Insulin detemir enhances proglucagon gene expression in the intestinal L cells via stimulating β-catenin and CREB activities

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Submitted 5 July 2011; accepted in final form 20 June 2012

Liu S, Liu R, Chiang Y, Song L, Li X, Jin T, Wang Q. Insulin detemir enhances proglucagon gene expression in the intestinal L cells via stimulating β-catenin and CREB activities. Am J Physiol Endocrinol Metab 303: E740–E751, 2012. First published July 17, 2012; doi:10.1152/ajpendo.00328.2011.—Insulin therapy using insulin detemir (d-INS) has demonstrated weight-sparing effects compared with other insulin formulations. Mechanisms underlying these effects, however, remain largely unknown. Here we postulate that the intestinal tissues’ selective preference allows d-INS to exert enhanced action on proglucagon (Gcg) expression and the production of glucagon-like peptide-1, an incretin hormone possessing both glycemia-lowering and weight loss effects. To test this hypothesis, we used obese type 2 diabetic db/db mice and conducted a 14-day intervention with daily injection of a therapeutic dose of d-INS or human insulin (h-INS) in these mice. The body weight of the mice after 14-day daily injection of d-INS (5 IU/kg) was decreased significantly compared with those injected with the same dose of h-INS or saline. The weight-sparing effect of d-INS was associated with significantly elevated circulating levels of total GLP-1 and reduced food intake. Histochemistry analysis demonstrated that d-INS induced rapid phosphorylation of protein kinase B (Akt) in the gut L cells of normal mice. Western blotting showed that d-INS stimulated Akt activation in a more rapid and enhanced fashion in the mouse distal ileum compared with those by h-INS. In vitro investigation in primary fetal rat intestinal cell (FRIC) cultures showed that d-INS increased Gcg mRNA expression as determined by Northern blotting and real-time RT-PCR. Consistent with these in vivo investigations, d-INS significantly increased GLP-1 secretion in FRIC cultures. Consistently, d-INS was also shown to induce rapid phosphorylation of Akt in the clonal gut cell line GLUTag. Furthermore, d-INS increased β-catenin phosphorylation, its nuclear translocation, and enhanced CAMP response element-binding protein (CREB) phosphorylation in a phosphatidylinositol 3-kinase and/or mitogen-activated protein kinase kinase/extracellular signal-regulated kinase-sensitive manner. We suggest that the weight-sparing benefit of d-INS in mice is related to its intestinal tissues preference that leads to profound stimulation of Gcg expression and enhanced GLP-1 secretion in intestinal L cells, potentially involving the activation of insulin/β-catenin/CREB signaling pathways.

Insulin; protein kinase B; glycogen synthase kinase-3; extracellular signal-regulated kinase; β-catenin; adenosine 3’,5’-cyclic monophosphate response element-binding protein; Wnt; proglucagon; gut; L cells

INSULIN THERAPY IS A CRITICAL treatment for type 1 diabetes patients, and it is also becoming increasingly accepted for type 2 diabetes for the reduction of diabetic complications (48). Weight gain is a primary adverse effect in both type 1 and type 2 diabetic patients on insulin therapy (7, 50). However, patients using long-acting insulin analogs, particularly insulin detemir (d-INS), often show decreased weight gain (16, 39).

Insulin exerts a variety of biological effects through activation of its cell surface receptor (43). Activation of phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (Akt) is a critical event that triggers diverse downstream signaling cascades and effector responses (23). In the periphery, activation of this pathway conveys insulin actions such as glucose transport, gene transcription, and protein synthesis (23, 53). In the brain, integral insulin signaling via the PI3-K/Akt pathway is related to nutrient homeostasis and appetite regulation (36). d-INS has a fatty acid chain addition at LysB29 that permits reversible albumin binding (15). Diabetic patients with d-INS therapy usually show both improved glycemic control and weight stability (16, 18) compared with other basal insulin therapies. The molecular mechanisms underlying the weight-sparing benefit of d-INS remain largely unknown. It has been proposed that d-INS could cross the blood-brain barrier faster and in higher quantities than other types of insulin to induce stronger effect in the brain (17). As a result, reduced appetite may contribute to its weight-sparing benefit (4).

We have demonstrated recently that insulin stimulates proglucagon (Gcg) expression in the intestinal endocrine L cells but not in the pancreatic α-cells (53). In colon cancer cells, insulin stimulates β-catenin (β-cat) Ser675 phosphorylation, which was associated with enhanced nuclear localization of β-cat and the binding of β-cat/TCF7L2 to Wnt target promoters (41). The bipartite transcription factor β-cat/TCF is the key downstream effector in the Wnt signaling pathway while the phosphorylation of β-cat at Ser675 increases its nuclear translocation (44). Interestingly, insulin appears to use the same cis- and trans-elements that are used by the Wnt signaling pathway to stimulate Gcg expression and glucagon-like peptide-1 (GLP-1) production (53). Consistent with a potential role of cross talk between insulin and Wnt signaling pathways in the regulation of the Gcg expression (53), it has been reported recently that insulin can directly enhance GLP-1 secretion from the enteroendocrine L cells (27).

GLP-1 is a 30-amino acid peptide hormone synthesized and released from the intestinal endocrine L cells in response to nutrient ingestion (2). GLP-1 exerts a variety of important physiological effects, including the stimulation of glucagon-dependent insulin secretion from the β-cell (20), inhibiting glucagon release (34), decreased gastric emptying (49), en-

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hanced satiety, and reduction of food intake (13, 32). GLP-1 mimetics and/or pharmacological reagents, which decrease the degradation and prolong the endogenous action of GLP-1, have been used in the treatment of diabetes (1, 3). In clinical studies, GLP-1 and GLP-1 mimetics, in adjunct to conventional diabetes therapies, have also demonstrated potent weight loss effects in overweight patients with type 2 diabetes (11, 33).

In the gut L cells, GLP-1 is generated by tissue-specific post-translational processing of Gcg (30). Elevation of blood GLP-1 level can be detected within 10–30 min after the meal ingestion and can persist for up to several hours (45). Nutrients, including glucose, fatty acids, and dietary fiber, are the primary stimuli that trigger GLP-1 secretion and the upregulation of the transcription of Gcg (27, 53). In type 2 diabetes, the overall incretin effect was impaired possibly because of decreased secretion of GLP-1 in the patients (3). It is possible that declined GLP-1 secretion under insulin-resistant conditions contributes to the impaired GLP-1 action in type 2 diabetes patients (20, 27).

We postulated that the weight-sparing benefit of d-INS is an alternative consequence of insulin action on the gut L cells by enhancing Gcg expression and consequent GLP-1 production. Here we show that, compared with human insulin (h-INS), d-INS exerted rapid and enhanced insulin action in the distal ileum of normal mice, and increased circulating GLP-1 levels in the obese diabetic db/db mouse model with reduced body weight and decreased food intake. In vitro studies using the GLP-1-secreting GLUTag cell line and fetal rat intestinal cell (FRIC) cultures suggested that d-INS stimulated GLP-1 secretion as a consequence of enhanced Gcg expression by a mechanism involving activation of Akt- and/or extracellular signal-regulated kinase (ERK)-dependent β-cat and CREB signaling pathways. These data collectively suggest that the gut tissues selectivity preference is among the mechanisms underlying the weight-sparing effects of this albumin-bound insulin.

MATERIALS AND METHODS

Reagents. h-INS and d-INS were provided by Novo Nordisk Canada (Cooksville, ON). Forskolin, isobutyl methylxanthine (IBMX), antibodies for Akt, phospho-Akt (Ser473), GLP-1, phospho-glycogen synthase kinase (GSK)-3α/b (Ser21/9), Erk1/2, phospho-Erk1/2, phospho-β-cat

![Figure 1](http://ajpendo.physiology.org/) Insulin detemir (d-INS) increases serum glucagon-like peptide (GLP)-1, decreases weight gain, and reduces food intake in db/db mice. A: ip glucose tolerance test (IPGTT) performed after 14 days intervention either with PBS [control (Ctrl)], human insulin (h-INS), and d-INS injections. The areas under the glycemic curves (AUC) are shown in B. C: total GLP-1 RIA conducted in serum from db/db mice treated with d-INS or h-INS at 5 IU/kg (ip daily) or saline for 14 days. D: cumulative changes of body wt were measured during the 14-day insulin therapy. E: cumulative changes of food intake during the 14-day intervention. NS, not significant. Data are means ± SE. *P < 0.05, n = 4–5.
(Ser<sup>75</sup>), phospho-cAMP response element-binding protein (CREB) (Ser<sup>133</sup>), histone H3, β-actin, and the horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Cedarlane, Ontario, Canada). Cell culture medium, FBS, and trypsin-EDTA solution were purchased from Invitrogen Life Technology (Burlington, ON, Canada). Mitogen-activated protein kinase kinase (MEK) inhibitor PD-98059 and protein kinase B inhibitor Akti-1/2 were purchased from Calbiochem (EMD Biosciences, San Diego, CA). The PI3-K inhibitor LY-294002 was purchased from Sigma-Aldrich (St. Louis, MO). Glyceraldehyde-3-phosphate dehydrogenase and β-cat (E-5) were purchased from Abcam (Cambridge, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

**Cell cultures.** The mouse L cell model GLUTag has been described previously (9, 21). Cells were grown in DMEM (high glucose) containing 10% (vol/vol) FBS and 1% penicillin/streptomycin at 37°C in an atmosphere of humidified air (95%) and CO<sub>2</sub> (5%). Cells in 12-well culture plates (60–80% confluence) were serum-starved overnight and were washed two times with PBS before 15 min incubation with serum-free DMEM containing pharmacological reagents. Cells were then treated with h-INS or d-INS for the indicated time points. Experiments were performed on different batches of cultures to ensure the reproducibility of the results.

FRIC cultures were prepared from fetal Wistar rats (Charles River Canada, St. Constant, Quebec, Canada) as described previously (35). Briefly, intestines from 19- to 21-day-gestation rats were pooled, and the cells were dispersed by sequential 15-min incubations with collagenase (45 mg/dl), hyaluronidase (50 mg/dl), and deoxyribonuclease I (5 mg/dl; Sigma). Dispersed cells were placed on six-well plates in DMEM culture medium for 16–24 h. Before the experiment, FRIC cultures were rinsed two times and then incubated with indicated agents for 2 h in 2 ml serum-free DMEM. In FRIC cultures, the endocrine L cells account for 1% of the cell numbers.

Fig. 2. d-INS induces more rapid and enhanced phosphorylation (p) of protein kinase B (Akt) in distal ileum in mice. Time course of Akt phosphorylation by Western blotting using isolated distal ileum (A), brain tissue (B), muscle (C), or liver tissue (D) from CD1 mice given injections of d-INS or h-INS (1 U/kg iv) for indicated times. A’–D’: scatter plots showing densitometry analysis. E: histochemistry of isolated distal ileum from mice injected with d-INS (1 U/kg iv) for 10 min or PBS as control, dual stained for GLP-1 (red) and p-Akt (green). Arrows indicate GLP-1 positively stained cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AU, arbitrary units. Data are means ± SE. *P < 0.05 (vs. control), n = 5.
Animal care and tissue process. Seven-week-old CD1 mice (Charles River Laboratories) and diabetic db/db mice (B6J; Jackson Laboratories, Bar Harbor, ME) were housed under controlled temperature conditions and a 12:12-h light-dark cycle in the St. Michael’s Hospital Animal facility with free access to food (normal rodent chow).

For in vivo Akt phosphorylation assay, 7-wk-old male CD1 mice (Charles River Laboratories), ketamine anesthetized, received injection of d-INS or h-INS (1 U/kg iv) for indicated times. Tissues (liver, muscle, brain) were removed at the indicated time points and flash-frozen in liquid nitrogen. The frozen tissues were minced and grinded into a powder in a precooled (~20°C) laboratory mortar and pestle and immediately homogenized and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 20 μg/ml leupeptin, 2 μg/ml apro tinin, 1 mM phenylmethylsulfonyl fluoride, 25 mM β-glycerophosphate, 5 mM sodium orthovanadate, and 5 mM NaF). Homogenates were allowed to be solubilized for 30 min on ice, and, after a 2 min-ice-cold bath sonication, the samples were clarified by centrifugation at 12,000 g for 20 min. Samples were used immediately for immunoblotting or kept in −80°C for further use.

The db/db mice were used at 30 wk of age, after they developed severe obesity (body wt: >75 g) and diabetes as confirmed by blood glucose measurement and intraperitoneal glucose tolerance test (IPGTT). Vehicle (control), d-INS, or h-INS was injected intraperitoneally (5 IU/kg) daily at 10:00 AM. Injection of insulin was omitted (IPGTT). Vehicle (control), d-INS, or h-INS was injected intraperitoneally (100 nM) for 20 min. Samples were used immediately for serum hormone measurement and intraperitoneal glucose tolerance test (IPGTT).

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GLP-1 measurement using a total and/or active GLP-1 RIA kit (Linco, St. Charles, MO), according to the manufacturer’s instructions. Animal handling complied with the protocols and guidelines of and was approved by the institutional animal care committee.

Immunostaining. Cells grown on glass chamber slides were stimulated with or without d-INS (100 nM) for 10 min, rinsed two times with ice-cold PBS, and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, followed by permeabilization (0.2% Triton X-100 in PBS, 5 min) and incubation with 1% BSA in PBS for 1 h. The cells were then incubated with monoclonal anti-GLP-1 antibody (5 μg/ml) overnight at 4°C, washed four times with PBS, and incubated with fluorescein-conjugated goat anti-mouse IgG (10 μg/ml) for 1 h at room temperature. Cells were costained for nuclei using TO-PRO-3 iodide (1 mM; Molecular Probes) for 5 min and were mounted using Dako Fluorescence Mounting Medium (Dako North America). For immunohistochemistry, a segment of distal ileum is isolated ~5 cm from the ileocecal valve. The gut tissues were formalin fixed, paraffin embedded, and then stained for GLP-1, phospho-Akt (1:100), and phospho-β-cat (1:100) using a standardized procedure as described previously (46). The images were taken using a Nikon fluorescence microscope.

Quantitative RT-PCR and Northern blotting. GLUTag cells were serum starved and treated with 10 μM forskolin/IBMX (F/I), 100 nM h-INS, d-INS, or medium only as control for 4 h. The total cellular RNA was isolated by the Trizol method (53) and subjected to electrophoresis using a 1% (wt/vol) agarose-formaldehyde gel containing ethidium bromide. The Gcg mRNA expression was determined by real-time RT-PCR or Northern blotting using the rat Gcg cDNA as the probe (10). DNA sequences of the primers used to detect Gcg mRNA by real-time RT-PCR were forward 5' -CCACTCA-
CAGGGCACATTCC-3’ and reverse 5’-CGGTCTCTTGGTGTTCA-3’, resulting in amplification of a 99-bp Gcg cDNA fragment.

**Western blotting.** Protein samples (25 μg) were subjected to SDS-PAGE analysis. Western blotting was performed using desired primary antibodies, and densitometry was used to quantify the Western blot results as described previously (40).

**Subcellular fractionation.** Cytoplasmic and nuclear protein fractions were prepared as described previously (41). Briefly, cells were treated with or without inhibitors and/or d-INS, washed with ice-cold PBS, and removed from the culture plate by gentle scraping with a cell scraper in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). After pelleting by centrifugation at 13,000 rpm for 1 min at 4°C, the supernatant was transferred (cytoplasmic fraction) to a new microcentrifuge tube and stored at 80°C. The nuclear pellets were washed repeatedly in 400 μl of ice-cold buffer A (13,000 rpm for 1 min) at 4°C three times and resuspended in buffer B (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 400 mM NaCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After rocking for 30 min at 4°C, the samples were centrifuged at 13,000 rpm for 10 min at 4°C to recover a nuclear fraction (supernatant).

**Statistical analysis.** The body weights are expressed as the net weight change vs. the body weight before the intervention. The food intake is expressed as the cumulative changes during the intervention. All data are presented as means ± SE. Statistical significance between experimental groups was assessed by ANOVA using n-1 post hoc custom hypothesis tests. Significance was determined at the P < 0.05 level in these comparisons.

**RESULTS**

**d-INS but not h-INS increases GLP-1 levels in db/db mice, associated with decreased weight gain and reduced food intake.** The db/db mice fed with normal rodent chow showed consistent body weight gain at a rate of ~1 g/wk. At 30 wk of age, their body weight increased to ~75 g associated with severe glucose intolerance as determined by IPGTT (Fig. 1A). During the intervention, mice are hosted in individual cages allowing the determination of food intake and body weight. d-INS and h-INS at a therapeutic dose (5 IU/kg) or saline as control were administrated by daily intraperitoneal injection. After 2 wk treatment, both insulins improved glycemic excursion during an IPGTT, and d-INS showed statistically significant effects (P < 0.05, n = 5; Fig. 1, A and B). Serum total...
GLP-1 levels were measured in blood samples from nonfasted mice after 2 wk of treatment, and RIA showed that d-INS but not h-INS significantly increased circulating GLP-1 levels compared with the control mice \( (P < 0.05, n = 5; \text{Fig. 1A}) \). d-INS-treated mice showed significantly decreased weight gain \( (P < 0.05, n = 5; \text{Fig. 1B}) \) that was associated with reduced food intake \( (P < 0.05, n = 5; \text{Fig. 1C}) \).

*d-INS exerts more rapid and enhanced effects than h-INS on phosphorylation of Akt in mouse distal ileum.* To test our working hypothesis that d-INS-enhanced GLP-1 secretion may underlie weight-sparing effects of d-INS, we sought to determine the impact of d-INS and h-INS on Akt phosphorylation in the distal ileum of mice. Western blotting was then used to assess the effects of d-INS and h-INS on Akt phosphorylation in the distal ileum from the mice treated with d-INS and h-INS for various time points. Remarkably, the results showed that Akt phosphorylation was rapid and enhanced by d-INS treatment rather than those by h-INS in the distal ileum \( (\text{Fig. 2, A and A'}) \), suggesting that the two insulins display different pharmacokinetic profiles in the gut tissues.

Western blotting was also conducted, in parallel, using brain, liver, and muscle tissues from the same mice. Compared with h-INS, rapid and enhanced effects of d-INS on Akt phosphorylation were also observed in the brain tissue \( (\text{Fig. 2, B and B'}) \), consistent with previous findings in brain cortical tissue \( (17) \). In contrast, d-INS displayed relatively slower but persistent action on Akt phosphorylation in the muscle and liver \( (\text{Fig. 2, C, D, C', and D'}) \).

Immunohistochemistry was conducted to determine whether d-INS-stimulated Akt phosphorylation occurred in the segment of the distal ileum. Dual staining of GLP-1 and phospho-Akt in isolated distal ileum from the CD1 mice treated with d-INS \( (1 \mu\text{kg iv, 10 min}) \) showed remarkable enhanced staining intensity of phospho-Akt in the GLP-1 positive cells compared with the samples collected from control animals \( (\text{Fig. 2E}) \).

The above results collectively suggest that d-INS has a unique pharmacological profile in mice such that it has more rapid and potent effects on Akt phosphorylation in the gut and brain.

*Insulin stimulates gcg expression.* In vitro studies were performed to determine the effect of insulin on Gcg expression in intestinal endocrine L cells. To accomplish this, GLUTag cells were serum starved overnight and treated with d-INS \( (100 \text{nM}) \) for indicated times, and mRNA extractions were subjected to real-time RT-PCR. As shown \( (\text{Fig. 3A}) \), d-INS enhanced the Gcg mRNA level in a time-dependent fashion. The maximal Gcg expression was observed at 4 h after the treatment \( (2.04 \pm 0.74\text{-fold}, P < 0.05) \). Northern blotting was then conducted to verify the effect of d-INS on Gcg expression in these cells, and it showed that the treatment of GLUTag cells with F/I, h-INS,
and d-INS similarly increased GLP-1 transcript expression by more than twofold (2.2 ± 0.47 vs. the control, \( P < 0.05 \)) (Fig. 3B). Consistently, real-time RT-PCR showed that d-INS and h-INS significantly increased Gcg expression in the primary FRIC cultures (Fig. 3C). Furthermore, GLP-1 RIA showed that d-INS but not h-INS stimulated GLP-1 secretion in the FRIC cultures (\( P < 0.05 \), \( n = 3 \); Fig. 3D).

**d-INS activates Akt signaling in GLUTag enteroendocrine cells.** To further explore molecular mechanisms underlying the d-INS-stimulated Gcg expression in the GLP-1-secreting L cells, we first assessed the activation of Akt by Western blotting in GLUTag cells using anti-phospho-Akt antibody (Ser\(^{473}\)). The results showed that 100 nM d-INS (Fig. 4A) or h-INS (Fig. 4B) rapidly (<5 min) stimulated Akt phosphorylation in these cells. Multidose study showed that both d-INS and h-INS, at concentration as low as 1 nM, significantly stimulated Akt activation (Fig. 4, C and D). We next conducted Western blotting to assess the Akt downstream molecule GSK-3 in response to d-INS and h-INS stimulation in GLUTag cells. As shown, phosphorylation levels of GSK-3 were increased significantly at ~5–10 min after the treatment with 100 nM d-INS (Fig. 4E) or h-INS (Fig. 4F). These results suggest that, under in vitro assay conditions, both d-INS and h-INS exert comparable effects and potencies on the activation of Akt signaling in the gut endocrine cell line.

**d-INS stimulates \( \beta \)-cat phosphorylation at Ser\(^{675} \) in GLUTag cells.** Given that \( \beta \)-cat plays a role in mediating the cross talk between insulin and Wnt signaling pathways (53), we sought to assess whether insulin-stimulated Gcg expression in the enteroendocrine cells is involved with \( \beta \)-cat activation. d-INS or h-INS was found to increase \( \beta \)-cat phosphorylation (Ser\(^{675} \)) of GLUTag cells in a time-dependent fashion; the phosphorylation of \( \beta \)-cat induced by both d-INS and h-INS, at 100 nM, peaked at 5–10 min after treatment (\( P < 0.05 \), \( n = 5 \); Fig. 5, A and B). Dose study showed that d-INS at concentrations as low as 1 nM significantly stimulated \( \beta \)-cat phosphorylation at this residue (\( P < 0.05 \), \( n = 4 \); Fig. 5C). Interestingly, an ~10 times higher concentration of h-INS was required to be able to reach a comparable extent of the \( \beta \)-cat phosphorylation as by 1 nM d-INS (\( P < 0.05 \), \( n = 4 \); Fig. 5D).

**d-INS enhances \( \beta \)-cat nuclear translocation in GLUTag cells.** The binding of \( \beta \)-cat/TCF7L2 with the G2 enhancer element of Gcg promoter is an important process for insulin-stimulated Gcg transcription (31, 53). We hence assessed the effect of d-INS on the nuclear \( \beta \)-cat level by Western blotting using an isolated nuclear fraction from GLUTag cells pre-treated with 100 nM d-INS or h-INS. The results showed that d-INS or h-INS markedly increased nuclear \( \beta \)-cat content (\( P < 0.05 \), \( n = 3 \); Fig. 5, E and F), suggesting that insulin may use...
a mechanism involving β-catenin phosphorylation and nuclear translocation to convey its action on Gcg transcription.

**d-INS enhances phosphorylation of ERK1/2 in GLUTag cells.** We then examined whether or not d-INS has an impact on ERK1/2 phosphorylation in the L cells. Western blot analysis was conducted using cell lysates of GLUTag cells treated with d-INS at various concentrations for different time periods. The results showed that d-INS stimulated ERK1/2 phosphorylation in a dose- and time-dependent fashion (Fig. 6, A and B). Similar results of Western blotting were obtained in cell lysates of GLUTag cells treated with h-INS at comparable concentrations for indicated time periods (data not shown). The cellular fractionation studies showed that d-INS time dependently stimulated ERK1/2 phosphorylation in both cytosolic and nuclear fractions (Fig. 6, C and D). In contrast, in the same fractioning preparations, d-INS-induced Akt phosphorylation was only mostly detected in the cytosolic fractions with only a small amount detected in the nuclear fractions (data not shown), suggesting specific detections.

**d-INS stimulates CREB phosphorylation in GLUTag cells.** CREB is a transcription factor that imparts cAMP responsiveness when it is activated. Recent studies suggested that CREB could be activated by kinases other than protein kinase A (PKA), including mitogen-activated protein kinase (MAPK) and GSK-3 (29, 47). We thus assessed whether activation of insulin signaling in the gut L cells affects CREB activity. As shown, d-INS at concentrations as low as 1 nM dose dependently stimulated CREB phosphorylation (Ser133) (Fig. 7A). d-INS-induced CREB phosphorylation at this residue occurred rapidly (∼5 min) and remained persistently high during the treatment period (Fig. 7B).

**d-INS-stimulated activation of β-catenin and CREB is PI3-K or ERK dependent.** Pharmacological inhibitors were then employed to dissect the signaling cascades that mediate d-INS-induced phosphorylation of β-catenin and CREB in the L cells. We found that insulin-stimulated β-catenin phosphorylation was blocked significantly in the GLUTag cells pretreated with the PI3-K inhibitor LY-294002 or the MEK inhibitor PD-98059.
Phosphorylation is mainly MEK/ERK dependent. MEK/ERK activities, whereas d-INS-stimulated CREB phosphorylation and nuclear translocation are dependent on PI3-K and Akt, whereas CREB phosphorylation is mainly MEK/ERK dependent.

**DISCUSSION**

The molecular mechanism underlying the weight-sparing effects of d-INS has not been clearly identified. In this study, we examined whether d-INS enhances Gcg expression and GLP-1 production in the intestinal L cells and whether this is among the mechanisms that underlie the weight-sparing effects of d-INS. Clinical studies have demonstrated that treatment with d-INS is associated with less weight gain than other insulin formulations. There are several proposed mechanisms underlying the lower weight gain observed among patients treated with d-INS, including faster transport across the blood-brain barrier, induction of stronger central nervous anorexigenic efficacy (14), and preferential inhibition of hepatic glucose production vs. peripheral glucose uptake (19). Our investigation using the obese diabetic db/db mouse model demonstrated that d-INS but not h-INS increased circulating GLP-1 levels, which were associated with decreased weight gain, reduced food intake, and improved glucose homeostasis. We hence suggest that the effects of the albumin-bound insulin on the L cells may be a reason among the mechanisms of weight-sparing effects of d-INS.

It is interesting to note, however, d-INS (but not h-INS) also increased plasma GLP-1 levels in high-fat-diet streptozotocin (STZ)-treated CD1 mice, but the weight loss effects were not observed (data not shown). Thus, the weight-sparing effects of d-INS in mice appear to be model dependent, given that STZ-induced diabetic mice are usually associated with the reduction in body weight, and it increases when their hyperglycemia is controlled (40). Therefore, this may potentially mask any weight-sparing effects by d-INS in these mice. Nevertheless, increased GLP-1 levels were observed in both models, confirming the gut preference of d-INS in mice.

Administration of d-INS or h-INS displayed different pharmacological properties in various tissues in normal mice. Remarkably, in the distal ileum, insulin-signaling kinetics determined by Akt phosphorylation were enhanced, and the maximum was shifted to earlier time points in the mice treated with d-INS compared with h-INS. These observations suggest that d-INS has altered pharmacological properties in the gastrointestinal tract.

It is interesting to note that both d-INS and h-INS enhanced Gcg gene expression in FRIC cultures and GLUTag cells. Treatment of d-INS or h-INS elicited comparable effects in the phosphorylation of Akt and other signaling molecules in the GLUTag cells. It is presumed that the receptor-binding affinities of the two insulins are equivalent (25). The lack of a blood-tissue barrier(s) in the in vitro assay system may contribute to this equal potency of the two insulins observed under the in vitro settings. Our in vitro findings are consistent with a previous study by Lim et al. (27), suggesting that insulin action on gut L cell secretion may be a mechanism underlying the weight-sparing effects of d-INS.

In the current study, we have also shown that an earlier and stronger Akt phosphorylation was observed in the brain tissue of the mice that received d-INS injection compared with those with h-INS injection, which is consistent with a previous study by Hennige et al. (17). However, in muscle and liver, d-INS-induced phosphorylation of Akt appeared to be relatively slower but more persistent compared with those by h-INS. Our observations on Akt phosphorylation in liver and muscle tissues by d-INS and h-INS are essentially consistent with the results from Hennige et al. (17). However, our time course of Akt phosphorylation was prolonged to 120 min vs. 30 min treatment by Hennige et al., which provided more comprehensive time-dependent observations.

The molecular mechanism underlying the more rapid and enhanced action of d-INS in the distal ileum is presently unclear. Previous studies suggested that the unique albumin-bound d-INS, while it has similar receptor-binding properties as those of regular insulins, may provide the ability of tissue selectivity preference and prolonged action (17, 28). The tissue-specific blood-tissue barriers attributed by local vascular endothelial properties are critical for maintaining tissue homeostasis. Although the molecular machinery behind blood-tissue barriers is yet to be identified, recent studies have suggested that specific proteins, including P-glycoprotein as well as the gap junction, play important roles in the maintenance of different regional blood-tissue barriers (12, 26). Further experimentation is warranted to investigate the molecular mechanism underlying the selective preference of d-INS in the gut tissues.

In GLUTag cells, d-INS-induced phosphorylation is followed by or associated with the activation of important signal-transduction pathways. Insulin also stimulates nuclear CREB phosphorylation via an MAPK/ERK-dependent fashion. The downstream effectors of CREB phosphorylation were enhanced, and the downstream effectors of CREB phosphorylation appeared to be relatively slower but more persistent compared with those by h-INS.

Fig. 8. Hypothetical model showing the roles of PI3-K and MAPK/ERK pathways in transducing the stimulatory insulin signal to Gcg expression. Activation of the insulin receptor stimulates the phosphorylation of β-catenin at Ser675 and its nuclear translocation via PI3-K- and MAPK/ERK-dependent pathways. Insulin also stimulates nuclear CREB phosphorylation via an MAPK/ERK-dependent fashion. The downstream effectors of β-catenin and CREB that initiate gene transcription are not shown. Solid lines, established pathways; dotted lines, putative pathways.
The findings that d-INS activates Wnt signaling pathway(s) involving activation of β-cat Ser675 phosphorylation and CREB transcription machinery to enhance Gcg expression provide molecular explanation of elevated GLP-1 levels in diabetic mice that received d-INS injections. It is noted that d-INS can exert its actions in other tissues or organs to contribute to its weight-sparing effects. One such example is that d-INS increases adiponectin levels, which is associated with both weight loss and reduced food intake in diabetic rats (19). A recent study by Schinner and colleagues (37) has demonstrated that adipocyte-derived Wnt signaling molecules could enhance β-cell proliferation and secretion, suggesting that cross talk between Wnt and insulin signaling could occur at intraorgan levels to exert physiological or pathophysiologic relevance.

Taken together, we have demonstrated that d-INS has weight loss effects associated with elevated circulating GLP-1 levels and reduced food intake in obese diabetic db/db mice. We suggest that the weight-sparing effect of d-INS is at least in part contributed by its preferable intestinal tissue selectivity, which allows the albumin-bound insulin to enhance Gcg expression in the enteroneodcrine L cells through phosphorylation of β-cat at Ser675, its nuclear translocation, and simultaneous activation of CREB-related transcription machinery, via a PI3-K- and/or MERK-dependent fashion.

ACKNOWLEDGMENTS

We thank Dr. Yimin Wang and Connie Wang for technique assistance and Fang Zhao for critical reading of the manuscript. We thank Dr. Daniel J. Drucker for providing the GLUTag cell line.

GRANTS

This study is supported by a research grant from Novo Nordisk (LIBRA). Research in Q. Wang’s laboratory was supported by grants from the Canadian Institute for Health Research (CIHR), Canadian Diabetes Association (CDA), and Juvenile Diabetes Research Foundation. Q. Wang is supported by CIHR New Investigator Program. T. Jin’s laboratory was supported by CIHR and CDA.

DISCLOSURES

The authors declare that there is no duality of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS


REFERENCES

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