K_d of 12.0 μM ($R = 0.877$). It is interesting to note that the conjugate was better able to stimulate lipogenesis than the parent **7** (maximal activity: 24% MIR, $K_d = 15.9$ μM; $R = 0.789$) possibly because **1** bears a better resemblance to the IPG pharmacophore[20] due to the presence of the sulfate groups distal to the inositol.

The ability of conjugate **1** to enter intact rat adipocytes was checked by two methods: qualitatively by fluorescence microscopy, and quantitatively by flow cytometry. Cells were isolated from epididymal fat pads from male Long–Evans rats by collagenase digestion,[19] then divided into two groups. One group of cells was incubated with 40 μM conjugate **1** at 37 °C for 1 h, then viewed by fluorescence microscopy or analyzed by flow cytometry. No incorporation of fluorescence into adipocytes was observed by either method. The second group of cells was checked for viability by assaying for stimulation of lipogenesis by either insulin or conjugate **1**. These cells showed results identical to those reported above, establishing that the cells were alive and metabolically active.



Scheme 1. Synthesis of a fluorescent IPG. Reagents and conditions: (a) TsCl, pyridine (89%); (b) BnSLi, THF, 0 °C–25 °C (77%); (c) TBAF, AcOH, THF (86%); (d) Cl_3CCN, K_2CO_3; (e) **4**, TMSOTf (10 mol%); (f) LiOH, H_2O, THF (29% over three steps); (g) CH_3OPOCl_2, pyridine; (h) Na, NH_3, −78 °C; NH_4Cl(s), −78 °C; CH_3OH, −78 °C to 25 °C, (76% over two steps); (i) lucifer yellow iodoacetamide, 25 mM aq HEPES, pH 7.1, TCEP, 2 h.

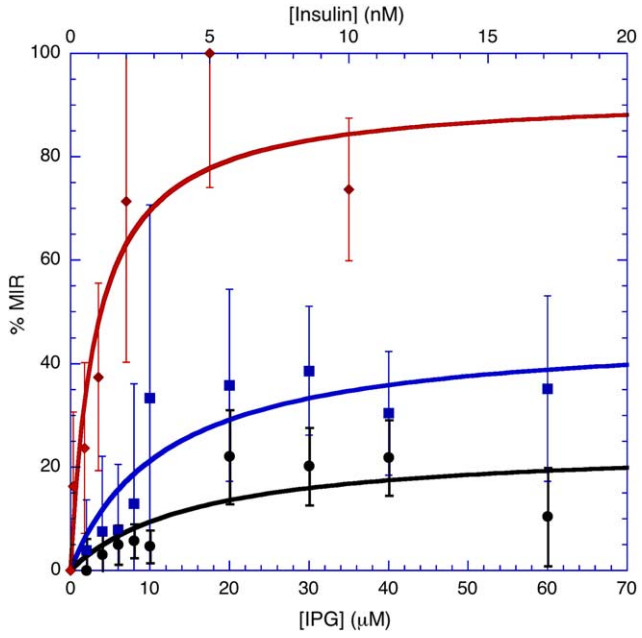


Figure 1. Stimulation of lipogenesis in rat adipocytes. Isolated native rat adipocytes were incubated with 6-[^{3}H]-glucose (0.55 mM) and various concentrations of the analyte for 1 h. Incorporation of tritium into lipids was measured and is expressed as a percent of the maximal insulin response (%MIR). Black circles: 2-deoxy-2-amino-glucopyranosyl-(α1–6)-*myo*-inositol 1,2-cyclic phosphate, **7**[11] (0–60 μM); blue squares: compound **1** (0–60 μM); red diamonds: insulin (0–500 nM). Each data point is the average of at least five replicates. Error bars represent ±1 SD.

To rule out the possibility that stimulation of lipogenesis by conjugate **1** was due to a degradation product produced during the incubation with adipocytes, the buffer used during the incubation was collected at the end of the incubation period and analyzed by HPLC on a G-10 sizing column, eluting with water. We found no evidence of any degradation product in the incubation buffer by this analysis. These chromatography conditions successfully separated conjugate **1** from both of the smaller compounds, that is, parent disaccharide **7** and unconjugated lucifer yellow iodoacetamide. We cannot rule out the possibility that a degradation product is formed at concentrations below the detection limit of this technique, but the possibility that a degradation product present at such a low concentration is responsible for the observed stimulation of lipogenesis is remote, particularly since the parent disaccharide **7** was less active than conjugate **1**, even when present at the same concentration.

We conclude that despite the known ability of an IPG to be actively transported into an insulin sensitive cell,[5] at least for conjugate **1**, such internalization is not required for stimulation of lipogenesis in rat adipocytes. We are currently exploring if larger synthetic IPGs are transported into rat adipocytes.[15]

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