Brief Communication

Insulin/IGF-1 enhances intestinal epithelial crypt proliferation through PI3K/Akt, and not ERK signaling in obese humans

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Impact statement

This study investigates if insulin or insulin-like growth factor-1 (IGF-1) induces intestinal epithelial proliferation in humans, and if insulin and IGF-1 receptor signaling is involved in this process in obesity. Although obesity-induced high levels of insulin and IGF-1 in the stem cell niche are found to impact the proliferation of intestinal epithelial stem cells in rodents, we are the first to investigate this effect in humans. We found that insulin and IGF-1 enhanced the proliferation of intestinal crypts (including stem cells and other crypt cells) isolated from obese humans, and PI3K/Akt, and not ERK signaling was involved in insulin or IGF-1-induced proliferation.

The imbalance in signaling between PI3K/Akt and ERK pathways may point to a pathway-specific impairment in insulin/IGF-1 receptor signaling. We propose that this may contribute to reciprocal relationships between insulin/IGF-1 receptor resistance and intestinal epithelial proliferation that leads to abnormal tissue renewal and function.

Abstract

The intestinal epithelium is continuously regenerated through proliferation and differentiation of stem cells located in the intestinal crypts. Obesity affects this process and results in greater stem cell proliferation and altered tissue growth and function. Obesity-induced high levels of insulin and insulin-like growth factor-1 in the stem cell niche are found to impact proliferation in rodents indicating that insulin and insulin-like growth factor-1 receptors may play a role in modulating intestinal epithelial stem cell proliferation. To determine whether insulin or insulin-like growth factor-1 can induce proliferation in human intestinal epithelial stem cells, and if two downstream insulin and insulin-like growth factor-1 receptor signaling pathways, PI3K/Akt and ERK, are involved, we used primary small intestinal epithelial crypts isolated from obese humans and investigated (1) the effect of insulin or insulin-like growth factor-1 on crypt proliferation, and (2) the effect of insulin and insulin-like growth factor-1 signaling inhibitors on insulin or insulin-like growth factor-1-induced proliferation. We found that insulin and insulin-like growth factor-1 enhanced the proliferation of crypt cells, including intestinal epithelial stem cells. Inhibition of the PI3K/Akt pathway attenuated insulin and insulin-like growth factor-1-induced proliferation, but inhibition of the ERK pathway had no effect. These results suggest that the classical metabolic PI3K pathway and not the canonical proliferation ERK pathway is involved in the insulin/insulin-like growth factor-1-induced increase in crypt proliferation in obese humans, which may contribute to abnormal tissue renewal and function.

Keywords: Insulin, insulin-like growth factor-1, intestinal epithelium, proliferation, obesity

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Introduction

The intestinal epithelium plays important roles in nutrient absorption, satiety hormone release, and immune barrier function. In order to ensure proper tissue actions are maintained, the cells are continuously regenerated through proliferation and differentiation of intestinal epithelial stem cells (IESCs) located in the intestinal crypts. Obesity affects this process and results in changes in tissue growth and function.[1] In particular, obesity increases IESC number and proliferation, total epithelial cell number, villus height or crypt depth *in vivo*, and causes lasting effects in IESCs, such that isolated IESCs from obese compared to lean mice grow at different rates *in vitro*.[2,3] These changes are associated with many negative adverse consequences in tissue function. The increase in proliferation leads to a greater chance of mutations and less DNA repair mechanisms which can lead to tumor growth and cancer,[4–6] and may be the cause of the obesity-associated increase in the risk of intestinal cancers.[7,8] Aberrant proliferation of IESCs specifically, and not other intestinal epithelial cell types,

also underlies both small intestinal[9] and colon[9–11] cancer. Moreover, greater proliferation leads to greater growth of the tissue, which is reflected as an increase in differentiated cell number and not just an increase in the number of stem or progenitor cells.[3] In particular, the increase in tissue size is reflected by an obesity-induced increase in enterocyte number.[12–14] This increase in enterocyte number reflects the greater absorptive capacity of the epithelial tissue in obesity, a quality that is not desirable and could result in greater body weight gain.[13,15]–17 Taken together, these findings suggest that obesity may alter the proliferation, growth and function of the intestinal epithelial tissue, and increase the risk of intestinal cancers through the modulation of IESCs. It is unclear, though, the mechanism underlying the obesity-induced alterations in IESCs.

Obesity induces high levels of circulating insulin and insulin-like growth factor-1 (IGF-1) released from the pancreas and liver, respectively, and high levels of local intestinal IGF-1 released from the mesenchymal cells that underlie the IESCs.[18] Insulin receptor isoforms A and B and IGF-1 receptor are expressed in IESCs.[3] Obesity-induced high levels of insulin and IGF-1 in the stem cell niche are found to impact proliferation in rodents indicating that insulin and IGF-1 receptors may play a role in modulating IESC proliferation.[3,19] Moreover, insulin and IGF-1 receptor resistance are a hallmark of obesity that can be driven by an imbalance between or abnormal signaling within two downstream pathways, phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinases (ERKs), in non-intestinal epithelial tissues.[20–22] It is unknown, though, if insulin or IGF-1 induces IESC proliferation in humans, and if PI3K/Akt and ERK pathways are involved in insulin or IGF-1-induced proliferation in obesity. Thus, we used primary small intestinal epithelial crypts, which include IESCs and other crypt cells, isolated from obese humans, and investigated proliferation in response to insulin or IGF-1, and insulin and IGF-1 receptor signaling inhibitors (i.e. PI3K/Akt pathway inhibitor Wortmannin, ERK pathway inhibitor PD98059).

Materials and methods

Human tissue

Human intestinal mucosal tissue samples (1 × 1 inch) were obtained from tissue normally discarded from obese patients undergoing Roux-en-Y gastric bypass surgery. All procedures were approved by the Institutional Review Board (IRB) at Carle Foundation Hospital and the University of Illinois at Urban-Champaign (IRB protocol #16068). Informed consent was obtained from the subjects and the privacy rights of the subjects were observed.

Crypt isolation and culture

Intestinal crypts were isolated as previously described.[23,24] Briefly, the villi were scraped off from the intestinal mucosal tissue with a curved forceps. The tissue was cut into 2–4 mm pieces with scissors and washed 5–10 times with cold 1× PBS until the supernatant was almost clear. Tissue fragments were incubated with 2 mM EDTA (Fisher Scientific, Pittsburgh, PA) chelation buffer (1× PBS containing 43.4 mM sucrose (Fisher Scientific) and 54.9 mM D-sorbitol (Fisher Scientific)) and gently rocked at 4°C for 30 min. After removal of EDTA, tissue fragments were vigorously washed with cold chelation buffer for eight times. The supernatant was then collected and centrifuged at 200*g* at 4°C for 5 min. Freshly isolated crypts were embedded in Matrigel (Corning, Corning, NY) at 200 crypts/10 μL, seeded on 96-well plates (replicates of 4 wells per group), and incubated in crypt culture medium (Advanced DMEM/F12 (Gibco, Grand Island, NY) containing 2 mM GlutaMax (Gibco), 10 mM HEPES (Gibco), 100 U/mL penicillin–streptomycin (Gibco), 1× N2 (Gibco), 1× B27 (Gibco), 1 mM N-Acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO), 1% bovine serum albumin (Sigma-Aldrich), 10 mM nicotidamide (Sigma-Aldrich), 50 ng/mL EGF (Gibco), 100 ng/mL Noggin (PeproTech, Rocky Hill, NJ), 500 ng/mL R-Spondin-1 (PeproTech), 10 nM gastrin (Sigma-Aldrich), 10 μM SB 202190 (Sigma-Aldrich), 500 nM A 83–01 (Sigma-Aldrich), and 100 ng/mL Wnt-3A (R&D, Minneapolis, MN)) overnight with 5% CO_2 at 37°C.

Cell proliferation measurements

For the determination of proliferation in response to insulin or IGF-1, crypt cultures were changed to insulin and IGF-1-free crypt culture medium (DMEM/F12 (Gibco) containing 2 mM GlutaMax, 10 mM HEPES, 100 U/mL penicillin–streptomycin, homemade N2 (DMEM/F12 containing 100 μg/mL transferrin, Holo (Sigma-Aldrich), 6.3 ng/mL progesterone (Sigma-Aldrich), 16.11 μg/mL putrescine (Sigma-Aldrich), and 5.2 ng/mL selenite (Sigma-Aldrich)), 1× B27, minus insulin (Gibco), 1 mM N-Acetyl-L-cysteine, 1% bovine serum albumin, 10 mM nicotidamide, 50 ng/mL EGF, 100 ng/mL Noggin, 500 ng/mL R-Spondin-1, 10 nM gastrin, 10 μM SB 202190, 500 nM A 83–01, and 100 ng/mL Wnt-3A), and incubated with 5% CO_2 at 37°C for one day. A subset of crypt cultures were then incubated in insulin and IGF-1-free crypt culture medium with 25 mM HEPES (control) or with different concentrations of insulin (Santa Cruz Biotechnology, Dallas, TX) (0.1, 10, 100 nM) with 5% CO_2 at 37°C for additional one day. Another subset of crypt cultures were then incubated in insulin and IGF-1-free crypt culture medium with 1× PBS (control) or with different concentrations of IGF-1 (R&D) (0.1, 10, 100 nM) with 5% CO_2 at 37°C for additional one day. These concentrations and time points were chosen based on previously published experiments testing insulin or IGF-1-induced proliferation of intestinal epithelial cells.[3,19,25] Cell proliferation was then measured using Cell Proliferation Reagent WST-1 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Proliferation was then measured using the WST-1 assay in response to the PI3K/Akt pathway inhibitor, Wortmannin (Cell Signaling Technology, Danvers, MA), or the ERK pathway inhibitor, PD98059 (Cell Signaling Technology). After one day incubation in insulin and IGF-1-free crypt culture medium, crypt cultures were pretreated with DMSO (control) or with different concentrations of Wortmannin (0.2, 1 μM) or PD98059 (20, 50 μM) for 1 h,

and then incubated with 10 nM insulin or IGF-1 for additional one day with 5% CO_2 at 37°C. These concentrations and time points were chosen based on previously published experiments testing Wortmannin or PD98059-induced changes in intracellular signaling of intestinal epithelial cells and other cell types.[25–27] Cell proliferation was then measured using Cell Proliferation Reagent WST-1.

Data analysis

Data are expressed as Mean ± SEM. Differences between groups were analyzed using a one-way ANOVA followed by a Fisher's LSD *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Insulin and IGF-1 enhanced crypt proliferation

Crypt proliferation was increased in the 0.1, 10, and 100 nM insulin conditions compared with the control group. Specifically, proliferation was increased in the 10 and 100 nM insulin conditions compared with the control and 0.1 nM groups, and in the 0.1 nM insulin condition compared with the control group (Figure 1(a); $P < 0.05$). Moreover, proliferation was increased in the 0.1, 10, and 100 nM IGF-1 conditions compared with the control group (Figure 1(b); $P < 0.05$).

Inhibition of PI3K/Akt pathway attenuated insulin/IGF-1-induced proliferation, but inhibition of ERK pathway had no effect

Crypt proliferation was decreased in the 0.2 and 1 μM Wortmannin conditions compared with the control group, and in the 0.2 μM Wortmannin condition compared with the 1 μM Wortmannin group (Figure 2(a); $P < 0.05$). Moreover, proliferation was decreased in the 1 μM Wortmannin condition compared with the control and 0.2 μM Wortmannin groups (Figure 2(b); $P < 0.05$). However, there were no differences in the insulin-induced (Figure 2(c)) and IGF-1-induced (Figure 2(d)) proliferation after PD98059 application.

Discussion

The aim of this study was to investigate the effect of insulin or IGF-1 on crypt proliferation and the effect of insulin and IGF-1 signaling inhibitors on insulin or IGF-1-induced proliferation in IESCs and other crypt cells isolated from obese humans. The major findings were (1) insulin and IGF-1 enhanced the proliferation of intestinal crypts, and (2) PI3K/Akt, and not ERK signaling, was involved in insulin/IGF-1-induced proliferation.

Despite extensive research on the roles of insulin and IGF-1 in coordinating systemic growth and development with nutrient homeostasis in normal versus obese conditions, little is known about their roles in controlling somatic stem cells and tissue regeneration. Non-diabetic obese humans and diet-induced obese rodent models exhibit high circulating levels of insulin and IGF-1, and local release of IGF-1 from mesenchymal cells that underlie the IESCs.[3,18,28] These factors have been found to impact IESC proliferation in rodents and is the underlying mechanism responsible for the differential IESC proliferation and growth potential of IESCs isolated from diet-induced obese compared with lean mice.[3,19] Consistent with previous findings in rodents,[3,19] our results showed that insulin and IGF-1 enhanced the proliferation of intestinal crypts (including IESCs and other crypt cells) in humans. This suggests that obesity-induced high levels of insulin and IGF-1 in the stem cell niche may impact IESC proliferation in obese humans via a similar mechanism as has been found in rodents and may drive abnormal proliferation as insulin resistance develops.

The degree of insulin and IGF-1-induced increases in proliferation differed (Figure 1(a) and (b)). It is likely that the varied effect reflects differences in the ligand-receptor binding affinity and signaling between insulin and IGF-1. The three highly homologous tyrosine kinase receptors by which insulin and IGF-1 mediate their biological effects, insulin receptor isoform A (IRA), insulin receptor isoform B (IRB), and IGF-1 receptor (IGF-1R), are expressed in IESCs.[3] Both insulin and IGF-1 are able to bind to each receptor, albeit at different affinities, and IR and IGF-1R can also form heterodimers that modulate the selectivity and affinity for insulin and IGF-1 in activating downstream signaling molecules.[22] Moreover, IGF-1, but not insulin,

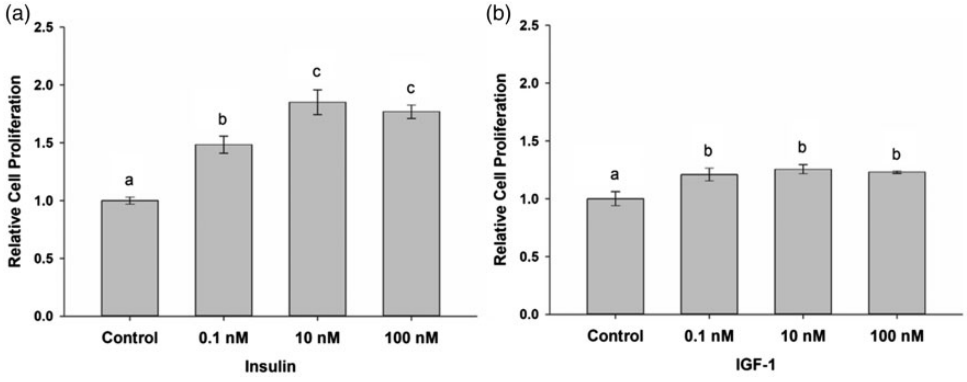

Figure 1. Cell proliferation in response to insulin (a) or IGF-1 (b). Data are expressed as Mean ± SEM of fold change relative to the control group ($n = 4$). Means with different letters indicate significant differences, $P < 0.05$.

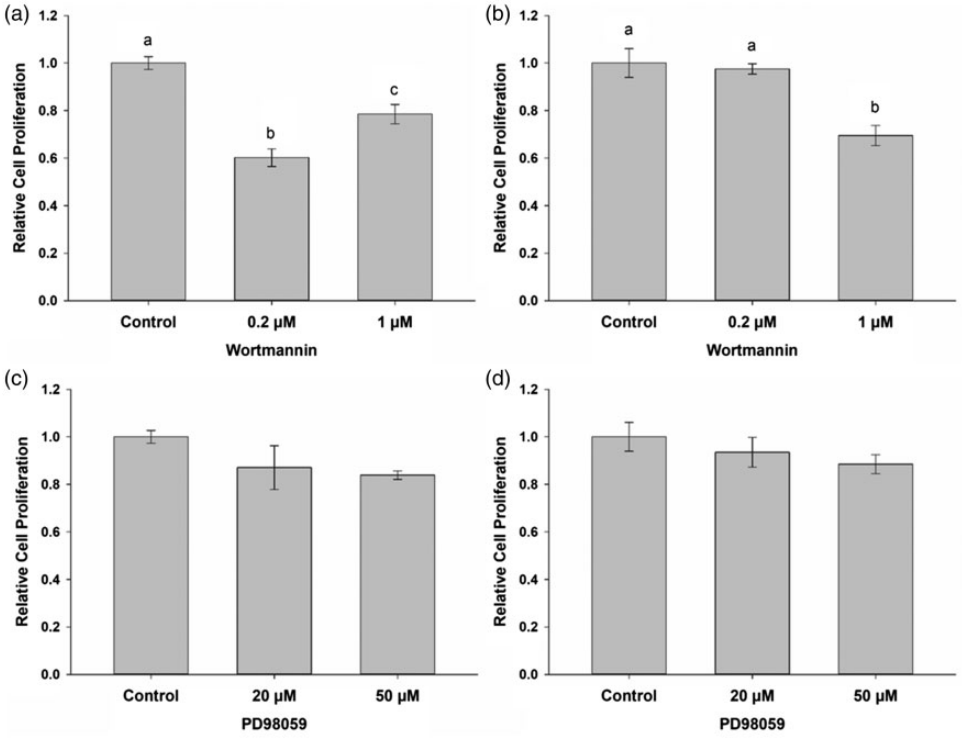

Figure 2. The effect of Wortmannin on insulin-induced (a) or IGF-1-induced (b) proliferation, and the effect of PD98059 on insulin-induced (c) or IGF-1-induced (d) proliferation. Data are expressed as Mean ± SEM of fold change relative to the control group ($n = 4$). Means with different letters indicate significant differences, $P < 0.05$.

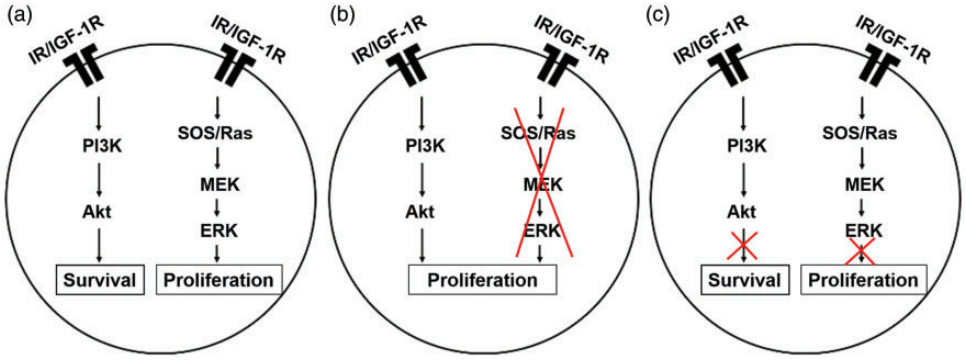

Figure 3. Proposed mechanisms of insulin receptor (IR) and IGF-1 receptor (IGF-1R)-mediated proliferation of IESCs under normal and resistant states. (a) A balance between the PI3K-dependent pathway regulating nutrient flow for cell survival and the ERK-dependent pathway regulating cell proliferation determines the cellular response to insulin and IGF-1 under normal conditions. (b) An imbalance in signaling between the two pathways may result in an uncoupling of the control mechanism between survival and proliferation in IESCs and lead to abnormal tissue renewal and function. (c) Inactivation of PI3K and/or ERK signaling molecules may induce insulin and IGF-1 resistance in IESCs and result in abnormal tissue renewal and function.

binds to IGF binding proteins (IGFBPs) in circulation; thus, the actions of IGF-1 are inhibited by IGFBPs, while insulin is not affected.[29] In addition, a variety of receptor substrates and signaling molecules have been identified to be differentially activated by insulin or IGF-1.[30] Taken together, these differences in the ligand-receptor binding affinity and signaling molecules between insulin and IGF-1 may contribute to their differential effects on metabolism and proliferation.

Insulin and IGF-1 receptor resistance is a hallmark of obesity, and insulin and IGF-1 receptor resistance in other tissues or organs (e.g. adipose tissue, liver) has been known to be driven by high levels of insulin and IGF-1.[31,32] It is traditionally thought that there is a balance between two downstream insulin and IGF-1 receptor signaling pathways, the PI3K-dependent pathway regulating nutrient flow for cell survival, and the ERK-dependent pathway regulating cell proliferation[22] (Figure 3(a)). The balance between these two pathways determines the cellular response to insulin and IGF-1.[33] Contrary to this traditional thought, we found that insulin and IGF-1 induced an increase in the proliferation of crypts isolated from obese humans through the PI3K pathway, and not the classical ERK mitogenic pathway (Figure 3(b)). An activation of PI3K signaling is known to prevent the action of cell cycle inhibitors (e.g. p21Cip1/WAF,[34] p27(kip1)[35]) and inhibits

pro-apoptotic proteins (e.g. Bcl-2-associated death promoter (BAD),[36] caspase-9[37]), and regulates the activity of a variety of transcription factors (e.g. Forkhead family of transcription factors (FoxO), murine double minute 2 (Mdm2))[37] that are responsible for the transcription of pro- and anti-apoptotic genes. An obesity-induced increase in PI3K signaling and activation of these mechanisms may not only increase proliferation, but lead to a greater chance of mutation or less DNA repair, which causes an increased cancer risk.[4–6]

Authors' contributions: WZ and MJD conceived and designed research; BMR supplied human tissues; WZ performed experiments; WZ analyzed data; WZ and MJD interpreted data; WZ generated figures; WZ and MJD drafted manuscript; all authors were involved in editing and revising the manuscript, and had final approval of the submitted and published versions.

DECLARATION OF CONFLICTING INTERESTS

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