THE ROLE OF INSULIN ON BETA-CELL PROLIFERATION

BY

JENNIFER LYNN BEITH
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ABSTRACT

A relative decrease in β-cell mass is key in the pathogenesis of type 1 diabetes, type 2 diabetes and in the failure of transplanted islet grafts. It is now clear that β-cell duplication plays a dominant role in the regulation of adult β-cell mass. Knowledge of the endogenous regulators of β-cell replication is therefore critical for understanding the physiological control of β-cell mass and for harnessing this process therapeutically. We have shown that physiological concentrations of insulin act directly on β-cells to promote survival. Whether insulin stimulates adult β-cell proliferation remains unclear. We tested this hypothesis using dispersed primary mouse islet cells double-labeled with BrdU and insulin antisera. Treating cells with 200 pM insulin significantly increased proliferation from a baseline rate of 0.15% per day. Elevating glucose from 5 mM to 15 mM did not significantly increase β-cell replication. β-cell proliferation was inhibited by somatostatin, as well as inhibitors of insulin signalling. Interestingly, inhibiting Raf-1 kinase blocked proliferation stimulated by physiological, but not super-physiological insulin doses. Insulin-stimulated MIN6 cell proliferation was dependent on both PI3-kinase/Akt and the Raf-1/MEK pathways. Examination of the effects of insulin and its receptor pathway on cell cycle molecules was inconclusive. Together, these results demonstrate for the first time that insulin, at physiological levels, can directly stimulate β-cell proliferation and that Raf-1 kinase is involved in this process. These findings have significant implications for the understanding of the regulation of β-cell mass in both the hyperinsulinemic and insulin-deficient states that occur in the various forms of diabetes

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Cause your love is so amazing,
Cause your love inspires me.
And when I need a friend,
You're always on my side
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INTRODUCTION

Diabetes

Diabetes is a devastating disease that is quickly becoming one of the most prevalent diseases, affecting over 200 million people worldwide (1, 2). In Canada alone, over two million people suffer from diabetes and this number is expected to almost double in the next few decades (1). Roughly 90-95% of all diabetes patients are classified as people with type 2 diabetes, whereas 5-10% are classified as type 1 diabetes. In addition, less common forms of diabetes have been identified, including the monogenic maturity onset diabetes of the young (MODY) syndromes, which account for approximately 5% of all type 2 diabetes cases (3). Thus, diabetes is a global epidemic that takes many forms.

Type 1 diabetes, formerly known as insulin-dependent diabetes mellitus (IDDM), occurs mainly in the young but can also occur in adulthood. The hallmark diagnosis in type 1 diabetes is hyperglycemia, which is caused by inadequate release of the blood sugar-lowering hormone insulin. Type 1 diabetes is characterized by a loss of insulin secreting β-cells resulting from autoimmune attack (4-6). Patients with type 1 diabetes are treated with injections of exogenous insulin to maintain a stable blood glucose level. Despite intensive treatment, diabetes is often accompanied by multiple complications such as heart disease, kidney failure and blindness (5, 6). Together with these complications, diabetes shortens the life of those afflicted by ~10 years. Therefore, although insulin is a treatment for diabetes, it does not cure the disease. A true cure would result from either increased endogenous β-cell mass or introduction of a cell type that perfectly mimics normal β-cells.

Type 2 diabetes, formerly called non-insulin-dependent diabetes mellitus (NIDDM), is characterized by both inadequate insulin sensitivity and β-cell dysfunction (7). Patients with type 2 diabetes exhibit hyperglycemia and often hyperinsulinemia, which is associated with insulin resistance. During the progression to type 2 diabetes, β-cells initially proliferate in an attempt to compensate for insulin resistance. However, in those progressing to diabetes, compensation fails and β-cell mass becomes insufficient to meet the demands of the body. Thus, a better understanding of how β-cells proliferate during this compensation period may offer insights into the pathogenesis of the disease and possible treatments that mimic this natural process. Type 2 diabetes is typically associated with advanced age, unbalanced diet and sedentary lifestyle but

also has a major genetic component (7, 8). People with type 2 diabetes are treated to various degrees depending on the severity and form of the disease; these treatments range from diet and exercise to insulin injections.

Genetic susceptibility to diabetes

Combinations of susceptibility genes and environmental factors have been shown to play a role in the pathogenesis of both type 1 diabetes and type 2 diabetes. Several diabetes susceptibility genes are known to affect β-cell mass (9-11). In type 1 diabetes, there are approximately 18 regions of the genome that have been shown to influence disease risk, called IDDM1-IDDM18 (10). The most characterized loci are IDDM1 and IDDM2, which contains HLA and insulin genes, respectively (10). Variation in the IDDM2 gene contributes to approximately 10% of type 1 diabetes susceptibility (12), but the mechanisms by which insulin affects disease progression is unclear. Although many investigators have focused on the role of insulin in thymic tolerance (13, 14), it is also possible that alterations in islet insulin expression could modulate β-cell mass. The remaining IDDM loci contain genes such as those involved in immunity, development and glucose metabolism (10).

A number of type 2 diabetes susceptibility genes have been found through whole-genome linkage studies that contribute to β-cell function and/or survival (15-17). These include TCF7L2, KIR6.2 and calpain 10, among others (15-17). Recent genome-wide analysis was carried out on approximately 1500 type 2 diabetic patients from Finland and Sweden. It was reported that single nucleotide polymorphisms (SNPs) on the coding region of known β-cell cycle inhibitors, p15 and p16, predisposed individuals to the disease (11). At this time, it is not known whether these polymorphisms result in gain-of-function or loss-of-function effects, or which cell types are most affected. Many factors contribute to the pathogenesis of diabetes. The observation that genes capable of influencing β-cell mass contribute susceptibility to diabetes, suggests the possibility that reduced β-cell mass or compensatory ability may play a causal role in the disease.

Proliferation of pancreatic β-cells

A relative loss of pancreatic β-cell mass is critical to the pathogenesis of diabetes. A loss of β-cell graft mass is a key limiting factor for successful islet transplantation therapy (18, 19). A steady β-cell mass is maintained throughout life by a balance of apoptosis and proliferation (20). Pancreatic β-cell mass expands during times of need, such as obesity (21) and pregnancy (22). There have been several hypotheses regarding the origin of newly formed post-natal β-cells (23), and many groups have attempted to generate β-cells *in vitro*. It has been suggested that new adult β-cells can arise from multipotent precursors found within the pancreas (24-26) in a manner that recapitulates development from embryonic stem cells (27-29). Hypotheses that new adult β-cells are formed by transdifferentiation of pancreatic ductal (30-32) and acinar (33) cells have also been proposed, but were later challenged by lineage tracing studies (34, 35). Furthermore, a study implying human islet epidermal to mesenchymal transition (31) was later challenged using lineage tracing of mouse β-cells *in vitro* (36). Nevertheless, it is now well established that proliferation of adult pancreatic β-cells is a major regulator of β-cell mass (34, 35, 37). *In vivo* genetic lineage tracing provided strong evidence that adult pancreatic β-cells are formed by self-duplication rather than differentiation from a stem cell (34). This was further supported by Teta *et al.*, who used a novel *in vivo* DNA analog-based lineage tracing technique to show that growth of adult β-cells does not involve specialized highly replicating progenitors (35). Thus, β-cell proliferation is a critical factor for maintaining a stable β-cell mass. Understanding the endogenous regulation of β-cell proliferation may lead to new therapeutic strategies to combat diabetes.

Various studies have sought to determine the proliferation rate of pancreatic β-cells *in vivo*. The earliest studies estimated a proliferation rate of roughly 6% and 1% per day in rats of one and three month(s) of age, respectively (38). Using a mathematical model, β-cell proliferation rates were estimated to be as high as 10% per day in adolescent mice (39). Recent reports indicate that β-cells replicate at a rate of approximately 0.07-2% per day in adult mice exposed to BrdU or related analogues via their drinking water and that this rate declines dramatically with age (35, 40). Proliferation measured in pancreatic sections using the proliferation marker Ki67 showed 0.04% and less than 1% of β-cells were replicating in human adult (21) and infant (41) pancreatic sections, respectively. Taken together with the lineage-

tracing studies described above, these investigations strongly suggest that β-cell replication, while very slow, plays a dominant role in the control of adult β-cell mass.

An overview of the cell cycle

The cell cycle is very tightly regulated in all cell types, including the β-cell. Eukaryotic cell division requires coordinated cycle events including DNA synthesis (S) and mitosis (M). These events are separated by two gap phases (G1 and G2) (42). During the G1 phase, the cells receive information from the extracellular environment to determine whether to proliferate or to adopt an alternate fate (42, 43). DNA synthesis requires a large amount of energy; therefore, prior to committing to replication, the cell must pass the G1 to S checkpoint. One of the key gatekeepers of this checkpoint is the tumour suppressor protein p53, which is activated by cellular stress (42, 44). p53 regulates multiple mechanisms such as DNA repair and cell death. p53 also transactivates cyclin inhibitory kinases (CIPs) such as p21, p27 and p57 (42, 44) (see Fig. 1). These inhibitory proteins, along with the inhibitory kinases (INKs) p15, p16 and p18, prevent cell cycle progression by binding to cyclins and cyclin dependent kinases (cdks) (45). Cyclin D and cyclin E bind cdk4 and cdk2, respectively, during the G1/S checkpoint (see Fig. 1). These complexes in turn phosphorylate the tumour suppressor protein retinoblastoma (Rb), leading to progression through the cell cycle (46). Rb is the final molecule maintaining cell cycle arrest and it does this by inhibiting E2F transcription factors and recruiting histone deacetylases (47). Once the cell proceeds through the G1/S checkpoint, DNA synthesis occurs. Upon completion of this process, the G2 phase allows the cell to make the necessary proteins and prepare for mitosis, the separation of sister chromosomes and daughter cells into individual cells (45). Although the main components of the eukaryote cell cycle have been mapped out, many details remain unclear.

There are also a number of proteins associated with the cell cycle that have not yet been fully characterized. For example, the functions of the cell cycle molecules cyclin G1 and cyclin G2 are poorly understood. They are atypical cyclins because they do not promote cell cycle progression, but induce cell cycle arrest (48-53). Cyclin G1 may inhibit the G2/M phase transition in response to DNA damage (50, 51), whereas cyclin G2 may arrest the cells at the G1/S checkpoint (48, 52, 53). Cell cycle regulation is very complex and tightly controlled. The

regulation of the β-cell cycle and β-cell replication is still poorly understood as the majority of studies on the cell cycle have employed other cell types.

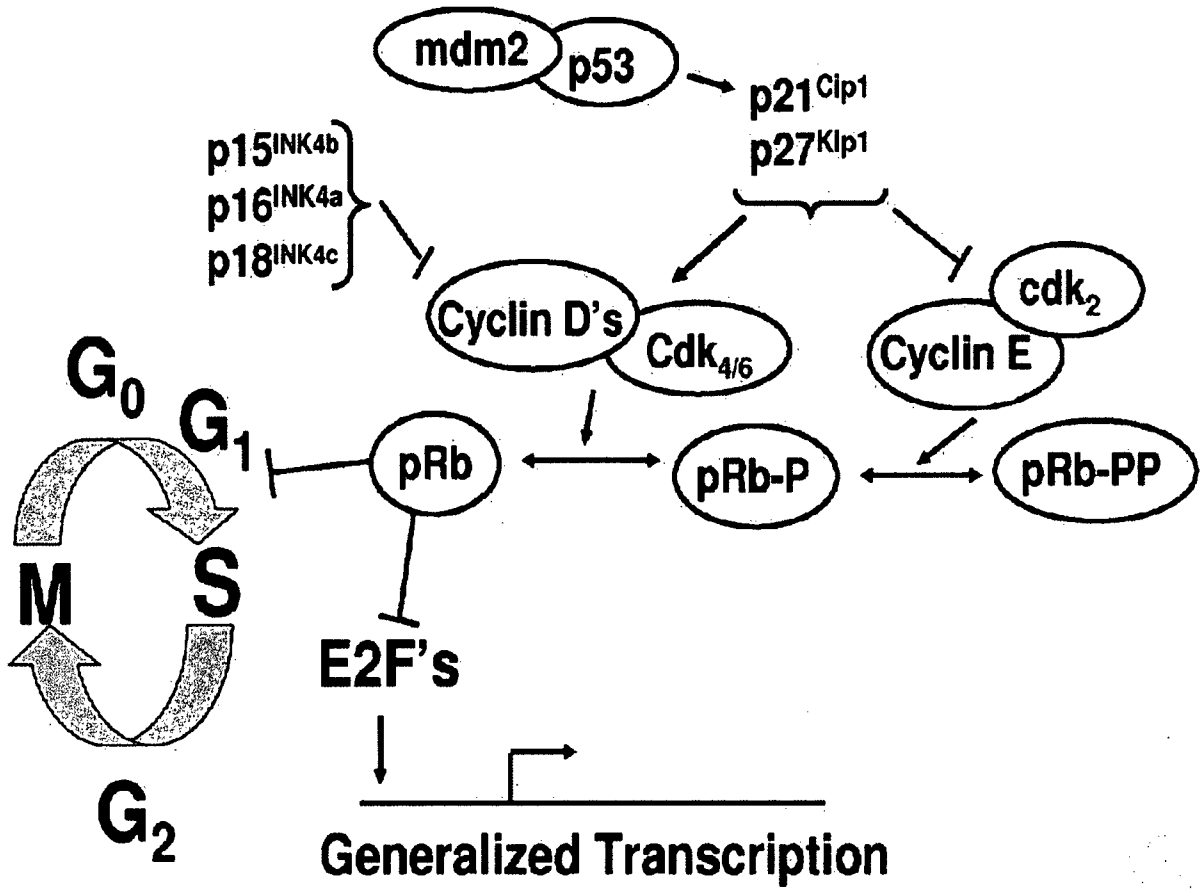


Fig. 1. Cell cycle molecules involved in G1/S phase in β-cells. The cell cycle is highly regulated by inhibitory molecules which act on cyclin and cyclin dependent kinases (cdks). Phosphorylation of retinoblastoma (Rb) results in DNA synthesis and cell cycle progression. (ref. 54)

Cell cycle control in the pancreatic β-cell

Understanding the molecular regulation of pancreatic β-cell replication is of critical importance for the prevention of diabetes and for possible islet replacement therapy. The G1/S checkpoint of the cell cycle has been shown to be highly regulated in islet cells (37, 54-70). Disruption of the β-cell cycle is exemplified in transgenic mice over-expressing the SV40 T-antigen, which increased β-cell proliferation and islet hyperplasia by inactivating p53 and Rb (68). Mice with heterozygous knockout of both Rb and p53 have islet hyperplasia and islet cell tumours (58, 66). However, β-cell specific knockout of Rb did not result in a robust difference in β-cell mass or replication (71), implying a role for other gatekeepers. These experiments showed the importance of the G1/S checkpoint in the pancreatic β-cell and encouraged further studies on the many molecules upstream of Rb, including the cyclin-dependent kinases. Global changes in cdk4 affected only three tissues, the ovaries, testes and pancreatic β-cell (62, 64). In mice with a whole body cdk4 null mutation, hypoplasia of β-cells occurs (64), while mice with a whole body constitutively active cdk4 have β-cell hyperplasia (62). Attention has also been focused on the D type cyclins. It has been demonstrated through germ line ablation of the D-type cyclins that both cyclin D1 and cyclin D2, but not cyclin D3, are required for postnatal β-cell proliferation (37, 61). Cyclin $D1^{-/-}$ mice have a normal β-cell mass whereas cyclin $D2^{-/-}$ mice have a decreased β-cell mass and reduced serum insulin levels, suggesting that cyclin D1 is not required for β-cell development (37, 61). Nevertheless, over-expression of cyclin D1 causes β-cell hyperplasia (67) and over-expression of both cyclin D1 and cdk4 increases phosphorylation of Rb with a 10-fold increase in both rat and human β-cell replication (55).

Other important regulators of the G1 phase in β-cells are the INK and CIP kinases. Initial studies showed a prominent role for p21 in maintaining β-cell cycle arrest when exposed to mitogenic stimuli *in vitro* (56). However, subsequent *in vivo* studies showed that mice with a p21 null mutation have no apparent change in islet mass or β-cell replication, even in the presence of additional mitogenic stimuli (69). Nevertheless, an increase in β-cell mass and proliferation was seen in mice with the p27 gene deleted (65). p27 was found to be critical during development and neonatal period for allowing quiescent cells to proliferate (70), not for maintenance of adult β-cell mass (63). Furthermore, expression of p16 in islets increases with age and diminishes the regenerative capacities of the islet (72). Studies on β-cell cycle molecules illustrate the

importance of β-cell cycle regulation in the control of islet mass and glucose homeostasis. The gaps in these studies also illustrate the need to further elucidate the complex mechanisms regulating β-cell proliferation in normal physiological conditions, diabetes progression and diabetes treatments. Moreover, these studies generally do not address the external cues that trigger β-cell mitogenesis.

Insulin and the insulin receptor signalling pathway

Insulin is both an anabolic hormone that regulates glucose metabolism and a growth factor. This 5.8 kDa protein was discovered in 1922 by Frederick Banting, Charles Best, James Macleod and James Collip. In pancreatic β-cells, the insulin mRNA transcript codes for an inactive protein called preproinsulin (73). Once inside the ER, preproinsulin is post-translationally modified into the proinsulin precursor molecule. Proinsulin is then cleaved by the proteolytic enzymes prohormone convertases (PC1/3 and PC2), and carboxypeptidase E (CPE) (73). This cleavage removes the middle portion of proinsulin, C-peptide. The C- and N-terminal ends (the A- and B- chains) are bound together by disulfide bonds to form the final 51 amino acid form of insulin (74). Insulin is then packaged into secretory granules, which are stored in the cytoplasm awaiting a signal to be released into the circulation via exocytosis.

Insulin secretion is triggered by an increase in blood glucose and, to varying degrees, other nutrients. After a meal, glucose is absorbed into the β-cell via plasma membrane type 2 glucose transporters (GLUT2) (75). Metabolism of glucose causes an increase in adenosine tri-phosphate (ATP) concentration (76). The rise in ATP/ADP ratio causes ATP-gated potassium channels to close, depolarizing the cell membrane. L-type voltage-gated calcium channels are then activated, transporting calcium ions into the cell. The increase in cytosolic calcium concentration triggers the insulin-containing secretory granules to be exocytosed (76). Zinc is also present inside these secretory granules, which facilitates the formation of insulin into an insoluble microcrystal hexamer structure (74). When the insoluble insulin is released from the granule into the bloodstream, it faces a change in pH from approximately 5.5 to 7.4. This change disrupts hydrogen bonds and converts insulin into a soluble monomer structure that can bind its receptors (74).

Insulin secretion occurs in a pulsatile fashion and in two phases (77). The first phase of insulin secretion is caused by the immediate release of insulin from the pre-packaged secretory granules. The second phase of insulin secretion occurs 5-15 minutes later and accounts for a combination of newly synthesized and pre-packaged insulin. Insulin secretion is strongly inhibited by somatostatin, which is secreted within the islet by δ-cells. Somatostatin binds to its G-protein coupled receptor on the β-cell leading to a reduction in cytosolic calcium concentration by opening the ATP-gated potassium channel and closing the L-type voltage calcium channel on the surface of the β-cell (78).

Insulin is first released into the portal circulation where it acts on the liver to increase glycogenesis and to decrease gluconeogenesis and glycogenolysis (79). Once in general circulation, insulin acts on muscle to increase glycogenesis and glucose uptake via increased concentrations and cell surface expression of GLUT4 (79). Adipose cells are also targeted by insulin, resulting in decreased lipolysis and increased fatty acid synthesis (79). The actions of insulin are countered by the hormone glucagon, which is secreted by pancreatic α-cells and acts to increase blood glucose between meals.

The actions of insulin are rapid, as it has a short half-life of only minutes and circulates in picomolar concentrations (80). Insulin actions are mediated by the binding of insulin to its receptor. The insulin receptor can be found on almost all tissues, but has a significantly higher number on adipose and liver cells (81). The insulin receptor is made up of two chains consisting of an extracellular α-subunit (ligand binding) and an intracellular β-subunit (tyrosine kinase activity), which are linked together by disulfide bonds. These disulfide bonds along with the cysteine-rich motifs in its extracellular α-subunit, classify the insulin receptor as a class II tyrosine kinase receptor (82). The insulin receptor has a dissociation constant (K_d) of between 0.2 to 1 nM (83). Unlike most hormone receptors, the insulin receptor binding affinity for its ligand is bell-shaped due to a process called negative cooperativity (i.e. affinity is lost when insulin concentrations are too high) (84). IGF-1 receptors have a K_d similar to the insulin receptor but with much lower binding affinity for insulin molecules (83). Therefore, only super-physiological concentrations of insulin (approximately 50 nM) can activate IGF-1 receptors (85, 86). The cross-reactivity between insulin and IGF-1 receptors makes it difficult to distinguish between the roles of each receptor when non-physiological doses of each ligand are used. While insulin's role in

regulating blood glucose is clearly defined, it's 'IGF-1-like' growth factor effects are less well understood.

Insulin binding to the extracellular domain of the insulin receptor initiates the activation of many downstream pathways. These signal transduction mechanisms are similar to those employed by the IGF-1 receptor. Upon insulin binding, the intracellular tyrosine kinase activity of the insulin receptor is activated causing autophosphorylation and phosphorylation of insulin receptor substrates (IRS1-4) and SH2-containing protein (Shc). IRS can activate two main signalling pathways; the phosphatidylinositol-3 kinase (PI3-K) and/or the mitogen-activated protein kinase (MAPK) pathways (see Fig. 2). These two main insulin signalling pathways are known to stimulate growth in various cell types; however, their role in β-cell proliferation remains unclear.

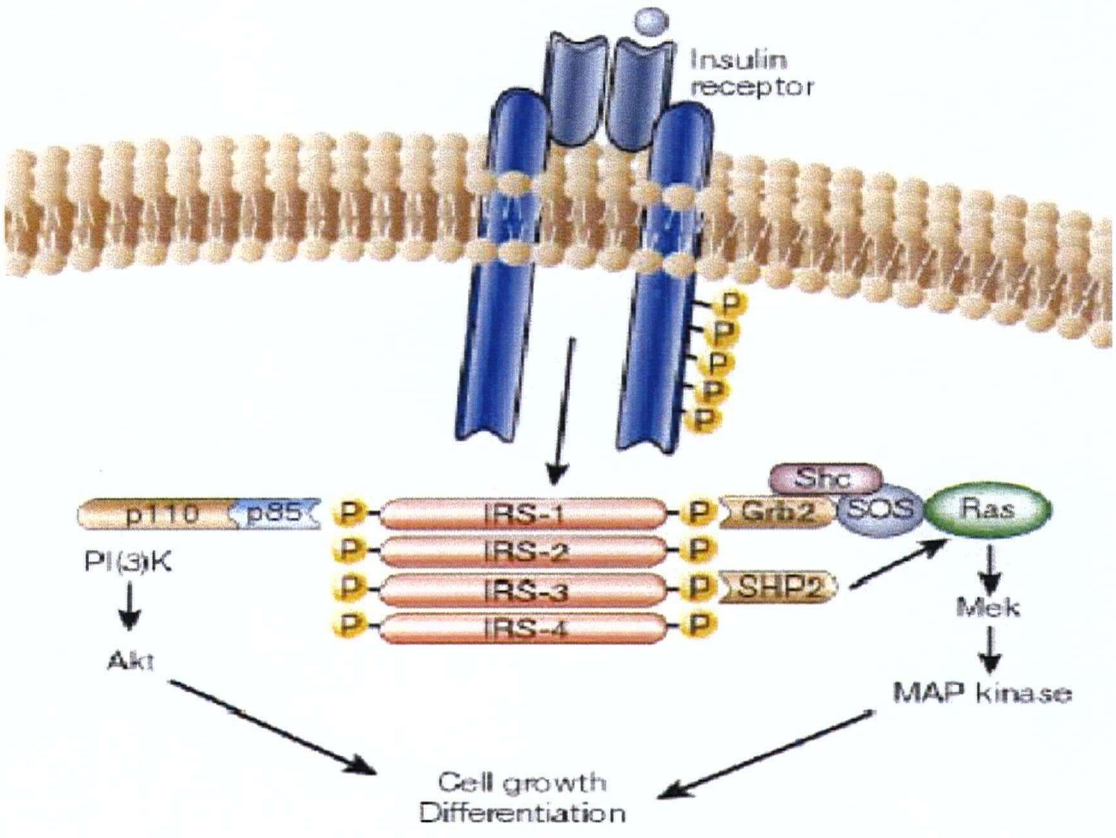


Fig. 2. Schematic of the major components of the insulin receptor pathway. Insulin receptor activation results in autophosphorylation of the receptor and activation of insulin receptor substrates (IRS). PI3-kinase binds to IRS proteins, leading to activation of Akt. Through growth factor receptor-bound protein-2 (Grb2) and Son of Sevenless (SOS), the Ras/Raf pathway is activated. Both arms of the insulin receptor pathway lead to cell survival. http://hmgc.mcw.edu/laboratories/olivier/olivierproject.html

The arm of the insulin receptor signalling pathway that has received the most attention in the β-cell is the PI3-kinase pathway. Activated IRS recruits the p85 subunit of PI3-kinase, leading to increased PI3-kinase activity (87). PI3-kinase causes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) from phosphatidylinositol bisphosphate (PIP_2). The formation of PIP_3 recruits proteins with a PH (pleckstrin-homology) domain to the membrane where they become activated, as such with phosphoinositde-dependent kinase-1 (PDK-1). PDK-1 activates the serine-threonine Akt by phosphorylation. Many of the downstream substrates of Akt are involved in cell survival and growth, including GSK-3, mTOR and Foxo1 (87). On the other hand, independent of the PI3-kinase pathway, IRS activation leads to the recruitment of the growth factor receptor-bound protein-2 (Grb2) protein. Grb2 recruits the mammalian Son of Sevenless (mSOS) which acts as a guanine exchange factor for Ras. Activation of the serine-threonine kinase Raf-1 by Ras leads to phosphorylation of MAP/Erk kinase (MEK). MEK then phosphorylates ERK1/2 leading to such events as cell proliferation (87). Therefore, the multiple signalling components employed by insulin are thought to play important roles in insulin's effect on β-cell survival both *in vivo* and *in vitro* (88-99).

Regulation of β-cell function and mass by insulin signalling

Over the past several years, a series of studies using mice with tissue-specific knockout of the insulin receptor in muscle, liver, fat, brain and other sites have sought to determine which tissues are critical targets of insulin (100, 101). Surprisingly, the β-cells themselves were among the most important tissues in these experiments (100, 101). Although there has been interest in the possible autocrine/paracrine effects of insulin for over 30 years, these studies dramatically increased attention in this area of investigation. For example, several investigators proposed that insulin may have an autocrine role in its own synthesis and secretion. Although controversial, a series of studies proposed that insulin increases insulin gene expression (102-105). However, others have found that insulin has little or no effect on its own insulin gene expression (106). While, long term studies showed the insulin receptor is required for glucose-stimulated insulin secretion (103, 104, 107-110), short term insulin exposure decreased or had no effect on insulin

secretion in human β-cells (102, 111). Further examination is needed to fully understand the effects of insulin its own secretion and synthesis.

A prominent role for insulin and its receptor pathway in regulating β-cell mass is becoming more apparent with recent experiments. Mice lacking insulin receptors on their pancreatic β-cells (βIRKO) exhibit an age-dependent decrease in β-cell mass and several similarities to human diabetes (108, 109). In contrast, β-cell specific IGF-1 receptor knockout mice do not have a decease in β-cell mass (112). Compound knockouts of β-cell IGF-1 and insulin receptors demonstrated that, between these two growth factors, insulin plays the dominant role in regulating β-cell mass in adult mice (110). It was also demonstrated that βIRKO mice fed a high fat diet failed to increase β-cell mass and had a lower proliferation rate than their wildtype counterparts (113). This suggests that the insulin receptor plays an essential role in the β-cell compensation to dietary stress. Experiments in MIN6 cells with 80% knockdown of the insulin receptor showed a decrease in β-cell replication (103). On the other hand, mice with increased expression of a mutant insulin receptor were noted to have more β-cell proliferation (114), although IRS-2 phosphorylation was unaffected. However, complete deletion of both insulin genes in mice caused no change in pancreas development (115). These studies suggest that compensation may be occurring through alternate growth pathways. *In vitro*, super-physiological doses of insulin increased MIN6 cell proliferation (116) and cell viability of human islets via Akt (117). However, the effects of physiological doses of insulin on β-cell proliferation have yet to be elucidated. It is important to note that β-cell proliferation can be stimulated by many growth factors, including prolactin, placental lactogen, hepatocyte growth factor, parathyroid hormone-related protein, GLP-1/exendin-4, IGF-1 and insulin (118-129). Although exogenous treatment with these growth factors is sufficient to increase β-cell replication, insulin is relatively unique in that it is released locally in the islets and therefore may have a physiological autocrine/paracrine role in the regulation of β-cell mass.

In addition to β-cell proliferation, apoptosis is one of the key factors determining total β-cell mass. We have previously shown that the insulin signalling pathway has a positive effect on β-cell apoptosis (130). β-cells treated with low concentrations of insulin decreased apoptosis in primary human and mouse islets, but higher concentrations of insulin (200 nM and above) did not have an anti-apoptotic effect (130). The pro-survival effects of insulin were shown to be mediated through Pdx-1 and its binding partner Bridge-1. Bridge-1 has been shown to regulate

β-cell survival (131). In conclusion, studies examining the role of insulin and its receptor have clearly shown an important role for insulin in regulating β-cell mass. This is further strengthened by studies examining downstream molecules of the insulin receptor pathway.

Between whole-body knockout mice lacking insulin receptor substrate-1 (IRS-1) or IRS-2, only IRS-2 knockout mice had such a defect in β-cell mass that they developed type 2 diabetes (132-134), verifying a dominant role of IRS-2 in β-cell proliferation (96). Judging from similar experiments, IRS-3 and IRS-4 do not have a major role in regulating β-cell mass (135, 136). Transgenic mice over-expressing Akt in the β-cell had improved glucose tolerance, hyperinsulinemia and were resistant to streptozotocin-induced diabetes (89, 90). The improved glucose homeostasis was apparently due to an increase in β-cell mass and proliferation, since insulin secretion was not changed with Akt over-expression (89, 90). Surprisingly, mice with 80% reduced islet Akt kinase activity did not have any changes in β-cell mass, even after high fat feeding (126, 137). This indicates that basal and compensatory β-cell proliferation may involve an alternate pathway, such as the Raf-1/ERK pathway. Hormones, such as GLP-1 and GIP, have been shown to stimulate growth of β-cells through both Akt- and Raf-dependent pathways (126). Over-expression of a Raf-1 inhibitory protein decreased proliferation in the HIT β-cell line (138), however, the importance of Raf-1 in primary β-cell proliferation requires further investigation. Although the significance of the insulin receptor pathway in regulating β-cell mass is acknowledged, the direct effects of physiological doses of insulin on β-cell proliferation have yet to be elucidated.

Effects of insulin on β-cell cycle components

Recent studies have convincingly shown that β-cell proliferation is critical in maintaining an adequate β-cell mass throughout life. What are the endogenous factors that act on islets to regulate this process? Since insulin receptor signalling is thought to regulate β-cell mass, several studies have begun to examine the effects of insulin signalling on cell cycle molecules. MIN6 cells stably expressing siRNA targeting the insulin receptor had significant changes in the expression of many cell cycle regulatory genes, notably cyclin D2, cyclin G2, p18 and p21 (139). In the β-cell, Akt phosphorylates glycogen synthase kinase 3 (GSK-3). This event results in cell cycle activation because GSK-3 normally inhibits cyclin D1, cyclin D2, p21 and cdk4 (57). Since

Akt is downstream of the insulin receptor, this suggests that the insulin-mediated pathway may play a role in regulating the β-cell cycle. Application of these results to the adult pancreas implicates an important role for insulin signalling in regulating cell cycle molecules. In addition, insulin itself has been shown to increase levels of cyclin D1, cyclin E, p21, increase degradation of p27, and increase cyclin D-cdk4 and cyclin E-cdk2 complex formations in a breast cancer cell line (140-142). Insulin has also been shown to increase proliferation of a breast cancer cell line via the MEK/ERK pathway (143). Consequently, the insulin pathway may be intimately involved in mediating cell cycle progression in the β-cell by regulation of cell cycle proteins.

Effects of glucose on β-cell proliferation

The presence of glucose is the main stimulus for insulin secretion from the β-cell. Several groups have also suggested that glucose is also the major stimulus for increased β-cell mass during times of high demand (144-146). Distinguishing between the roles of glucose and insulin on β-cell mass is difficult, especially *in vivo*, since elevated glucose increases insulin secretion. *In vivo* glucose infusion studies on rats showed that high glucose increased β-cell mass and proliferation (92, 144-146) and had anti-apoptotic effects (92, 144, 147). *In vitro* experiments using MIN6 and mouse islet cells revealed that the pro-survival effects of high glucose were dependent on PI3-kinase and Akt, but not MAPK (92). Several groups have suggested that the effects of glucose are independent of secreted insulin. For example, glucose activates the Ras/MAP-kinase arm of the insulin pathway, possibly through phosphorylation of Raf-1, by increasing intracellular calcium and activating PKA (106, 148-153). However, these studies were not carried out in primary cells. A recent study found an increase in β-cell proliferation *in vivo* due to hyperglycemia and hyperinsulinemia via increased cyclin D2 abundance and cellular localization, implicating both insulin and glucose as potential growth factors (154). In addition, MIN6 cells with an 80% knockdown of the insulin receptor showed that the effects of glucose on Akt phosphorylation and the expression of a large percentage of genes required the insulin receptor (103). In conclusion, it has been difficult to demonstrate that the effects of glucose on β-cell proliferation do not involve secreted insulin.

Significance of β-cell proliferation in diabetes

A loss of β-cell mass is critical to the pathogenesis of all forms of diabetes. An understanding of what triggers β-cell proliferation may shed light on treatment options designed to increase β-cell mass. Further comprehension of the mechanisms involved in β-cell replication will be useful for a variety of reasons. For example, understanding the signalling pathways involved in β-cell proliferation will enhance our knowledge of possible mechanisms underlying the pathogenesis of diabetes. In addition, having the ability to generate a replenishable source of insulin-secreting β-cells would be of significant importance for islet transplantation. Thus, an efficient inducer of β-cell proliferation may benefit diabetes treatment options. The present studies will influence our understanding of how β-cells proliferate, which may guide our efforts to treat diabetes.

In the current study, we tested four hypotheses regarding the role of insulin in β-cell proliferation . Our first hypothsis postulated that physiological doses of insulin can increase β-cell proliferation at a rate comparable to serum conditions. We determined the basal rate of proliferation of cultured primary mouse β-cells and found that both physiological and super-physiological doses of insulin can stimulate β-cell division. Our second hypothesis stated that insulin's effects on β-cell proliferation are mediated through the Raf-1/MEK pathway. We found that only physiological doses of insulin require Raf-1 kinase in primary cells. Our third hypothesis states that the effects of elevated glucose on β-cell proliferation are dependent on secreted insulin. We found that β-cell proliferation in the presence of both low and high glucose concentrations likely involves insulin signalling. Together, these results support the concept that insulin is a critical regulator of β-cell proliferation and establishes the mechanism of this effect. Lastly, our fourth hypothesis states that physiological doses of insulin effects β-cell cycle proteins. We attempted to examine the specific β-cell cycle molecules that may be regulated by insulin signalling in the rare population of proliferating β-cells, however no conclusive results were obtained. Our findings have significance for the understanding of β-cell mass alterations in type 1 diabetes, type 2 diabetes and during graft failure in islet transplantation.

MATERIALS AND METHODS

Reagents

Exogenous human recombinant insulin, dissolved in 25 mM HEPES, was purchased from Sigma (St. Louis, MO). HNMPA-AM (hydroxy-2-naphthalenylmethylphosphonic acid), LY294002, Raf-1 Kinase Inhibitor, and Akt Inhibitor VIII were purchased from Calbiochem (La Jolla, CA). The MEK inhibitor U0o126 was purchased from Cell Signalling (Beverly, MA). Somatostatin, and all other reagents were from Sigma (St. Louis, MO). See Table 1 for a list of antibodies used.

Table 1

Antibody (Ab)	Size (kDa)	Manufacturer	Source/Type	1° Ab Dilution	2° Ab Dilution
Western Blot					
CCNB	55	Santa Cruz, CA	Rabbit/Polyclonal	1:1000	1:2000
CCND1	36	Santa Cruz, CA	Rabbit/Polyclonal	1:1000	1:2000
CCNE	45	Santa Cruz, CA	Rabbit/Polyclonal	1:750	1:2000
CCNG2	39	Abcam, MA	Rabbit/Polyclonal	1:10000	1:2000
p15	15	Santa Cruz, CA	Goat/Polyclonal	1:1000	1:2000
p16	16	Santa Cruz, CA	Mouse/Monoclonal	1:1000	1:2000
p21	18	Abcam, MA	Rabbit/Polyclonal	1:1000	1:2000
p27	27	Santa Cruz, CA	Rabbit/Polyclonal	1:750	1:2000
Rb (Ser780)	110	Cell Signalling, MA	Rabbit/Polyclonal	1:1000	1:2000
Rb (4H1)	110	Cell Signalling, MA	Mouse/Monoclonal	1:2000	1:2000
β-actin	43	Novus Biologicals, CO	Mouse/Monoclonal	1:30000	1:6000
Immunofluorescence					
Insulin		Linco, MI	Guinea Pig	1:750	
BrdU		Roche, QC	Mouse/Monoclonal	1:100	
CCNB		Santa Cruz, CA	Rabbit/Polyclonal	1:100	
CCND1		Santa Cruz, CA	Rabbit/Polyclonal	1:100	
CCNE		Santa Cruz, CA	Rabbit/Polyclonal	1:100	
CCNG2		Abcam, MA	Rabbit/Polyclonal	1:100	
p21		Abcam, MA	Rabbit/Polyclonal	1:100	
p27		Santa Cruz, CA	Rabbit/Polyclonal	1:100	
Texas Red		Jackson, PA	Rabbit α Guinea Pig		1:200
Texas Red		Jackson, PA	Goat α Guinea Pig		1:200
FITC		Jackson, PA	Goat α Mouse		1:200

Pancreatic islet isolation

Islets were isolated from six to ten week-old male C57Bl6/J mice (The Jackson Laboratory, Bar Harbour, MA) using collagenase and filtration as follows. Mice were sacrificed using a CO_2 chamber and cervical dislocation. Mouse pancreata were exposed and the bile duct ligated. The pancreas was then inflated via the pancreatic duct with 2-5 mL collagenase (0.375 mg/mL, Sigma Type XI) in Hanks solution (137 mM NaCl, 5.4 mM KCl, 4.2 mM NaH_2PO_4, 4.1 KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, and 5 mM glucose). The pancreas was then removed and placed in a 50 mL conical tube (BD Falcon, Franklin Lakes, NJ) containing 5 mL collagenase in Hanks solution. The pancreas was then incubated for 13 minutes in a 37°C water bath after which ten vigorous shakes took place to homogenous the pancreas in solution. The solution was then washed with 20 mL Hanks solution containing 1 mM $CaCl_2$. The mixture was centrifuged at 1200 rpm for 30 seconds and the supernatant was aspired. This step was repeated twice, replacing Hanks solution with Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Grand Island, NY) on the last resuspension. The new solution was filtered using a pre-wet 70 μm nylon filter (BD Biosciences, Franklin Lakes, NJ) and the captured islets were transferred into a 35x10 mm Nunc suspension dish (Nalge, Rochester, NY) containing complete RPMI 1640. The islets were then hand picked and cultured overnight at 37°C 95% O_2 and 5% CO_2 in RPMI 1640 media with 11.1 mM glucose. Media was supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY). Fetal bovine serum (10%; Gibco, Grand Island, NY) was added when indicated. Where applicable, islets were treated as described.

MIN6 cell culture

MIN6 (mouse insulinoma) cells were obtained from Dr. Timothy Kieffer's laboratory (Life Sciences Centre, UBC) under Material Transfer Agreement from Dr. Jun-ichi Miyazaki (Osaka, Japan). MIN6 cells were originally derived from transgenic mouse insulinomas as previously described (155). MIN6 cells were cultured in DMEM (Gibco, Grand Island, NY) containing 25 mM glucose, 10% fetal bovine serum (Gibco, Grand Island, NY) and 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY). MIN6 cells were passaged every 2-3 days using 0.5% Trypsin-EDTA (Gibco, Grand Island, NY) to maintain an overall confluency of 70-80%. For Western blot assays, MIN6 cells were seeded in 12 well culture

plates (Nunc, Rochester NY) 24 hours before treatment to ensure a final treatment confluency of 80%. For immunofluorescent assays, MIN6 were seeded onto coverslips and allowed to adhere overnight.

Human islets

Human islets were generously provided by Dr. Garth Warnock (Ike Barber Transplantation Unit, Department of Surgery, Vancouver General Hospital, BC) as part of the MSFHR-funded Center for Human Islet Transplant and β-cell Regeneration. Islets were isolated from both men and women ranging from 40-70 years of age with no history of diabetes. Human islets were cultured in 11.1 mM glucose RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 1100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY) and 10% fetal bovine serum (Gibco, Grand Island, NY). Cells were cultured at 37°C and 5% CO_2 in 35x10 mm Nunc suspension dishes (Nunc, Rochester NY) until treatment. Approximately 1,000 islets were transferred to each suspension dish for treatment on the same day they were received.

Islet dispersion

Islet dispersion was carried out by means of four consecutive washes with Ca^{2+}/Mg^{2+}-free MEM (Mediatech, Herndon, VA) followed by a gentle repetitive pipetting in the presence of a trypsin-EDTA solution (Gibco, Grand Island, NY) diluted 1:5 in MEM. Afterwards, the cells were washed with Ca^{2+}/Mg^{2+}-free MEM, and then transferred to glass coverslips in completed RPMI 1640 media (Gibco, Grand Island, NY). Following dispersion, the cells were allowed to adhere to the coverslips for 3 hours before the addition of media. The cells were allowed to adhere further by culturing overnight at 37°C and 5% CO_2.

Immunofluorescence staining

For experiments using exogenous insulin, primary dispersed islet cells were washed three times with serum free RPMI media before treatment as indicated in each experiment. For

proliferation studies, 10 μM BrdU (5-bromo-2-deoxyuridine) labeling medium (Roche, Laval, QC) was added to the media of each treatment. Primary cells were incubated in treatment media for three days before immunofluorescence analyses. All experiments were done in 5 mM glucose unless otherwise stated to reduce endogenous insulin secretion. MIN6 cells were treated as indicated for 6 hours and 10 μM BrdU labeling medium was added 2 hours before cells were fixed. Time points were tested to determine an optimal window for proliferation studies.

Following treatment, dispersed cells on coverslips were washed three times with PBS then fixed with an ethanol fixative (30 mL 50mM glycin, 70 mL ethanol) for 20 minutes at -20°C. Coverslips were then washed three times with PBS before co-incubation of primary antibody (see Table 1) for 40 min at 37°C. Primary antibodies are diluted accordingly (see Table 1) with Dakocytomation Antibody Diluent (Dako, Carpinteria, CA). For proliferation studies, primary antibodies were incubated separately to prevent cross-binding of the antibodies. An extra coverslip with dispersed cells attached was incubated with Dakocytomation Antibody Diluent alone as a negative control where the only fluorescence should be cellular autofluorescence (which is considerable in β-cells). All coverslips were washed three times with PBS before incubation with the proper secondary antibody (see Table 1) for 45 minutes in a dark humid chamber at 37°C. Coverslips were then washed three times with PBS and mounted on slides in VECTASHIELD Mounting Medium for Fluorescence with DAPI (Vector Labs, Burlingame, CA) and sealed with clear nail polish. Slides were kept overnight at 4°C and then cells were visualized on an Axiovert 200M inverted microscope (Zeiss, Thornwood NY) with a 10X and an oil immersion 100X objective equipped with filters for Cyanine (Cy3; typically used to visualize insulin), fluorescein (FITC; typically used to visualize the protein of interest), and 4',6-diamidino-2 phenylindole (DAPI) to visualize nuclei. Images were captured using a Coolsnap HQII CCD camera (Photometric, Tucson AZ) and were analyzed/quantified using SlideBook software (Intelligent Imaging Innovations, Boulder, CO). For proliferation studies, the total number of cells in each image was quantified by masking all nuclei (DAPI staining) as an individual object with Slidebook software. Masking DAPI allowed all cells to be calculated, however, cell clusters were masked as one object. Therefore, to obtain the total number of cells, the total object area of masked DAPI was calculated and divided by the area of an average cell to get an estimate of the number of cells in each image. In addition, cells co-stained for both insulin and BrdU were manually counted to ensure β-cell proliferation.

Immunoblot analysis

Primary islets were hand picked into a siliconized 1.5 mL tube, centrifuged and washed once with PBS. The islets were then placed into 35x10 mm Nunc suspension dishes (Nunc, Rochester NY) containing treatment media. Islets were cultured for either 24 or 48 hours. MIN6 cells were seeded in 12 well culture plates (BD Falcon, Franklin Lakes, NJ) overnight to ensure final confluency of approximately 80%. The cells were then treated with insulin alone or in combination with a drug for 6 hours.

Post treatment, MIN6 cells were washed twice with ice cold phosphate buffered saline (PBS) and lysed with 80 μL/well of cell lysis buffer (Cell Signalling, Danvers MA) supplemented with 1% phenylmethanesulphonylfluoride (PMSF; Cell Signalling, Danvers MA). Cell lysates were then collected into 1.5 mL tubes. Post treatment, human or mouse islets were individually transferred into 1.5 mL tubes for centrifugation. The media was aspirated and 50 μL/tube of cell lysis buffer supplemented with 1% PMSF was added to the tube.

Cell lysates were then subjected to cellular fragmentation by rapidly freezing and thawing the lysates three times and centrifuging for 15 minutes at 15000 rpm at 4°C. Total protein content in lysates was measured using a biocinchoninic acid (BCA) protein assay (Pierce, Rockford IL) with a BSA (bovine serum albumin) standard solution (Pierce, Rockford IL). Total protein (15-30 μg) was mixed with an appropriate volume of sample buffer containing sodium dodecyl sulphate (SDS; Cell Signalling, Danvers MA) and heated to 95°C for 5 minutes, placed on ice for 5 minutes and centrifuged at 12000 rpm for 1 minute. The total volume of each sample was loaded into the wells of a 12% SDS polyacrylamide gel along with 10 μL of the SM0671 PageRuler™ Pre-stained Protein Ladder (Fermentas, Burlington ON). Gels were run at 120V for 60-90 minutes in an SDS supplemented running buffer system. Gels were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-rad, Hercules CA) (activated with methanol) using a Trans-blot SD Semi-dry Cell (Bio-rad, Hercules CA) at 22V for 85 minutes. Membranes were washed with 0.2% w/w I-block (Tropix, Bedford MA) in PBS supplemented with 0.1% Tween-20 (Sigma-Aldrich, Oakville ON) for 30 minutes. Primary antibodies were diluted in 4 mL of 0.2% w/w I-block in PBS supplemented with 0.1% Tween-20 and incubated along with the membrane for two hours at room temperature or overnight at 4°C with gentle shaking.

Primary antibodies with dilutions used can be seen in Table 1. Membranes were washed three times for 6 minutes in a PBS solution supplemented with 0.1% Tween-20. Membranes were then incubated in horseradish peroxidase IgG secondary antibody (as seen in Table 1) diluted in 4 mL of 0.2% w/w I-block in PBS supplemented with 0.1% Tween-20 for 90 minutes at room temperature. Membranes were washed three times for 10 minutes in a PBS solution supplemented with 0.1% Tween-20. A chemiluminescent substrate solution (Amersham, Piscataway NJ) was added to the membranes in a 1:1 ratio of reagent 1 and reagent 2 and incubated with gentle shaking for 5 minutes. Membranes were then exposed to Blue Lite Autoradiography Film (ISC Bioexpress, Kaysville UT) for an adequate amount of time to ensure a gray band. Films were developed, scanned and band intensities were analyzed by densitometry Image J (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2004). Band intensities for the proteins of interest were normalized to the respective β-actin lane loading control band intensities. The ratios of the normalized intensities for the treated samples to the normalized intensities for the control samples were taken yielding the relative intensities with respect to the control.

Gene expression analysis

Primary mouse islets were treated with exogenous insulin for 48 hours before being collected for RNA extraction. Gene expression experiments using pharmacological inhibitors were carried out for 24 hours before collection of the islets. Total RNA was isolated from primary mouse islets using a RNeasy kit (Qiagen, Mississauga, ON) according to the manufacturers protocol. Following RNA isolation, cDNA was reverse transcribed using Superscript III (Invitrogen, Burlington, ON), DNTP's (Invitrogen, Burlington, ON) and Taq polymerase (Fermentas, Hanover, MD). Semi-quantative PCR amplification was carried out for: 1 cycle of 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec; and 1 cycle of 72°C for 7 min. Primer sequences used for Cyclin A, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin G1, Cyclin G2 and p21 were supplied by Integrated DNA Technologies and can be seen in Table 2. Cycle tests were performed on the primers to determine optimal primer annealing and amplification of gene products, as well as to ensure the amplification used was in the linear range.

Table 2

Gene	Forward Sequence	Reverse Sequence	Size (bp's)
CCNA1	ATC GCC CAG ACA GAG AAG AA	ATC GGA TTG CTG TGA TCT CC	244
CCND1	GGC ACC TGG ATT GTT CTG TT	CAG CTT GCT AGG GAA CTT GG	231
CCND2	CTG AGT CTG GTT GGT GCT GA	ACA CCC GAG ACC ACA GAA AC	238
CCND3	GCT CCA ACC TTC TCA GTT GC	AGC TAA GCA GCA AGC AAA GC	208
CCNG1	AGG TCT GCG GCT TGA AAC TA	TCA GTC CAA CAC ACC CAA GA	209
CCNG2	GCA CCT GTG TGA AAG CAG AA	CCA TCA CCA CAC AGA ATT GC	213
p21	GCC TTA GCC CTC ACT CTG TG	AGG GCC CTA CCG TCC TACT A	179
β-actin	TGC GTG ACA TCA AAG AGA AG	GAT GCC ACA GGA TTC CAT A	495

Insulin secretion

To assess the relative contribution of endogenous and exogenous insulin in our cultures, samples were taken from the media of dispersed cell cultures every 24 hours starting at day zero. Insulin was measured using radioimmunoassay (Rat Insulin RIA Kit, Linco Research, St. Charles, MO, USA). Samples taken from cultures treated with 20 nM and 200 nM insulin were diluted by 10 and 100, respectively to ensure concentrations fall within the standard curve.

Statistics

At least 3 independent mouse islet isolations were used for each experiment. Results are presented as means ± SEM. Data were analyzed by Student's t-test or ANOVA (Analysis of Variance) with the post hoc LSD (Lease Significant Difference) when comparing groups. ANOVA statistics were carried out using SPSS (Statistical Package for the Social Sciences). Statistical significance was noted when P values were <0.05.

RESULTS

Measurement of the β-cell proliferation rate in vitro

The proliferation rate of rarely dividing primary β-cells is difficult to estimate. To establish the baseline rate of mitogenesis in dispersed C57Bl6 mouse β-cells, islet cultures were triple-labeled with insulin, BrdU and DAPI (Fig. 3A,B). Under these baseline conditions, primary mouse β-cells replicated at a very slow rate of 0.16 ± 0.03 percent per day. By contrast, the proliferation rate of the MIN6 β-cell line was calculated to be 283.1 ± 33.9 percent per day after adjusting for the length of BrdU exposure (Fig. 3C,D). Thus, although it is extremely slow, the replication of primary mouse β-cells can be reliably measured in our cultures using BrdU and insulin staining. Efforts to perform similar analysis on human islet cells were hampered by the low relative β-cell number in these preparations and the overgrowth of rapidly-dividing fibroblast-like cells capable of taking up stainable insulin (19).

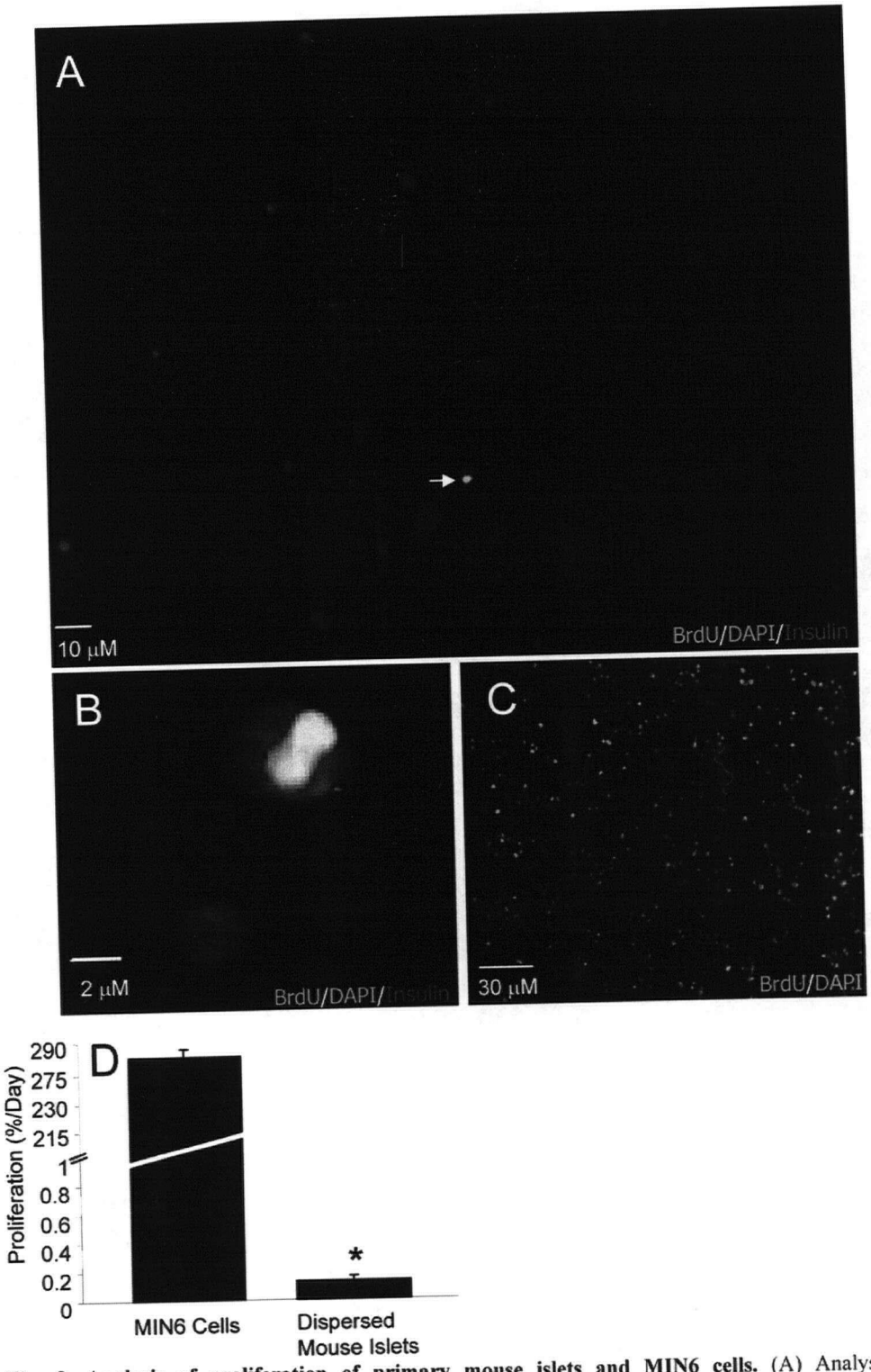


Fig. 3. Analysis of proliferation of primary mouse islets and MIN6 cells. (A) Analysis of proliferation in cultures of dispersed primary mouse islet cells under basal (serum-containing) conditions. BrdU was added to cultures for 3 days and is identified with green staining. Cells were co-stained with insulin (red) and DAPI (blue) to identify nuclei. (B) Magnified image of β-cells that have proliferated. (C) MIN6 cells; BrdU (green) was added for 2 hours. Cells were stained with DAPI (blue). (D) Quantification of proliferation of MIN6 cells (n = 6 experiments) and primary mouse islets (n = 15 experiments). *P < 0.05 MIN6 cells versus primary mouse islets.

Insulin stimulates primary β-cell proliferation

High levels of insulin are mitogenic to many cell types, but the effects of physiological (picomolar) insulin doses on primary β-cell replication are unclear. Previous studies have established that physiological doses of insulin can initiate specific signalling cascades in primary β-cells (102, 130) and multiple studies have implicated insulin receptor signalling in the control of β-cell mass (103, 108-110, 113, 116). To assess the direct effects of insulin on β-cell proliferation, dispersed islet cells were treated with a range of insulin doses for three days and β-cell proliferation was quantified using BrdU incorporation. Over 25,000 cells per group were counted throughout these experiments and only cells clearly co-stained with insulin and BrdU were considered in the analysis of β-cell proliferation (e.g. Fig. 3A, B). There was a significant increase in β-cell proliferation in serum-containing cultures, compared with serum-free controls (Fig. 4A). More notably, β-cells treated with the physiological dose of insulin (200 pM) had a significantly higher rate of replication compared with serum-free control cultures (Fig. 4). β-cells treated with insulin concentration of 2 nM had a more modest rate of replication that did not achieve statistical significance despite a high number of replicate experiments (n = 15). Proliferation was significantly increased in β-cells treated with 20 nM and 200 nM insulin (Fig. 4A), concentrations of insulin that have not been measured *in vivo* and doses that likely act primarily through activation of IGF-1 receptors (86). Together, these results suggest that β-cell replication can be stimulated with physiological levels of insulin.

In these experiments, care was taken to reduce the contribution of endogenous insulin in the media. For example, cells (~2,500 per coverslip) were cultured in a relatively large volume of media (3 ml). To determine the relative levels of endogenous and exogenous insulin in our culture medium, insulin was measured at the start of the experiment and every day thereafter. The first measurement should estimate only the exogenous insulin applied since these cultures had been washed thoroughly, whereas the subsequent measurements should reveal any insulin accumulation in the media over the culture period. As expected, insulin accumulation in the media only increased proportionally to the exogenous insulin added. Under these culture conditions, our results show that the majority of the ambient insulin is exogenous and that there is relatively little contribution from insulin release, even at very low insulin doses (0.2 nM and 2 nM) (Fig. 4B). We did not observe significant effects of high exogenous insulin on endogenous

insulin release over this time frame. These experiments illustrate our control over the insulin levels in our cultures over time. The ability of picomolar insulin to alter β-cell function is consistent with previous experiments where effects were seen at this dose (102, 130)

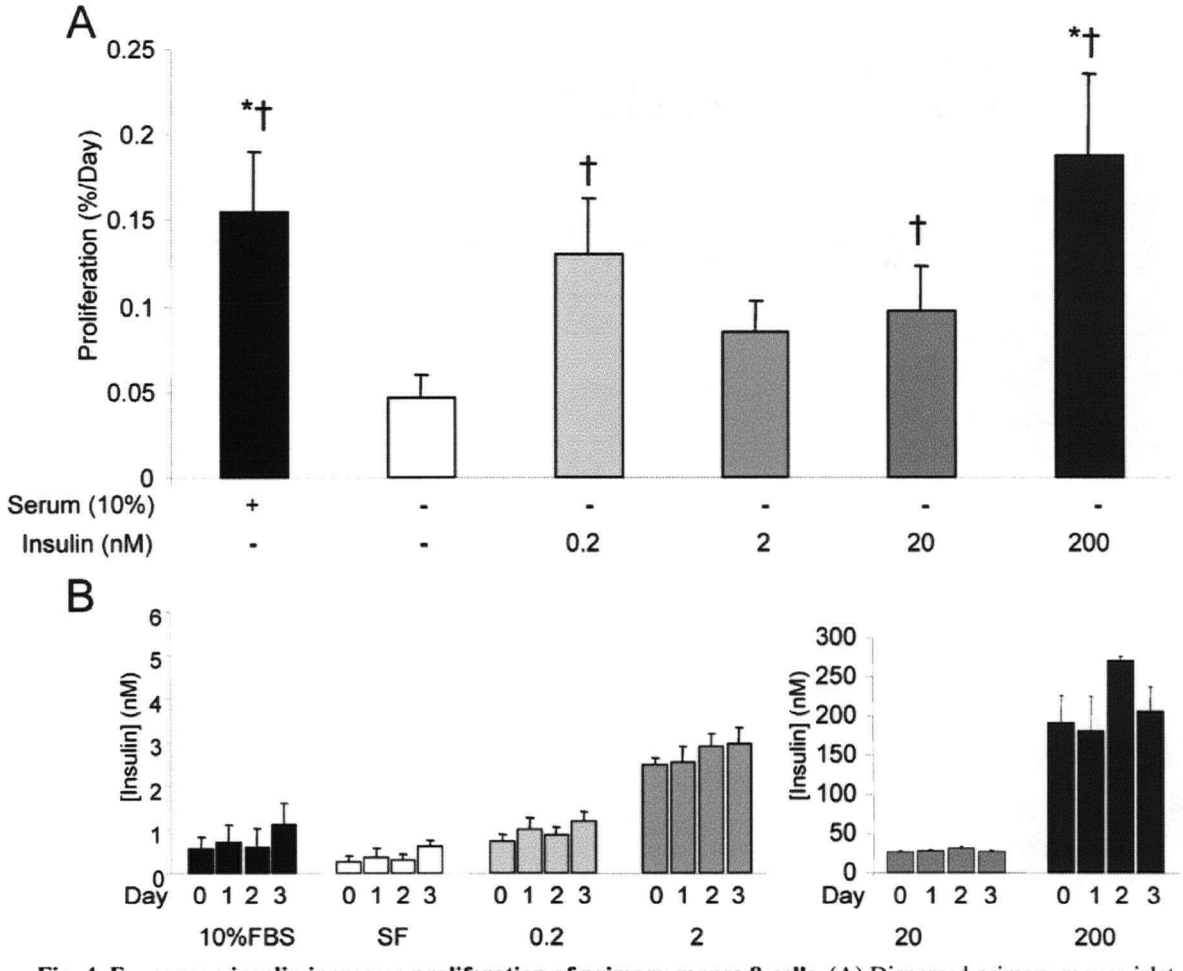


Fig. 4. Exogenous insulin increases proliferation of primary mouse β-cells. (A) Dispersed primary mouse islets treated with various doses of insulin as indicated for 3 days. Serum-containing media was used as a positive control. Proliferation was quantified using immunofluorescent BrdU staining as in Figure 1. (n = 14-16 experiments; >25,000 islet cells studied per group). *P < 0.05 versus serum-free control. †P < 0.05 versus serum-free control when normalized to 10% serum. (B) Media samples were extracted every 24 hours starting at day 0 from culture media to assess insulin secretion by radioimmunoassay (n = 4-13).

Role of autocrine insulin signalling in β-cell proliferation

The results described above demonstrate that exogenous insulin, when applied at specific doses, can induce proliferation in primary β-cells. However, it remained unclear whether endogenous insulin, acting in an autocrine or paracrine manner, contributed to the basal proliferation rate in β-cells. To address this question, tyrosine auto-phosphorylation of the insulin receptor was inhibited pharmacologically using HNMPA-AM. This drug is a specific inhibitor of the insulin receptor at doses below 100 μM, although higher concentrations of HNMPA-AM can inhibit IGF-1 receptor activation (156). The results of this experiment illustrate that insulin receptor (or IGF-1 receptor) activation was required for a significant proportion of the β-cell proliferation seen under basal conditions (Fig. 5). Moreover, the ability of insulin to stimulate β-cell proliferation was lost in HNMPA-AM-treated cells (Fig. 5), confirming a receptor-mediated mechanism. Together, these observations further suggest an important role for insulin signalling in β-cell proliferation.

It has been suggested by others that glucose itself can increase β-cell proliferation (146), although recent studies have indicated a dominant role for insulin receptor signalling in the effects of high glucose on β-cell function (103). Our *in vitro* system, where glucose and insulin levels are better controlled compared to the *in vivo* situation, provided an ideal model to test this hypothesis. Dispersed mouse islet cells were cultured in 5 mM glucose or 15 mM glucose, in both serum containing and serum-free conditions. β-cell proliferation was not significantly increased with high glucose culture, although a trend towards a modest increase was observed (Fig. 6A). To test whether the putative effects of glucose could be attributed to autocrine/paracrine insulin signalling, 1 μM somatostatin was added to these cultures to effectively block endogenous insulin release (Fig. 6B). Somatostatin caused a significant reduction in β-cell proliferation in cells incubated in 15 mM glucose and serum. A similar, though non-significant, trend was observed in high glucose without serum. Somatostatin also appeared to reduce proliferation at basal (5 mM) glucose, although this was also not statistically significant. Therefore, any putative effects of glucose can be explained by stimulated insulin release and autocrine/paracrine insulin signalling. Together with the findings using HNMPA-

AM, these results are consistent with a role for basal insulin secretion and paracrine insulin action in the control of β-cell proliferation.

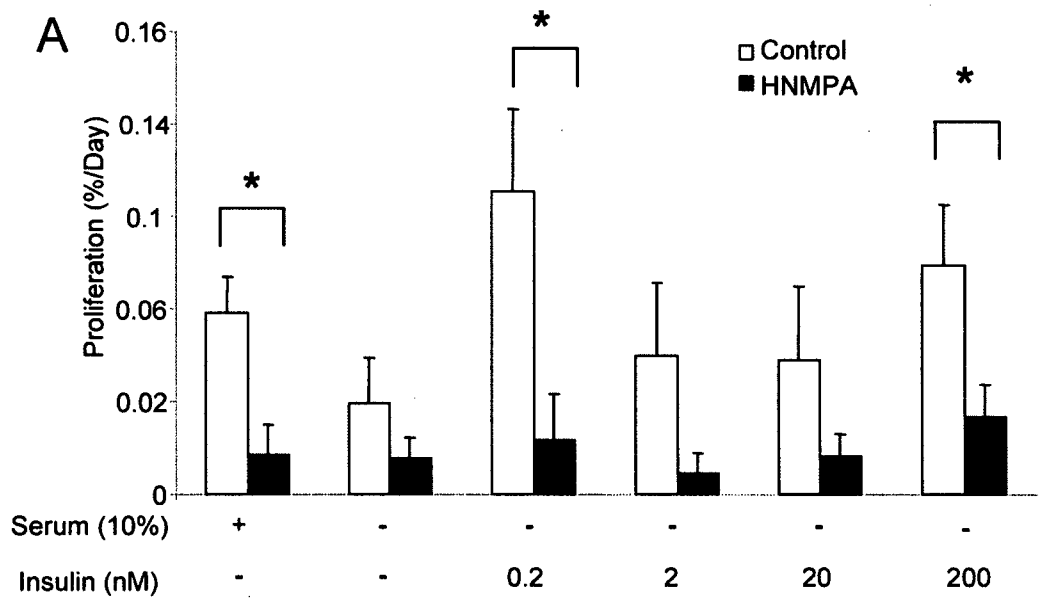


Fig. 5. Insulin receptor inhibition decreases β-cell proliferation. (A) Dispersed mouse islets were cultured with or without insulin as shown in the absence (white bars) or presence of 100 μM HNMPA-AM (black bars) for three days. $*P < 0.05$ versus control (n = 4-5).

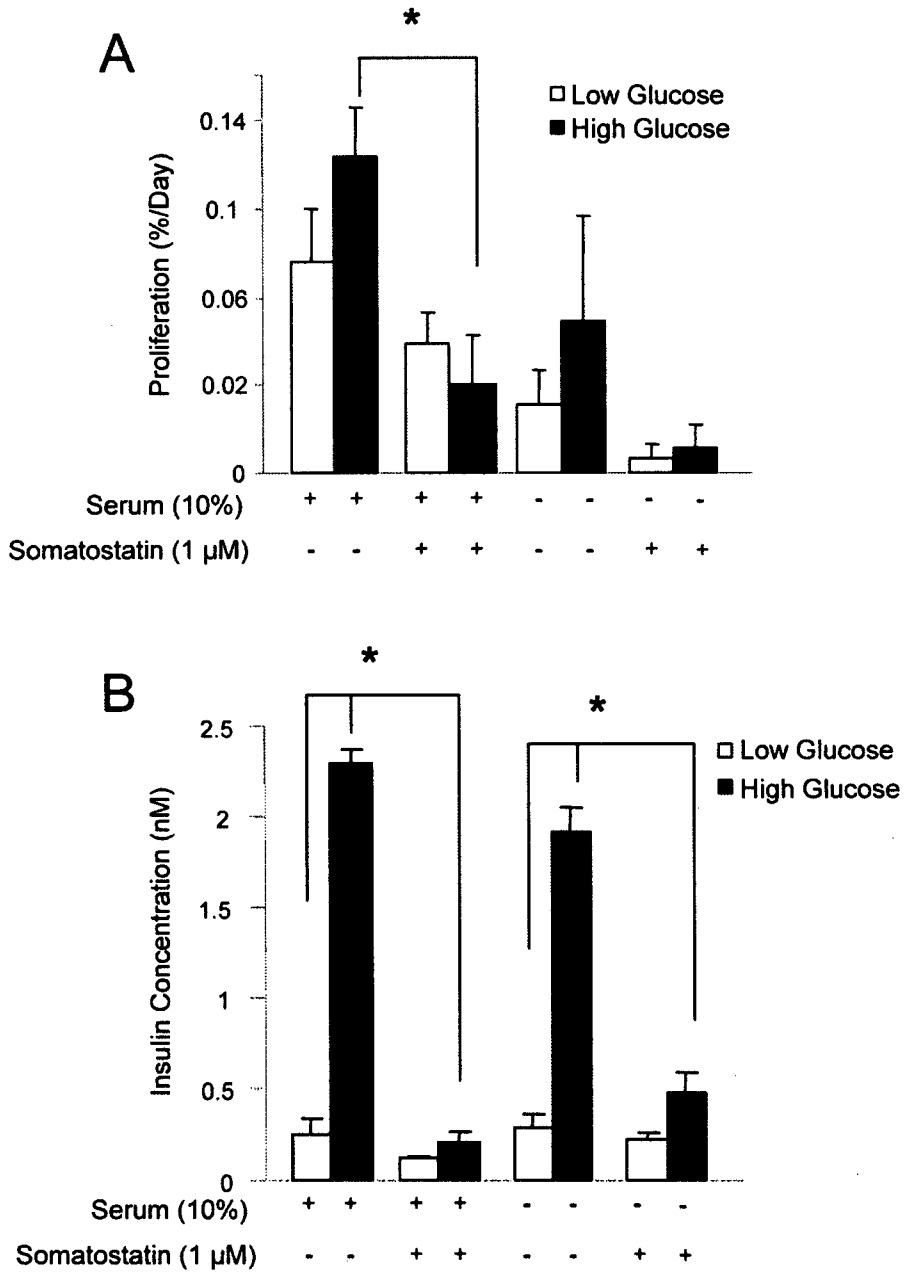


Fig. 6. Effects of elevated glucose on β-cell replication and insulin secretion. (A) Dispersed primary mouse islets treated with 5 mM glucose (white bars) or 15 mM glucose (black bars) for three days with or without 1 μM somatostatin. *P < 0.05 versus serum control (n = 4). (B) Insulin levels were assayed at 24 hours (n = 3).

Mechanisms of insulin-stimulated proliferation of primary β-cells

Previous investigations have focused on specific downstream targets of the insulin receptor in the regulation of β-cell mass, mostly notably IRS-2 (134) and Akt (57, 89, 137, 157). Experiments in a β-cell line showed that high, super-physiological doses of insulin can protect islets from apoptosis and increase proliferation via PI3-kinase (116). However, the role of these proteins in physiological insulin signalling in primary β-cells remains poorly understood. In the present study, the mechanism of insulin-stimulated β-cell proliferation was investigated using LY294002, an inhibitor of PI3-kinase-dependent signalling. We found that blocking PI3-kinase significantly prevented β-cell proliferation in the presence of 0.2 nM and 200 nM insulin. There were non-significant trends towards reduced β-cell proliferation in all other treatments, including the baseline serum-free control (Fig. 7A). Together, these experiments point to an important role for PI3-kinase in primary β-cell proliferation

While the PI3-kinase/Akt-dependent signalling pathway has been well studied in β-cells, insulin triggers its mitogenic effects via Raf-1 and ERK in many cell types (87). A highly-selective small molecule Raf-1 kinase inhibitor (158) was used to further address the mechanism of insulin-stimulated β-cell proliferation. This inhibitor decreased proliferation of primary β-cells cultured in 0.2 nM insulin, without affecting basal proliferation (i.e. serum-free conditions) (Fig. 7B). Remarkably, Raf-1 kinase inhibitor did not lower β-cell replication induced by high concentrations of insulin (Fig. 7B). This is the first demonstration, to our knowledge, of a kinase that is selectively involved in the effects of physiological insulin doses, but not higher doses of the hormone. Together, these results demonstrate that both PI3-kinase and Raf-1 kinase pathways are important for the proliferative effects of insulin on primary mouse β-cells, although Raf-1 appears to be specifically involved in mediating physiological insulin signals..

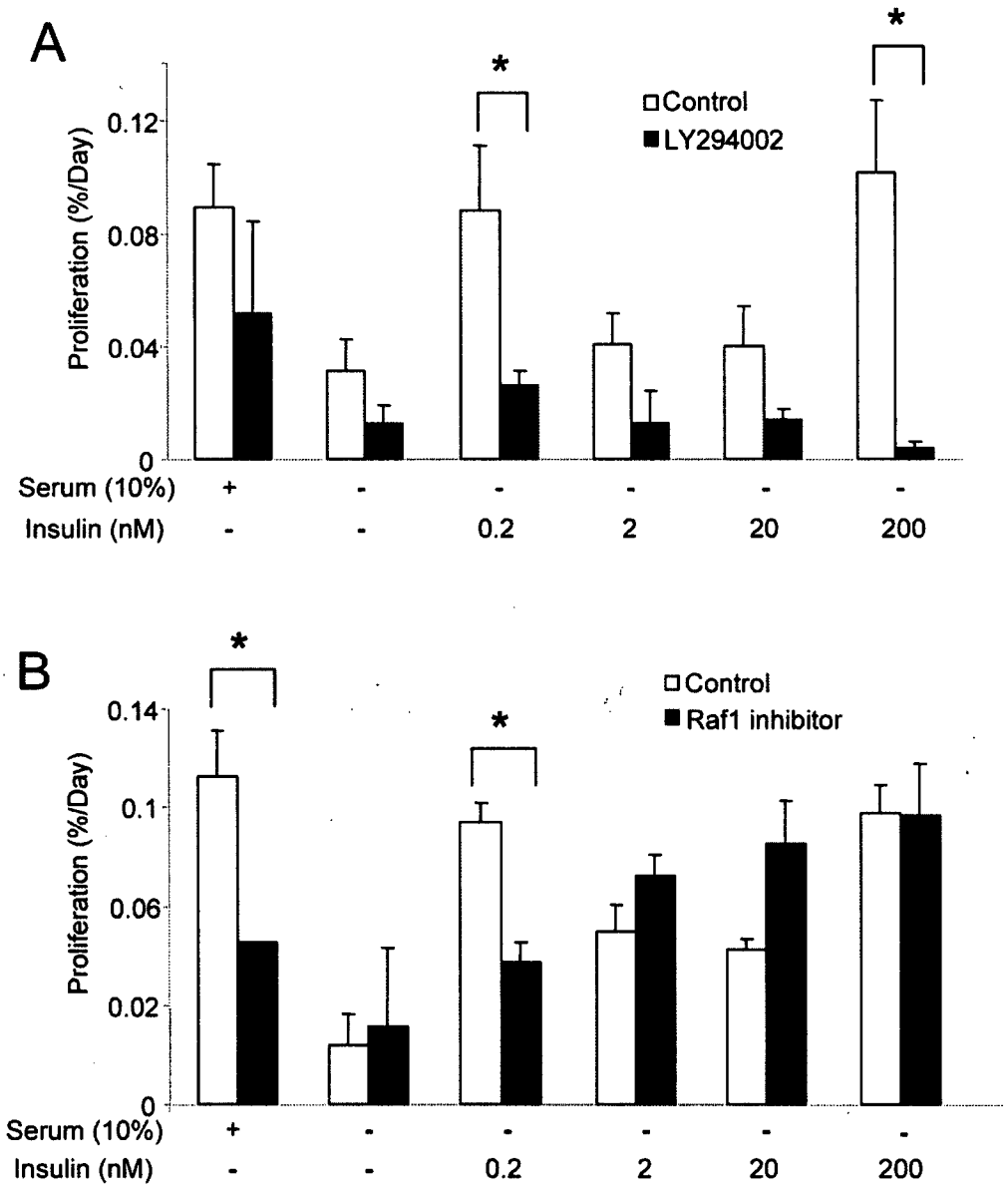
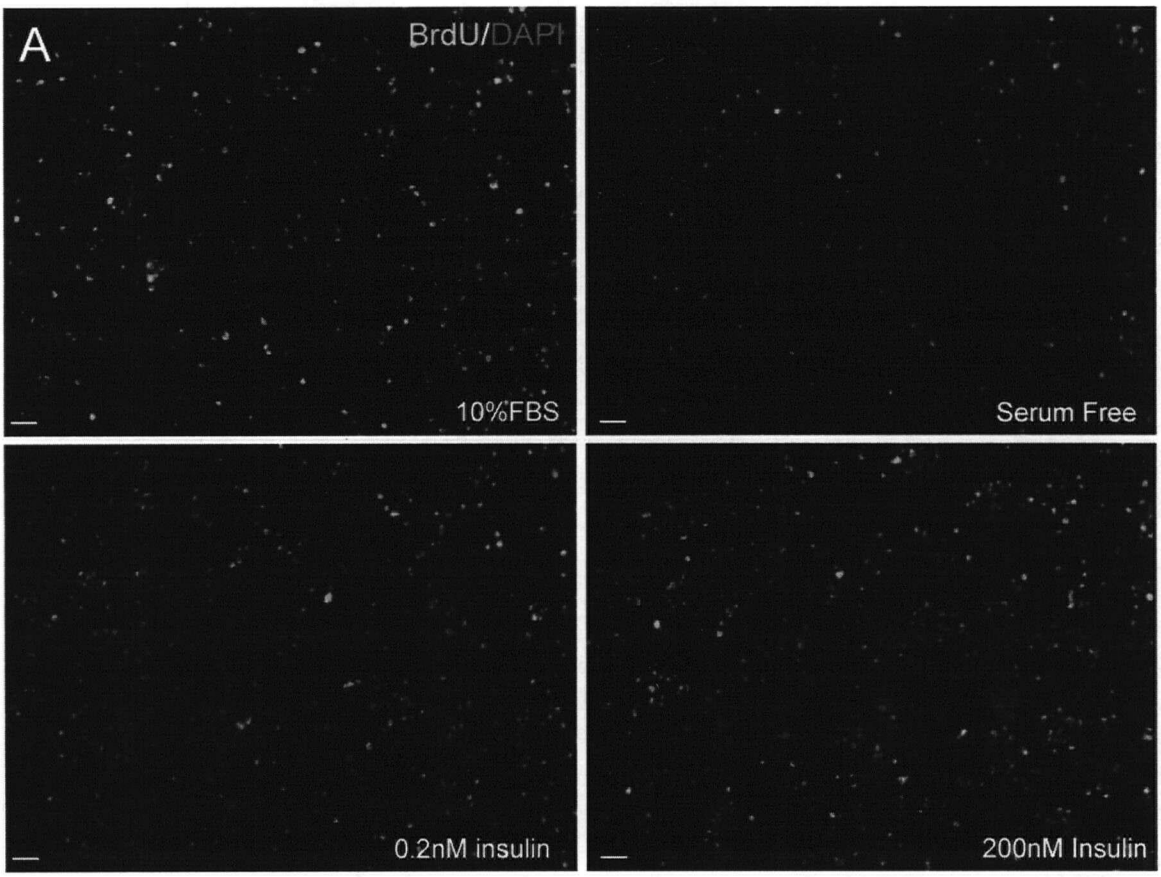


Fig. 7. Roles of PI3-kinase and Raf-1 in insulin-stimulated β-cell proliferation. (A) Dispersed mouse islets were cultured with or without insulin as indicated and in the presence (white bars) or absence (black bars) of 50 μM of the PI3-kinase inhibitor LY294002 for three days. *$P < 0.05$ versus control (n = 3). (B) Dispersed mouse islets were cultured as in A. in the presence (black bars) or absence (white bars) of Raf-1 kinase inhibitor (5 μM). *$P < 0.05$ versus control (n = 4).

Effects of insulin on proliferation of transformed β-cells

Although MIN6 cells proliferate at a very fast rate compared to primary β-cells (Fig. 3D), we examined whether the effect of insulin would be similar between these two models. The replication of MIN6 cells in the presence of insulin for six hours was determined by the incorporation of BrdU present in the media for the last 2 hours of the culture. Preliminary time course experiments determined that a six-hour treatment of insulin was optimal for observing the effects of insulin on β-cell proliferation (data not shown). As with the primary β-cells, both physiological and super-physiological doses of insulin significantly increased MIN6 cell proliferation (Fig. 8A,B). In the MIN6 cell model, the effects of insulin could be blocked using inhibitors of PI3-kinase or Akt (Fig. 9A). Inhibiting Raf-1 kinase or ERK (with U0126) reduced proliferation in response to physiological levels of insulin. Unlike the case in primary cells, Raf-1 kinase and ERK inhibitors also reduced proliferation induced with the super-physiological dose of insulin (Fig. 9B). Substantial proliferation in serum-free media was still observed in the presence of these inhibitors, indicating that these transformed cells are relatively freed from the requirement of growth factor support for basal growth (Fig. 9A,B). Together, these results indicate that insulin can also regulate the proliferation of transformed β-cells.



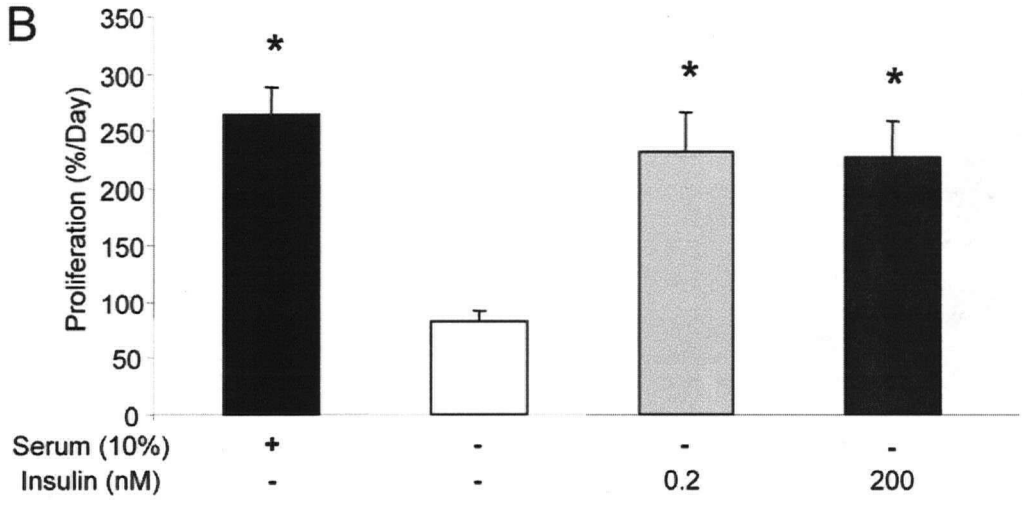


Fig. 8. Effects of insulin on MIN6 cell proliferation. (A) MIN6 cells were treated with exogenous insulin as indicated for six hours with BrdU (green) added for two hours. Scale bar equals 10 μm (B) Percent proliferation was quantified using BrdU immunofluorescence seen in A. $*P < 0.05$ versus serum free control (n = 6).

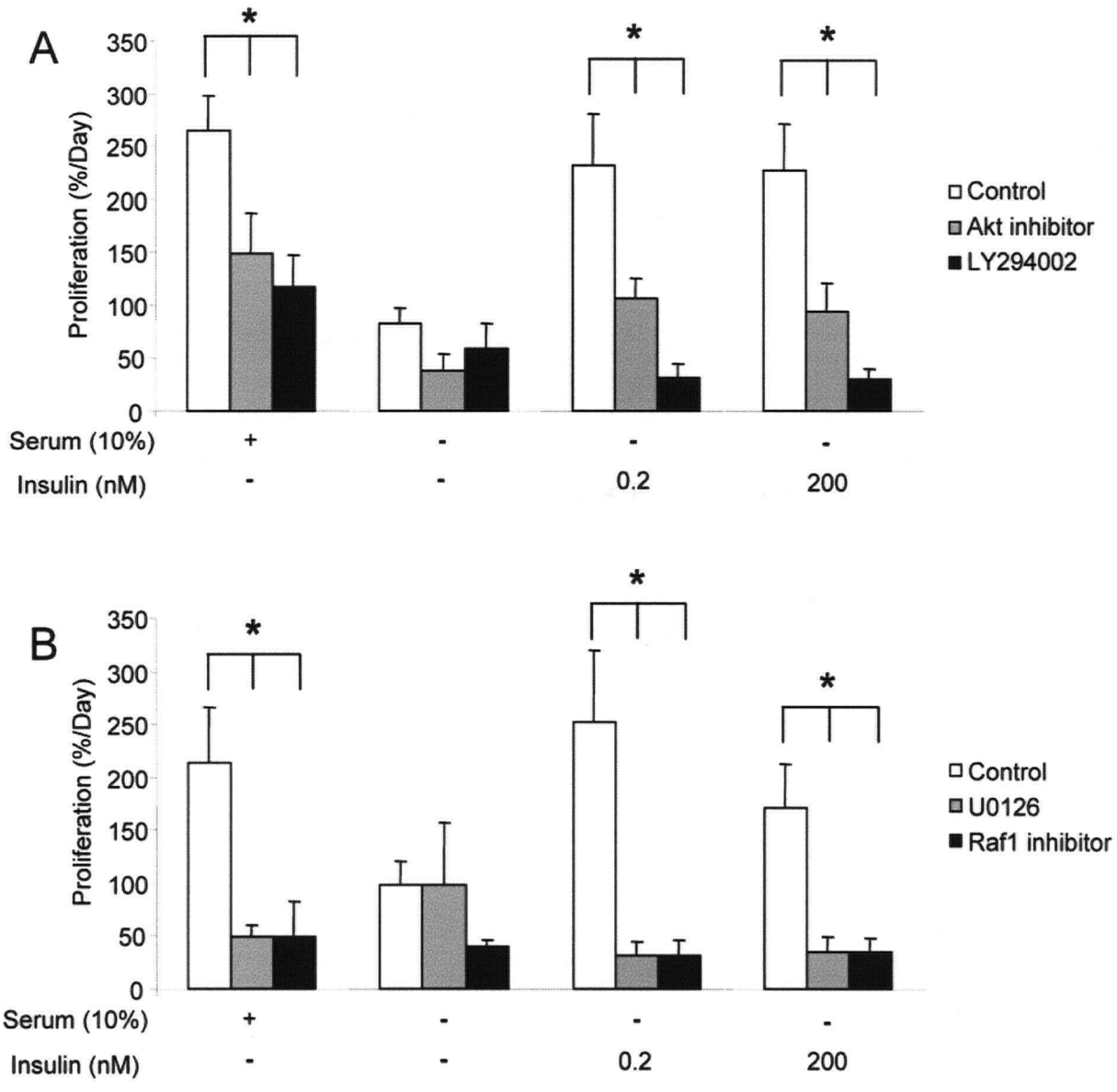


Fig. 9. Role of PI3-kinase and Raf-1 pathways in insulin-stimulated β-cell proliferation. (A) MIN6 cells were cultured as in Fig. 8 or treated with an Akt inhibitor (100 nM) or LY294002 (50 μM). *P < 0.05 versus control (n = 3). (B) MIN6 cells were cultured as in A or treated with UO126 (10 μM) or the Raf-1 inhibitor (5 μM). *P < 0.05 versus control (n = 3).

Effect of insulin receptor pathways on β-cell insulin secretion

We have shown above that insulin secretion from primary β-cells does not significantly accumulate in media over a three day culture period and confirmed previous studies (102) suggesting that insulin does not have robust effects on its own secretion in dispersed cells (Fig. 4B). However, whether inhibitors of insulin signalling have effects on insulin secretion remained unclear. Thus, to examine the effects of the drugs used in this study, media samples were collected from dispersed primary mouse islets every 24 hours (Fig. 10). Over the three-day culture period there were no apparent changes in insulin secretion with HNMPA, LY294002 or Raf-1 inhibitor (Fig. 10). However, an insufficient number of experiments limited the ability to perform statistical analysis on all data.

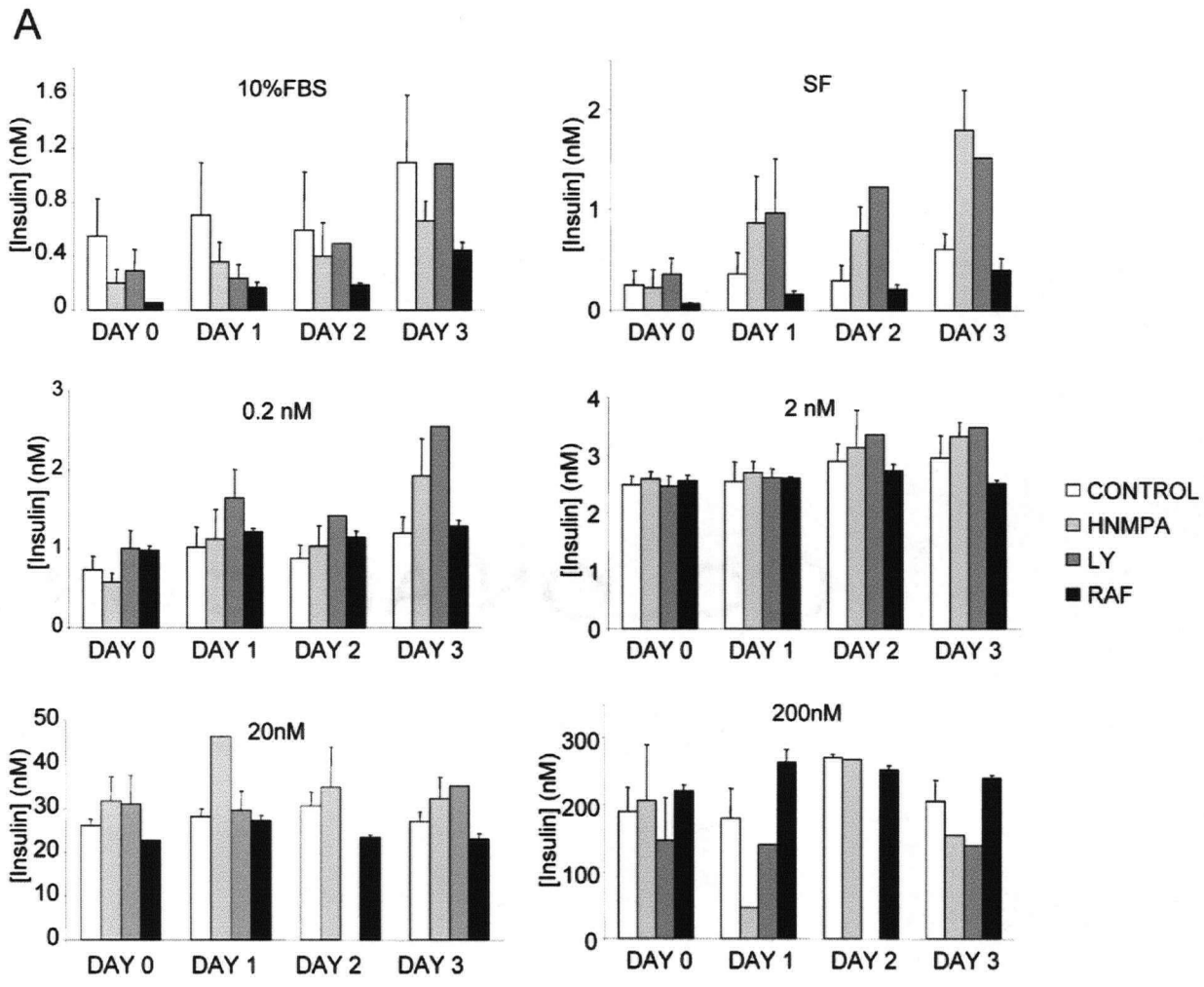


Fig. 10. Effects of insulin receptor pathway inhibitors on insulin secretion. (A) Media samples were extracted every 24 hours starting at day 0 from culture media of dispersed primary islets to assess insulin secretion by radioimmunoassay. Treatments are as indicated; HNMPA (100 μM; n=4-14), LY294002 (50 μM; n=2-5), and Raf-1 inhibitor (5 μM; n=3).

Analysis of cell cycle genes under conditions of β-cell growth

We have shown that exogenous insulin can stimulate primary β-cell proliferation *in vitro* (Fig. 4) through both PI3 kinase and Raf-1 pathways (Fig. 7). To study possible targets of insulin signalling that are farther downstream, we examined the expression of known β-cell cycle molecules. The effects of exogenous insulin on gene expression of the D-type cyclins and p21 in primary islets were examined using reverse transcriptase PCR (RT-PCR). No striking differences

in cyclin D1 and D3 were apparent between insulin treatments (Fig. 11A,C). There was a significant increase in cyclin D2 expression in serum containing cultures compared to serum free cultures (Fig. 11B). This agrees with previous studies showing a dominant role for cyclin D2 in β-cell proliferation (37, 61). There was also an increasing trend in cyclin D2 expression with 0.2 nM insulin (Fig. 11B). Gene expression of p21 was significantly higher with the lower doses of insulin (0.2 nM and 2 nM); however the lack of even loading may have contributed to the trend observed (Fig. 11D). No changes were observed using super-physiological doses of insulin on gene expression of the D-type cyclins or p21 (Fig. 11A-D). Together, these hint that cell cycle genes can be regulated by external cues in primary islets. However, a number of technical problems make the data difficult to interpret. Future studies should employ quantitative real-time PCR.

Cyclin G1 and G2 are novel proteins with roles that are still ill-defined, although they have been shown to arrest cell cycle progression (48-53). Gene expression of cyclin G2 in MIN6 cells was increased in response to insulin receptor knock down (103). To address the effects of exogenous insulin on gene expression of cyclin G1 and cyclin G2, RT-PCR was carried out in primary mouse islets. Gene expression of either of the G-type cyclins was not altered in the presence of insulin (Fig. 11E,F), although cyclin G2 was slightly increased in serum conditions and treatment with 0.2 nM insulin (Fig. 11F). These preliminary experiments suggest further studies are required to determine which cell cycle molecules are regulated during β-cell mitogenesis. It may not be possible to accurately determine gene expression in the very rare dividing β-cells (~0.15% per day) using RT-PCR of whole islets. Thus, future studies should attempt to employ single-cell analysis of gene expression within the specific population of dividing cells.

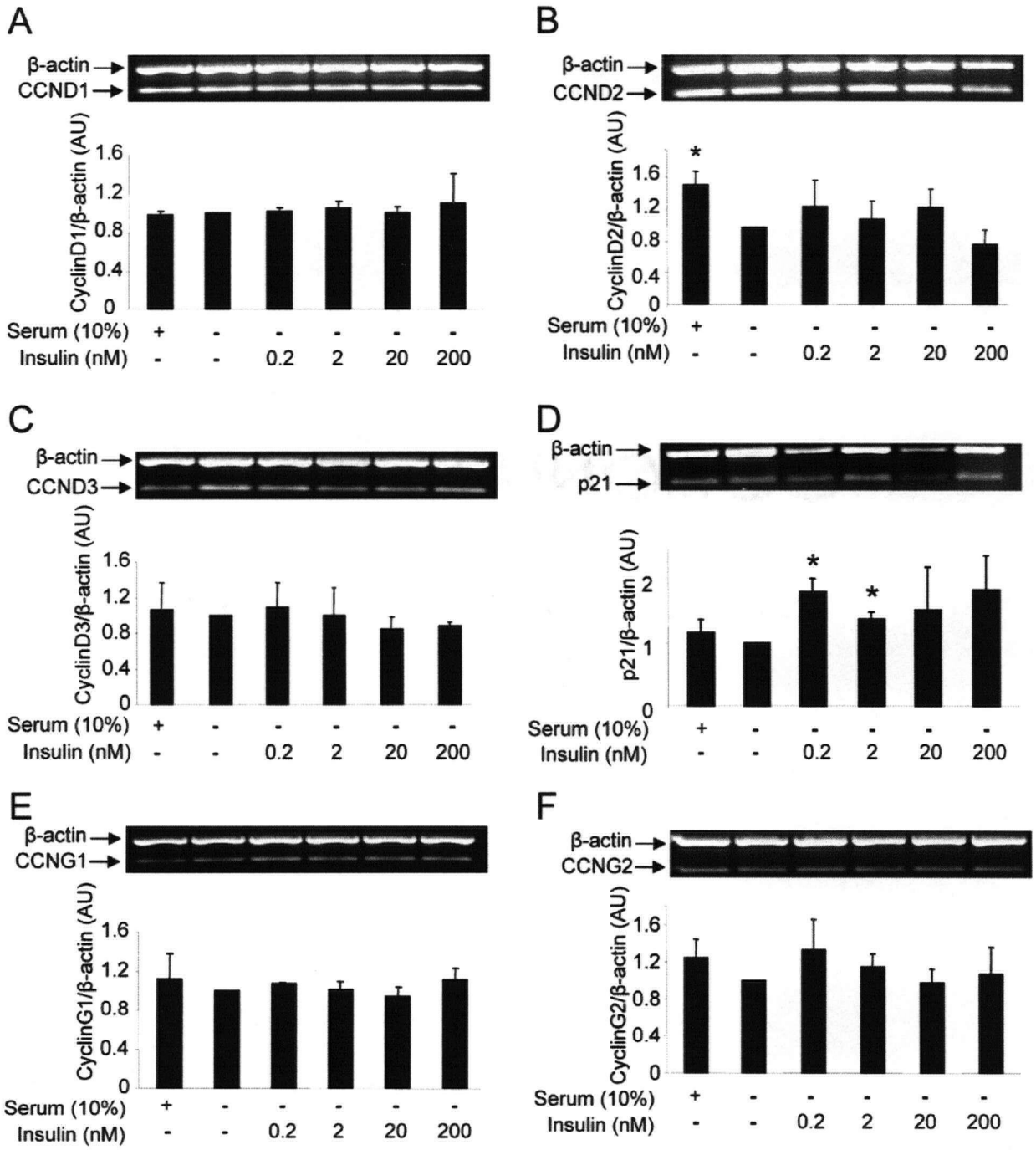


Fig. 11. Effects of insulin on gene expression of cell cycle molecules. (A) Reverse transcriptase PCR (RT-PCR) was carried out on primary mouse islets treated with insulin for 48 hours. Gene expression of cyclin D1 (n=3). (B) Gene expression of cyclin D2 $*P < 0.05$ versus serum-free control (n = 5). (C) Gene expression of cyclin D3 (n=3). (D) Gene expression of p21 $*P < 0.05$ versus serum free-control (n = 4). (E) Gene expression of cyclin G1 (n=3). (F) Gene expression of cyclin G2 (n=3).

Role of autocrine insulin signalling and glucose on cell cycle gene expression

Although we did not observe robust changes in gene expression using exogenous insulin, it remained unclear whether basal endogenous insulin signalling could regulate β-cell cycle molecules. To address this question primary mouse islets were treated with HNMPA-AM, a drug that induced profound reductions in proliferation. Alterations in the gene expression of cyclin D2, p21 and cyclin G2 were examined using RT-PCR. Compared to control, treatment with HNMPA-AM decreased the expression of cyclin D2 (Fig. 12A), whereas both p21 and cyclin G2 gene expression were increased (Fig. 12B,C). Although these differences were not significant, they suggest the insulin receptor pathway may increase proliferation by influencing key cell cycle molecules, in agreement with previous studies (103). However, preliminary studies did not find changes in the gene expression of p21, cyclin D2 or cyclin G2 in the presence of LY294002 (data not shown). Additional work will be required to determine whether specific cell cycle molecules can be controlled by components of the insulin signalling pathway in primary β-cells.

We have previously shown that high concentrations of glucose do not robustly increase β-cell proliferation, conflicting with previous reports (106, 144-146). Nevertheless, we examined increasing concentrations of glucose on gene expression of cyclin G2 using RT-PCR on primary mouse islets treated with 2, 10 or 20 mM glucose. A trend towards increase in gene expression with 10 mM and 20 mM glucose was observed (Fig. 12D), although no statistical analysis could be completed because the experiment was only carried out twice.

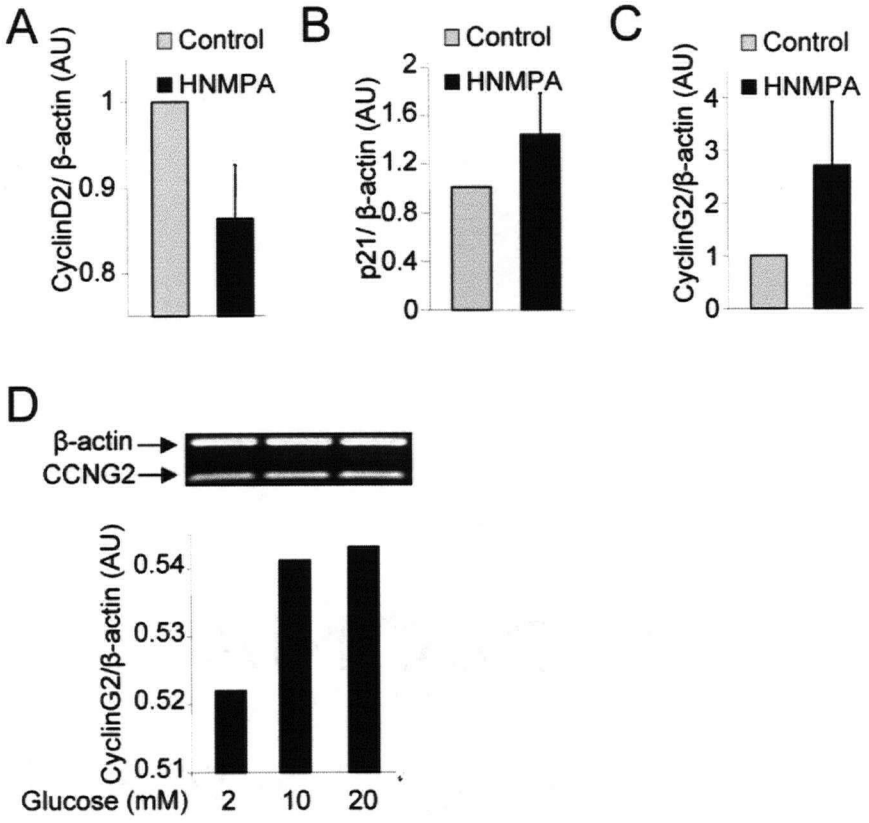


Fig. 12. Role of autocrine insulin on gene expression of cell cycle molecules. Primary mouse islets were treated with HNMPA (100 μM) for 24 hours. RT-PCR was carried out to assess gene expression of (A) cyclin D2 (n=3) (B) p21 (n=3) (C) cyclin G2 (n=3). (D) Expression of cyclin G2 mRNA in primary mouse islets cultured in elevated glucose concentrations (as indicated) (n=2).

Role of insulin and the insulin receptor pathway on cell cycle molecules

We have shown above that exogenous insulin can stimulate β-cell proliferation in primary mouse islets (Fig. 4) and MIN6 cells (Fig. 8). In order to further understand the mechanisms by which insulin exerts proliferative actions, we used primary human and MIN6 cells to quantify protein expression levels of known β-cell cycle molecules. Retinoblastoma protein (Rb) induces cell cycle arrest at the G1/S checkpoint and becomes inactive once it is phosphorylated (46, 47). We used an antibody that binds specifically to Rb that is phosphorylated at serine 780. This is a site that is phosphorylated by various cell cycle kinases such as cdk4 and cdk6 (47, 54, 159). Western blot analysis on human islets treated with

exogenous insulin showed no differences in Rb phosphorylation when compared with control islets (Fig. 13A). Given that MIN6 cells proliferate at much higher rate than primary cells (Fig. 7) and are a good model to study β-cell proliferation (Fig. 8), western blot analysis was performed to examine Rb phosphorylation in this β-cell line. MIN6 cells treated with exogenous insulin for six hours did not display a significant increase in phosphorylation of Rb, although there was an increasing trend in serum-containing conditions (Fig. 13B,C).

We have shown that both PI3-kinase and Raf-1 pathways are essential for β-cell proliferation in both primary mouse cells (Fig. 7) and MIN6 cells (Fig. 9). However, the importance of these pathways to Rb phosphorylation remained unclear. To answer this question, MIN6 cells were treated with insulin, with or without a pharmacological inhibitor of the insulin receptor pathway. Blocking PI3-kinase caused a slight reduction in Rb phosphorylation with 200 nM insulin, although this was not statistically significant (Fig. 14A,B). No other trends were identified on Rb phosphorylation by inhibiting PI3-kinase or Akt in MIN6 cells (Fig. 14A-D). We also examined the effects of Raf-1 and MEK inhibition. The Raf-1 inhibitor did not alter Rb phosphorylation in MIN6 cells treated with insulin (Fig. 14E,F). However, inhibiting Raf-1 activity did significantly decrease Rb phosphorylation in serum treated cells when compared to non-treated controls (Fig. 14E,F). In contrast, blocking MEK did not affect phosphorylation of Rb with or without insulin treatments (Fig. 14G,H). In general, results from these studies were ambiguous and this area requires further study (71).

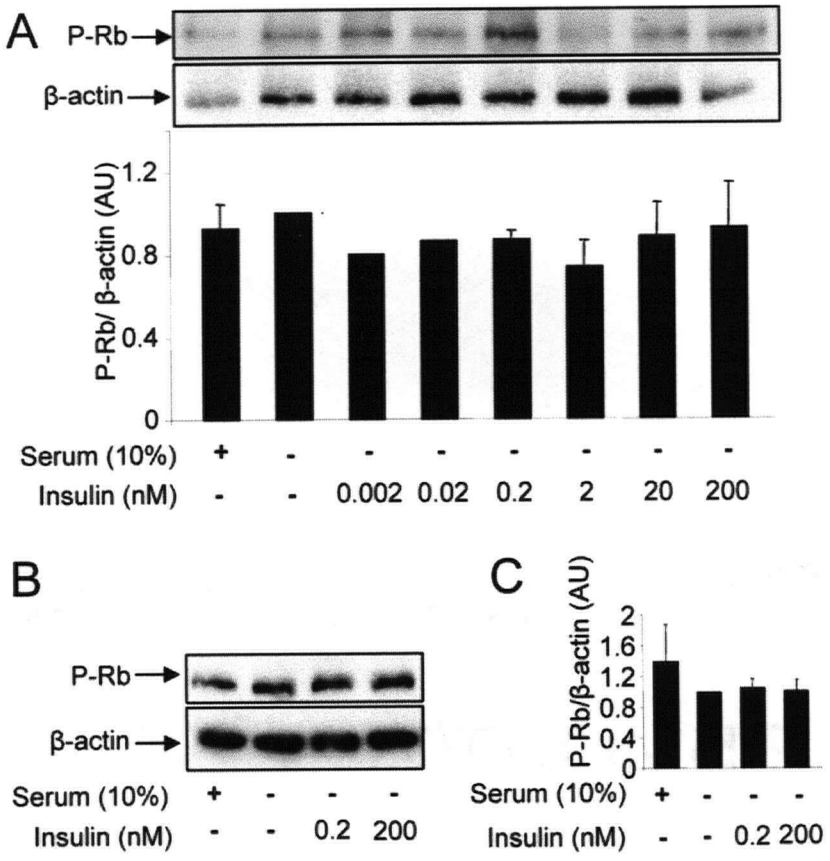


Fig. 13. Effect of insulin on phosphorylation of retinoblastoma protein. (A) Phosphorylation of retinoblastoma (ser780) was detected by Western blot in human islets treated with insulin for 24 hours (n=2-5). (B) MIN6 cells were treated with insulin for 6 hours before phosphorylation of retinoblastoma was assessed (n=9). (C) Quantification of Western blot shown in B (n=9).

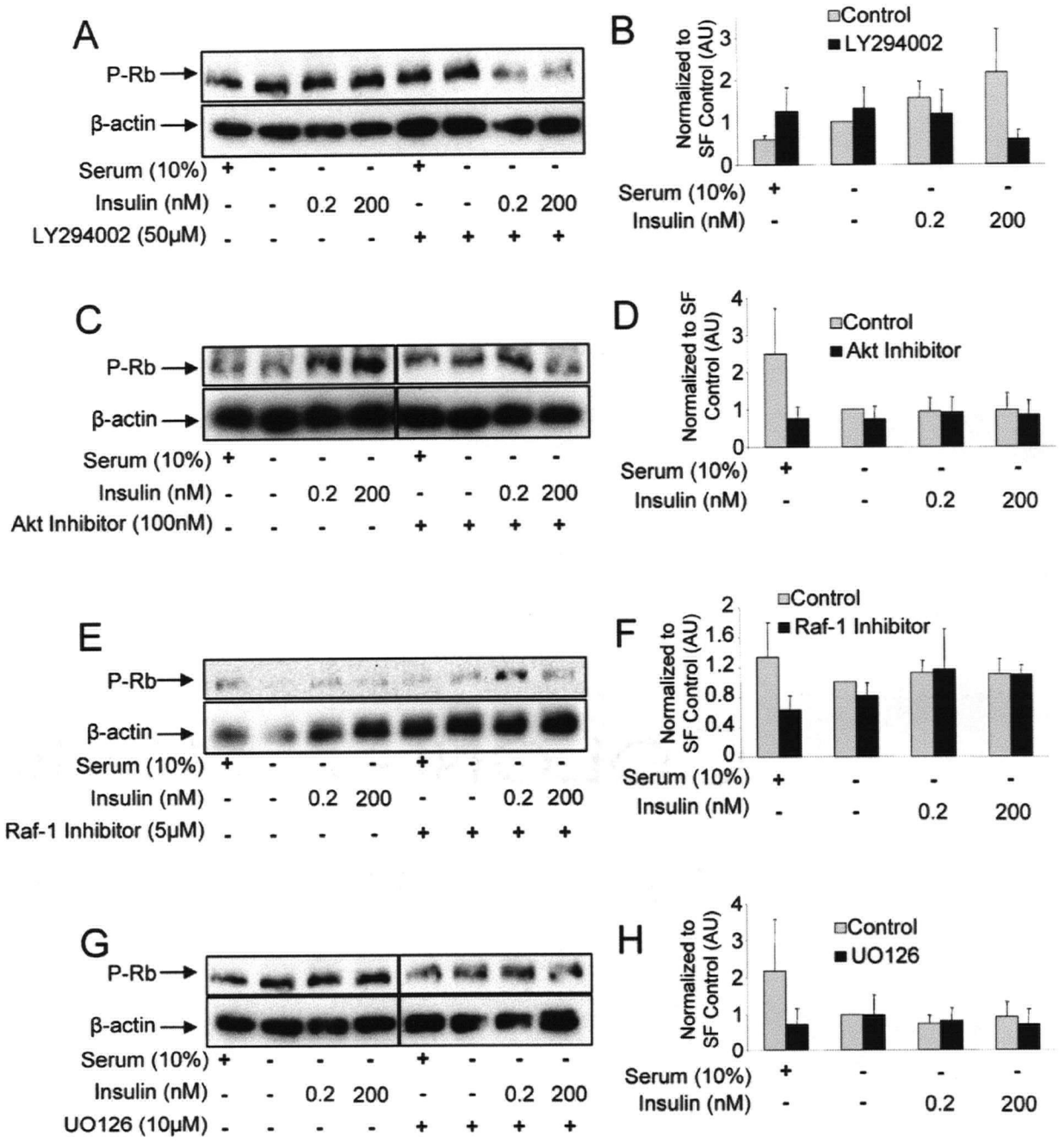


Fig. 14. Role of insulin and its receptor pathways on retinoblastoma phosphorylation. (A) Phosphorylation of retinoblastoma (ser780) was examined in MIN6 cells treated with insulin in the presence or absence of LY294002 for six hours (50 μM; n=5). (B) Quantification of results found in A. (C) MIN6 cells cultured as in A with or without Akt inhibitor (100 nM; n=3). (D) Quantification of results found in C. (E) MIN6 cells cultured as in A in the presence or absence of Raf-1 inhibitor (5 μM; n=4). (F) Quantification of results found in E. (G) MIN6 cells cultured as in A in the presence or absence of UO126 (10 μM; n=3). (H) Quantification of results found in G.

Although we did not observe robust changes in gene expression of p21 and cyclin D1 in primary mouse islets, it is possible that these proteins may have been regulated post-transcriptionally (e.g. protein degradation). Using western blot analysis on MIN6 cells, the effect of insulin on p21 and cyclin D1 protein levels with and without the presence of insulin receptor pathway inhibitors was examined. Both physiological and super-physiological doses of exogenous insulin and serum containing cultures increased protein levels of p21 when compared to serum free conditions (Fig. 15A-H), yet this trend was not significant. Inhibiting PI3-kinase did not change p21 levels with or without insulin, however, a slight decrease at 200 nM insulin was observed (Fig 15A,B). In a preliminary set of experiments, a decrease in p21 levels was seen at 200 nM insulin with both Raf-1 and MEK inhibitors (Fig. 15C-F), but statistical analysis could not be carried out due to lack of sufficient experiments (n=2). Furthermore, inhibiting MEK increased p21 protein level in serum free conditions compared with control (Fig. 15E,F). The relative importance of p21 β-cell proliferation remains unclear (69). Next, we also found that exogenous insulin did not affect protein levels of cyclin D1 in MIN6 cells, although an increase in cyclin D1 levels was seen in serum containing conditions (Fig. 15G,H). Blocking PI3-kinase showed an overall decrease in protein level of cyclin D1 in control and insulin treatments (Fig. 15G,H). However, a significant decrease in cyclin D1 levels was seen by inhibiting PI3-kinase at 200 nM insulin (Fig. 15G,H). Other cell cycle molecules (cyclin B, cyclin E, cyclin G2, p15, p16 and p27) were examined in the presence of insulin and insulin receptor pathway blockers, however, no changes were observed (data not shown).

One would expect that changes in cell cycle gene expression and protein level might be extremely difficult to distinguish in whole islets given that the percentage of dividing cells is miniscule and expression of the cell cycle molecules is transient. Therefore, we attempted to use MIN6 cells to examine translocation of various cell cycle molecules including p21, p27, cyclin D1, cyclin G2, cyclin E, and cyclin B. Unfortunately, the immunofluorescence staining was unsuccessful due to non-specific staining (data not shown).

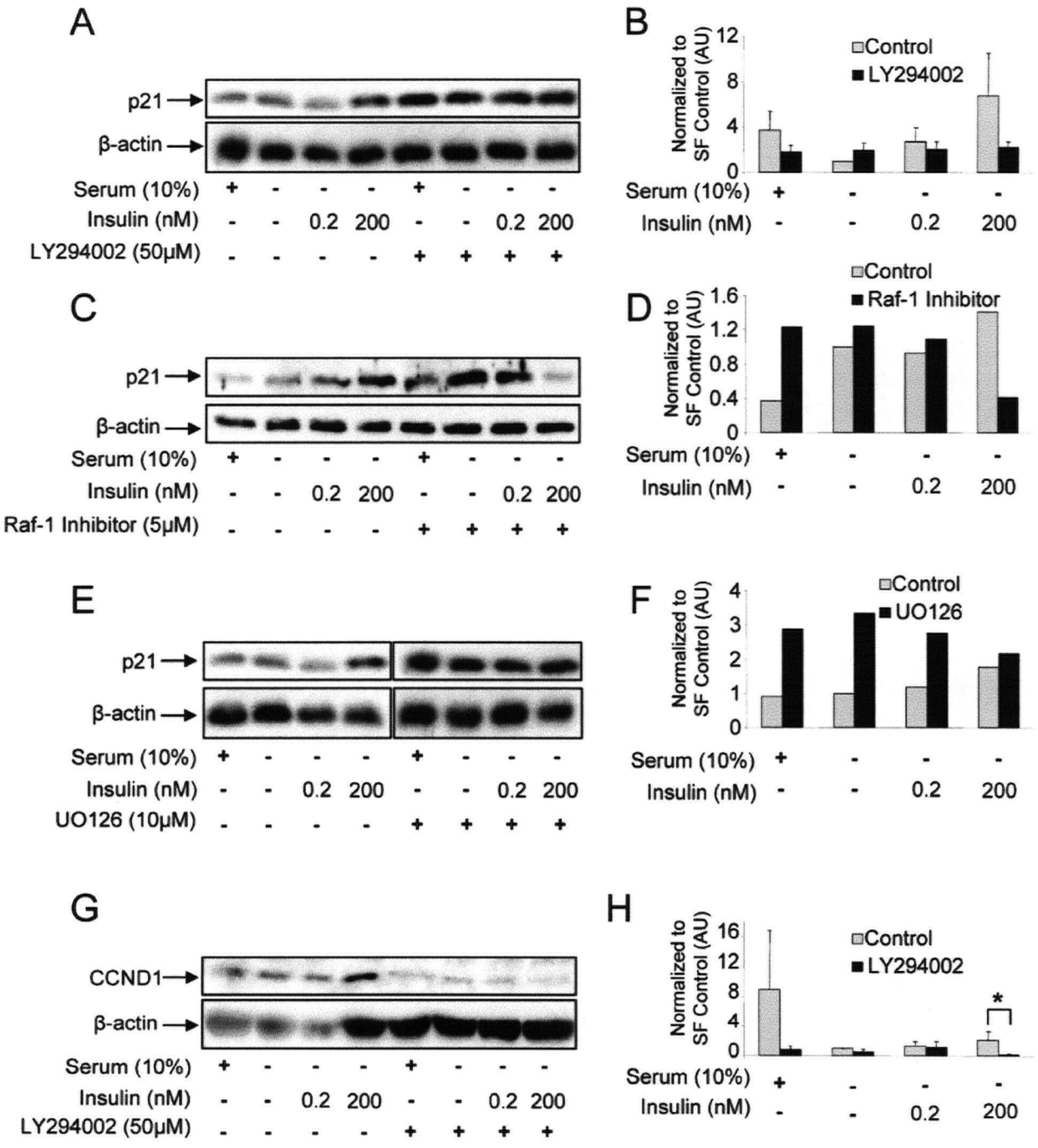


Fig. 15. Role of insulin and its receptor pathways on protein expression of cell cycle molecules. (A) Protein expression of p21 levels in MIN6 cells cultured with insulin in the presence or absence of LY294002 for six hours was assessed using Western blot analysis (50 μM; n=5). (B) Quantification of results found in A. (C) MIN6 cells cultured as in A with or without Raf-1 inhibitor (5 μM; n=2). (D) Quantification of results found in C. (E) MIN6 cells cultured as in A with or without UO126 (10 μM; n=2). (F) Quantification of results found in E. (G) Protein expression of cyclin D1 was assessed as seen in A in the presence or absence of LY294002 (50 μM; n=3). (H) Quantification of results found in G. *$P < 0.05$ versus control

DISCUSSION

This thesis study investigated the role of insulin signalling in pancreatic β-cell proliferation. Insulin is known to be a potent growth factor in other cell types, however its importance in the β-cell has only been recently appreciated. Now that pancreatic β-cell duplication is recognized as a major regulator of adult β-cell mass (34, 35, 40), new attention is being focused on endogenous regulators of β-cell growth. The βIRKO mice have highlighted the importance of insulin signalling in regulating adult β-cell mass *in vivo* (108-110, 113). *In vitro* studies on MIN6 cells have further supported a role for insulin in β-cell growth (103). Whether physiological doses of insulin can stimulate β-cell proliferation remained unclear. Thus, the present study was undertaken to test the hypothesis that insulin can stimulate the proliferation of primary β-cells and investigated the mechanisms involved. We tested this hypothesis in dispersed primary mouse islets. Both physiological and super-physiological concentrations of insulin increased replication of β-cells. Glucose did not significantly stimulate β-cell proliferation. We found the PI3-kinase pathway to be a general regulator of β-cell replication; however, the Raf-1 pathway was only critical at physiological doses of insulin. Further downstream mechanisms of insulin stimulated β-cell proliferation remains undefined. Understanding how β-cell mass is regulated is an important advance in our understanding of the molecular pathways involved in the progression of diabetes and in efforts to combat the loss of β-cell mass.

Estimation of the β-cell proliferation rate

Several studies have sought to estimate the proliferation rate of pancreatic β-cells. Early reports, some of which employed mathematical modeling, estimated an *in vivo* proliferation rate of between 1% and 10% per day (38, 39). However, recent analyses on pancreas sections from mice fed BrdU or related analogues in their drinking water estimated that *in vivo* β-cell proliferation is roughly between 0.07% and 2% per day (35, 40). *In vitro*, we found the replication rate of primary β-cells to be approximately 0.15% per day. While our results are in the same range as the newer studies, the reasons for the discrepancy with the earlier reports are not clear. It is notable that the earlier experiments did not fully distinguish between BrdU

labeling of non-insulin-positive cells and β-cells (38). By comparison, our *in vitro* dispersed cell culture system permitted us to verify that every proliferating β-cell was indeed co-labeled with both BrdU and insulin. Nevertheless, our results are from cultured β-cells that have been taken out of their natural environment, and perhaps it is not surprising that our proliferation rate may be slightly lower than the *in vivo* situation.

Insulin and β-cell proliferation

Many exogenous and endogenous growth factors have been shown to increase β-cell proliferation *in vivo* and *in vitro*, including prolactin, placental lactogen, hepatocyte growth factor, parathyroid hormone-related protein, GLP-1/exendin-4, and IGF-1 (118-129). However, it is not clear which, if any, of these factors plays a role in the endogenous control of β-cell mass. On the other hand, the role of insulin signalling in regulating β-cell survival and mass has now been well studied. However, the direct effects of acute insulin on primary β-cell proliferation had yet to be elucidated. We and others present evidence that autocrine insulin signalling may be an important endogenous regulator of β-cell proliferation. Of the known β-cell growth factors, insulin appears to be unique, as the only factor secreted in substantial amounts from within the islet. Importantly, studies with the βIRKO mice have conclusively demonstrated the importance of insulin signalling in regulating β-cell mass in the adult and under conditions of compensation in response to obesity and insulin resistance (103, 108-110, 113). Studies completed on the downstream targets of the insulin receptor, such as IRS-2, have also supported the concept that insulin may play an important role in regulating β-cell mass (89, 134, 137). Furthermore, autocrine insulin signalling has been shown to have a positive effect on β-cell survival and proliferation in both human islets and MIN6 cells (116, 117), yet only super-physiological concentrations of insulin were used in those experiments. We have recently shown that physiological doses of insulin can protect β-cells from apoptosis (130). In obesity and pre-type 2 diabetes, the endocrine pancreas attempts to compensate for insulin resistance by increasing its β-cell mass (160). Our results are consistent with a model whereby modest hyperinsulinemia could drive this response, a hypothesis that is supported by *in vivo* studies. Indeed, mice lacking insulin receptors in their β-cells have an age-dependent decrease in β-cell mass and the compensatory β-cell proliferation response to insulin resistance is completely absent (108, 109).

Another example supporting the possible stimulatory effects of insulin on β-cell proliferation *in vivo* comes from a line of mice in which the over-expression of an insulin transgene led to increased β-cell mitogenesis (161). Although these studies highlighted the importance of insulin signalling in the control of β-cell mass, they could not address the possibility of direct acute effects of insulin on β-cell proliferation or determine the relative effectiveness of specific insulin doses. Using a physiological concentration of insulin, we aimed to clarify the role of insulin in primary β-cell proliferation.

In the present study, we demonstrated for the first time that insulin stimulates primary β-cell division. However, insulin's proliferative effect was only seen at insulin concentrations of 0.2 nM, 20 nM and 200 nM, and not 2 nM. This is an atypical dose-response profile, but one that has been observed in previous studies (130, 162). For example, insulin stimulates Ca^{2+} release in ~80% of primary human and mouse islets cells at 0.2 nM, but only 30% of cells at 2 nM, 20 nM and 200 nM insulin (111, 162). Although the exact cause of this dose-response relationship remains to be elucidated, one can speculate that this profile is formed by multiple components (Fig. 16). Unlike most hormone receptors, the binding affinity of insulin to its receptor is bell-shaped due to negative cooperativity. As a result, the affinity for insulin binding to its receptor is lowered when insulin concentrations are well beyond the physiological range (84). Concentrations such as 2 nM insulin are probably too high to maximally activate the insulin receptor, but too low to robustly activate the IGF-1 receptor (84). Therefore, perhaps it is not surprising that a lack of proliferation was observed at intermediate concentrations. We speculate that the proliferation observed at super-physiological doses of insulin were due to activation of IGF-1 receptors (84).

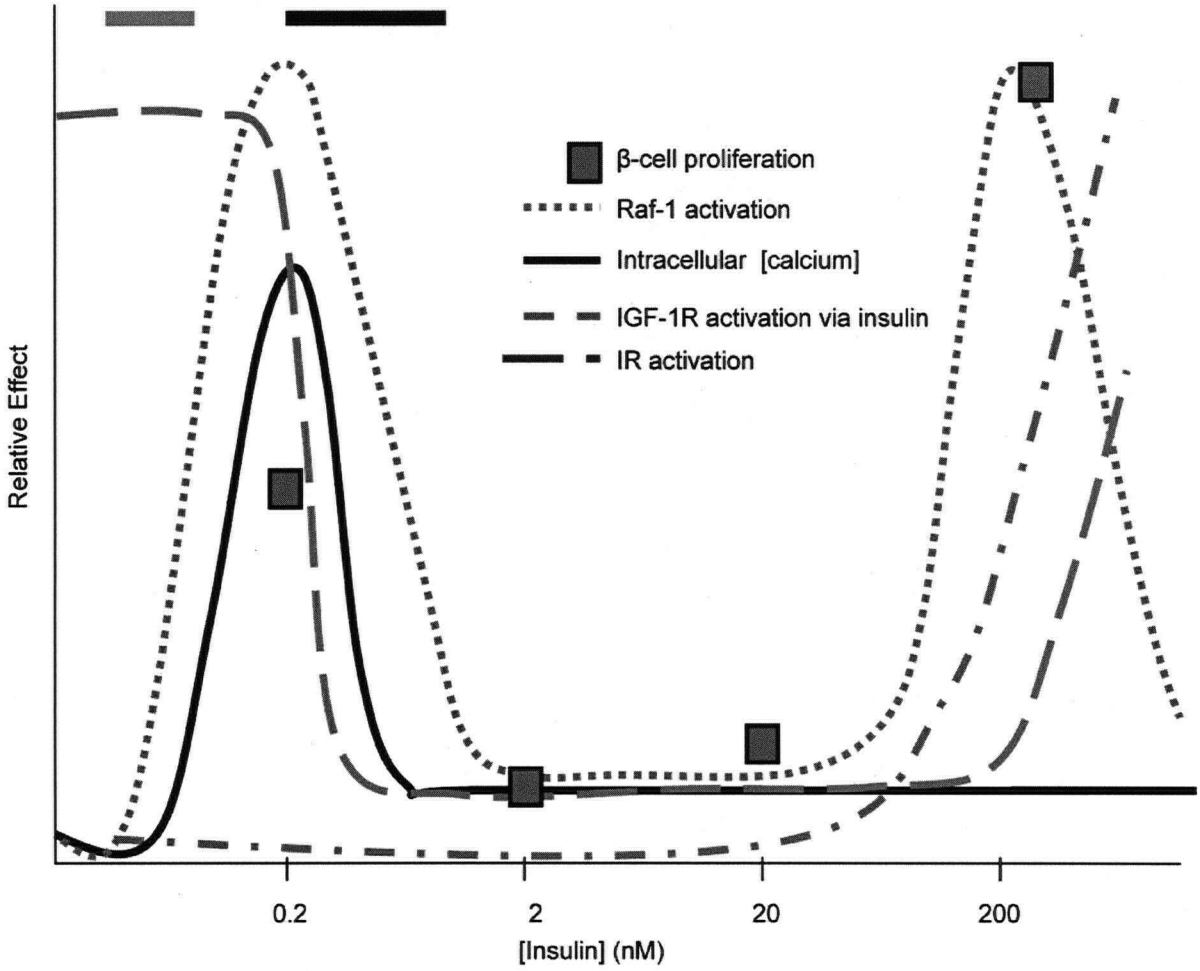


Fig. 16. Schematic effect of insulin on proliferation, calcium, Raf-1 activation, and insulin and IGF-1 receptor binding affinities. Insulin concentration found in general (gray bar) and portal (blue bar) circulation. β-cell proliferation is represented by blue boxes (ref. 77). Insulin (percent bound; red-dotted line) and IGF-1 (percent bound; blue-dotted line) receptor binding affinity for insulin at specific doses (ref. 84). Cytosolic calcium (nM; black line) response to exogenous insulin in human β-cells as shown through live-cell calcium imaging. Activation of Raf-1 shown by Western blot analysis of ser 259 phosphorylation in human islets (green-dotted line).

An important consideration for studies of physiological insulin signalling is the levels of insulin present *in vivo*. Insulin is secreted in a pulsatile manner with portal concentrations ranging from 200-1200 pM and basal systemic concentrations of 40-200 pM in humans (77, 163). Following a meal or hyperglycemic clamp in humans, insulin concentrations in portal and systemic circulation can rise to approximately 500 pM and 3000 pM, respectively (77, 163). Our results therefore imply that insulin stimulates significant β-cell proliferation at concentrations expected in the portal circulation or during periods of elevated insulin release. Although it has

never been directly measured, others have speculated that insulin levels surrounding β-cells may be higher than those observed in the portal circulation. Unfortunately, efforts to estimate the local insulin concentrations within islets are complicated by two major factors. First, insulin is released from β-cells as an insoluble microcrystal which only becomes a soluble monomer capable of binding to its receptor after exposure to the bloodstream pH (74). Second, within the complex islet microvasculature, β-cells are exposed first to the arterial blood supply, meaning that a population of β-cells would not be exposed to insulin secreted from neighboring cells. These factors suggest that β-cells may be exposed to insulin at levels that are similar to or only slightly higher than those measured in the circulation. Together, with previous studies (17, 111, 130, 162), our current results suggest a 'sweet spot' in the dose response profile of autocrine insulin signalling at ~200 pM. Together, with the *in vivo* evidence from βIRKO mice, these studies indicated that changes in insulin concentrations within the physiological range may modulate β-cell proliferation. Our study demonstrates that insulin can rapidly (within days) stimulate β-cell proliferation in the absence of other putative stimuli, such as hyperglycemia.

Effects of glucose on β-cell proliferation

Several *in vivo* and *in vitro* studies have shown that high glucose can increase β-cell survival (144-147). However, in suggesting that glucose is the major driver of β-cell growth, these studies fail to fully acknowledge the role of glucose-stimulated insulin secretion and the possibility that paracrine insulin signalling may mediate some or all of the effects of glucose. For example, a previous study demonstrated that the increase in MIN6 cell proliferation in response to high glucose was inhibited by nifedipine, a treatment that is well known to block glucose-stimulated insulin secretion (116). In addition, a separate study using microarray analysis demonstrated that ~80% of gene expression changes induced by glucose in MIN6 cells were no longer observed when insulin receptors were knocked down by 80% (103). These experiments illustrate the importance of autocrine insulin signalling in glucose-stimulated events. We examined the effects of glucose on primary β-cell proliferation and directly tested whether glucose's effects were dependent on insulin secretion. We did not observe significant stimulatory effects of glucose on β-cell proliferation, although a trend towards increase proliferation was detected. Notwithstanding, somatostatin significantly reduced β-cell proliferation in the presence

of high glucose. Therefore, any increase in proliferation observed with elevated glucose could be fully accounted for by autocrine insulin signalling. Previous reports suggest that glucose can activate signalling molecules downstream of the insulin receptor independent of insulin receptor activation. Glucose increased gene and protein expression of IRS-2 in primary rat islets, and this was proposed to be independent of autocrine insulin or Akt signalling (152, 153). Further studies reported an independent role for glucose-stimulated activation of ERK1/2 and the ribosomal-S6 kinase ($p70^{S6K}$) and suggested that this was possible by Raf-1 phosphorylation (148-150). However, these studies assumed the effects of glucose on β-cell proliferation were insulin-independent based on observations that IGF-1 did not augment glucose-stimulated proliferation and the direct effects of autocrine insulin signalling on β-cell proliferation were not examined (148-150). In our study, we directly examined the effects on insulin and glucose, independently of each other. Our results point to a dominant role for insulin signalling in primary and transformed β-cell proliferation.

Mechanisms of insulin-induced β-cell proliferation – post-receptor kinase cascades

Understanding the mechanisms by which insulin stimulates β-cell proliferation could potentially lead to new strategies to correct deficient β-cell mass in diabetes. It has been well established from work in other cell types that insulin activates two main signalling pathways, driven respectively by PI3-kinase/Akt and Raf-1/MEK kinases (87). Evidence from over-expression studies led many investigators to focus on Akt in the control of β-cell mass (57, 89), however, it is notable that mice lacking ~80% of islet Akt activity have normal β-cell mass, even in response to high fat feeding (137). Moreover, we have previously reported that 200 pM insulin does not stimulate Akt phosphorylation in primary mouse or human islets (130). The stimulatory effects of insulin on β-cell proliferation we observed in this study may be mediating its effects through an alternate pathway, possibly the Raf-1/ERK pathway. A previous study showed that over-expression of a Raf-1 kinase inhibitory protein in HIT-T15 cells decreased β-cell proliferation (138). Preliminary data completed in our laboratory suggests that Raf-1 is activated in the presence of 0.2 nM and 200 nM insulin (but not 2 nM and 20 nM insulin) in mouse islets, human islets and MIN6 cells (E. Alejandro and J. Johnson, unpublished observations). In the present study, we found that Raf-1 is required specifically for the

proliferative effects of physiological concentrations of insulin. Our results are the first to implicate the Raf/ERK/MEK pathway in insulin-stimulated β-cell proliferation. The present study suggests the role of the PI3-kinase pathway is broader, also mediating the effects of super-physiological concentrations of insulin. The difference observed between PI3-kinase and Raf-1 inhibition on β-cell proliferation may reflect the relative response of specific insulin doses on insulin and IGF-1 receptor activation. This is further complicated by the possibility of insulin/IGF-1 receptor heterodimerization.

There is also considerable cross-talk between these two arms of the insulin signalling pathway. This may help explain why Raf-1 inhibition had no effect on super-physiological insulin signalling in primary cells. Specifically, active Akt has been shown to decrease phosphorylation of ERK1/2 (151). Perhaps the inhibition of Raf-1 failed to effect β-cell proliferation at 200 nM insulin because Akt had inhibited the Raf-1 pathway via ERK1/2. The role of ERK1/2 in β-cell proliferation remains understudied. Although some aspects of the anti-apoptotic Raf-1 signalling are independent of ERK1/2 (164), this question has not been addressed in detail in regards to primary β-cell proliferation. In conclusion, further examination is required to fully understand the molecules and pathways involved in regulating β-cell proliferation.

Mechanisms of insulin-induced β-cell proliferation – cell cycle components

Molecules that regulate the β-cell cycle have been extensively studied (37, 54-70), but their regulation by endogenous or exogenous stimuli has not been examined in detail. In the present study, we performed preliminary experiments that will hopefully lead to future comprehensive investigations. In a few cases, we did observe noticeable differences in the expression of cell cycle mRNAs or proteins. For example, blocking endogenous insulin signalling with HNMPA-AM did show a trend which agrees with the changes in gene expression of cyclin D2, cyclin G2 and p21 observed with 80% knockdown of the insulin receptor in MIN6 cells (103). Unfortunately, efforts to further characterize the gene expression of cell cycle regulatory proteins (cyclin D1, cyclin D2, cyclin D3, cyclin G1, cyclin G2 and p21) were unsuccessful. The gene expression experiments were carried out in primary mouse islets, which have approximately 80% β-cells with only an extremely small population of those β-cells

dividing. We may have observed more robust changes using cells that divide more frequently such as MIN6 cells, and more delicate methods such as real time PCR or single cell analysis on a replicating β-cell. Further attempts to elucidate the effects of insulin on β-cell cycle machinery were carried out by examining protein expression levels. Experiments completed on human islets revealed no significant effect of insulin on phosphorylation of Rb, however most of the human islets we receive are less than 60% pure with only a tiny fraction of those β-cells dividing. Even though MIN6 cells were used because of their high rate of proliferation, it is possible that we failed to see significant changes because the cells were not synchronized. It is also possible that the cyclic nature of the proteins involved complicates analysis or that protein translocation is more critical than total protein levels of these cell cycle regulators in β-cells. Nevertheless, a recent study also suggests that Rb may not a critical regulator of β-cell mass (71) and cell cycle regulation may occur through an alternate pathway in β-cells. One possible alternate mechanism that has been recently described is the Gcn2/eIF2 pathway (165). Examining protein expression of other cell cycle molecules (cyclin B, cyclin D1, cyclin E, cyclin G2, p15, p16, p21 and p27) treated with varying doses of insulin and pharmacological inhibitors of the insulin receptor pathway were also unsuccessful. Non-specific protein bands observed on the western blots hindered much of the analysis (data not shown; cyclin B, cyclin E, p15, p16, and p27). Future studies must take into account the complexity of the molecules regulating the cell cycle. Cell cycle molecule activity is complex because of the transient nature of cell cycle proteins and the many ways in which they can be altered. Gene expression does not always correlate with protein expression because of phosphorylation and ubiquitination status of the cell cycle molecules (57). In addition, activation of many cell cycle proteins requires the formation of complexes and a change in cellular localization, as seen with p21 (69). Although attempts were made to further elucidate endogenous regulation of various β-cell cycle molecules, more work is required.

Effect of autocrine insulin on insulin secretion

Insulin secretion from the β-cell is regulated at a variety of steps, including insulin transcription, translation and exocytosis. It was initially reported that βIRKO mice had decreased insulin content (but not insulin gene expression) and a decrease in glucose-stimulated insulin secretion (108-110). However, it later became clear that many of these defects could be

accounted for by a significant reduction in β-cell mass, due to a combination of increased β-cell apoptosis and decreased β-cell proliferation (108-110) . While these studies examined the long-term effects of insulin on the β-cell, the present study measured acute insulin action (0-3 days). In our hands, we find significant differences in β-cell proliferation under these conditions, but no changes in insulin secretion. While studies have shown that autocrine insulin signalling can activate insulin gene transcription under specific circumstances, insulin secretion has not been well characterized (105, 166-168). Furthermore, the role of physiological doses of insulin on human β-cells showed autocrine insulin increased insulin content (transiently at 90 minutes) but not secretion (102), consistent with the current study. In the present investigation, we showed that blocking the insulin receptor pathways had no additional effect on insulin secretion, in accordance to previous studies (89, 137, 148). Although insulin has been shown to increase cytosolic calcium levels in β-cells, this occurs though release from intracellular stores (102, 111), a site that would not be expected to favour insulin exocytosis. On the other hand, glucose-stimulated insulin secretion is caused by an influx of calcium from extracellular space through voltage-gated calcium channels which are located in close proximity to insulin granules (169). Thus, the distinct calcium release sites used by insulin signalling and glucose signalling can potentially explain the differences seen in the regulation of insulin secretion. In conclusion, our work and the work of others points to a dominant effect of insulin on β-cell fate, not on the day-to-day function of these cells.

Conclusion and significance

A loss of β-cell mass is a hallmark of type 1 diabetes and increasing evidence points to a relative β-cell deficiency in type 2 diabetes compared to age- and weight-matched controls (21). Inadequate β-cell mass is a also major limiting factor of islet transplantation (19). It is now clear that β-cell proliferation is critical for maintaining a stable and adaptable β-cell mass (34, 35, 37, 60-62, 113). In the present study, our findings suggest that physiological and super-physiological doses of insulin can directly stimulate β-cell proliferation via Raf-1 and PI3-kinase. However, a complete understanding of the insulin signalling pathway remains undefined because of the complexity of the signalling pathways involved. The characteristics of the insulin and IGF-1 receptor pathways on β-cell proliferation, specifically downstream signalling molecules, require

further clarification in order to improve our knowledge of basic β-cell physiology. Understanding the endogenous regulators of β-cell proliferation has the potential to increase β-cell mass in diabetes and islet transplantation. Since tipping the balance in favour of β-cell mass growth comes with the potential unwanted danger of creating pancreatic islet cell tumors, a detailed understanding of the mechanisms involved is crucial. The downstream targets and cell cycle molecules involved in increasing β-cell proliferation with physiological doses of insulin warrant further investigation. Overall, the findings in this study are intriguing and suggest critical links between insulin and its receptor pathway in the regulation of β-cell proliferation.

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