Chapter 7
Work Towards Identifying Stimuli That Induce CREB Glycosylation

Summary. *O*-GlcNAc glycosylation levels in a number of cell types have been shown to dynamically respond to glucose concentrations. This observation, along with the identification of hyper-*O*-GlcNAc glycosylation in a number of mammalian models of hyperglycemia, inspired the hypothesis that abnormal *O*-GlcNAc glycosylation may contribute to the pathology of diabetes, where hyperglycemia is a hallmark of the disease. Using the ketone labeling approach developed in the Hsieh-Wilson lab, the level of CREB glycosylation in pancreatic islets and cultured cells was measured in a number of model systems. While cellular *O*-GlcNAc levels were indeed sensitive to changes in glucose levels, no parallel change in CREB glycosylation was observed. Whether CREB glycosylation is unresponsive to changes in glucose concentrations or whether the methods employed here are incapable of capturing glycosylation dynamics remains a question still unresolved. Preliminary studies have shown that high glucose increases IRS2 protein levels in direct opposition to GlcN treatment, implicating different mechanisms underlying the effect of glucose and GlcN. Treatment of pancreatic islets with the cytokines interferon-γ, interleukin-1β and tumor necrosis factor-α suggest that they inhibit CREB activity. However, the possible contribution of CREB glycosylation to this inhibition has yet to be elucidated.

Type II Diabetes and *O*-GlcNAc Glycosylation

Once uncommon, diabetes mellitus - a disease that is marked by the body's inability to regulate blood glucose levels - has reached epidemic proportions in the past few decades [1]. Type II diabetes is a complicated disease with no single effector responsible for its development. It is known that obesity, which has also seen a dramatic rise in recent years, can exacerbate weaknesses in metabolic regulation that lead to diabetes. The increased stress that obesity places on organs such as the pancreas in effect lowers the bar for the onset of type II diabetes [2]. Type II diabetes begins with the development of insulin resistance in skeletal muscle and adipose tissue, characterized by the inability of physiological levels of insulin to cause these peripheral tissues to sequester glucose from the blood (Fig. 7-1B). The situation is worsened by increased hepatic gluconeogenesis increasing blood glucose levels. Normally, the body is able to compensate for the insulin resistance by increasing the production of insulin through a combination of pancreatic β-cell hyperplasia and hypertrophy (Fig. 51B). As insulin resistance persists in late-stage type II diabetes, hyperglycemia develops as a result of a loss of β-cell mass, due to increased β-cell apoptosis (Fig. 7-1C) [3, 4]. There are a number of possible contributors to the increase in apoptosis, including metabolic stress, increased adipose-secreted cytokine secretion, lipotoxicity and glucose toxicity [5, 6]. Hyper-*O*-GlcNAc glycosylation has been posited as a possible cause of glucose toxicity [3, 7].

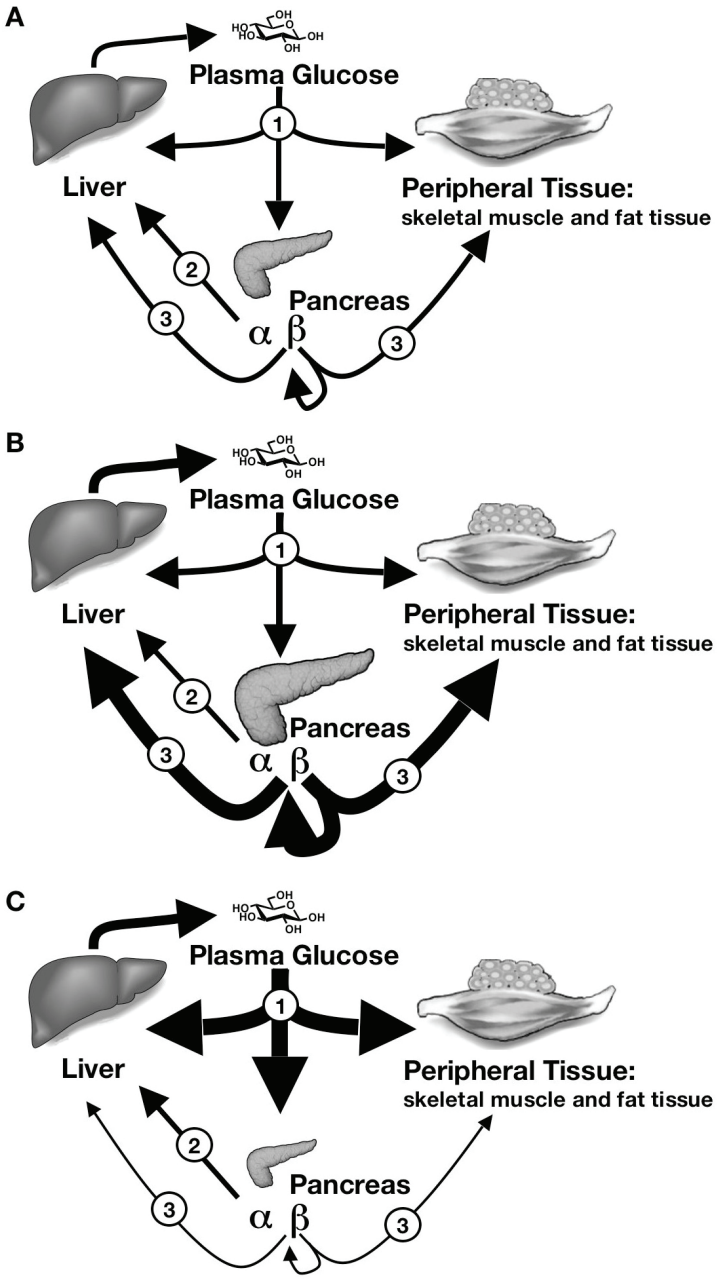

Figure 7-1. Type II Diabetes and β-Cell Apoptosis. **(A)** Under normal conditions, endocrine cells monitor plasma glucose levels and stimulate either the sequestration of glucose with the secretion of insulin by β-cells (2) or gluconeogenesis by the liver with the secretion of glucagon by α-cells (3). **(B)** In the early stages of type II diabetes, peripheral tissues display insulin resistance. The body compensates with hyperinsulemia (3) resulting from an increase in β-cell mass via a combination of hyperplasia and hypertrophy and an increase in insulin production. **(C**) In late-stage diabetes, hyperglycemia (1) develops as a result of persistent insulin resistance and the loss of β-cell mass. The loss of β-cell mass results from apoptosis caused by glucose-toxicity, metabolic stress and extracellular apoptotic signaling [1-4].

One of the hallmarks of diabetes is an inability of the body to process glucose properly. Approximately 2-5% of intracellular glucose reportedly travels through the hexosamine biosynthesis pathway, and increased glucose flux through this pathway results in increased production of UDP-GlcNAc, which serves as a substrate for *O*-GlcNAc modification [8]. Excess *O*-linked glycosylation has been detected in the pancreas and corneas of diabetic rats and has recently been implicated in cardiac dysfunction in pancreatic β-cells and the diabetic heart [7-11]. A number of proteins along the anti-apoptotic insulin/IGF-1 signaling cascade are modified with *O*-GlcNAc, including IRS1/2, PI3K and Akt [12, 13]. It is likely that the siRNA knockdown of OGT also relieves the inhibitory effects that OGT has on insulin/IGF-1 signaling. However, our work with the wild-type and AAA mutant CREB suggests that CREB glycosylation is a major contributor to the potentiation of pancreatic β-cells to apoptosis. Although it is premature to state a direct link between *O*-GlcNAc glycosylation and β-cell failure in type II diabetes, the role of *O*-GlcNAc in inducing pancreatic β-cell death through CREB glycosylation merits further investigation (Fig. 7-2).

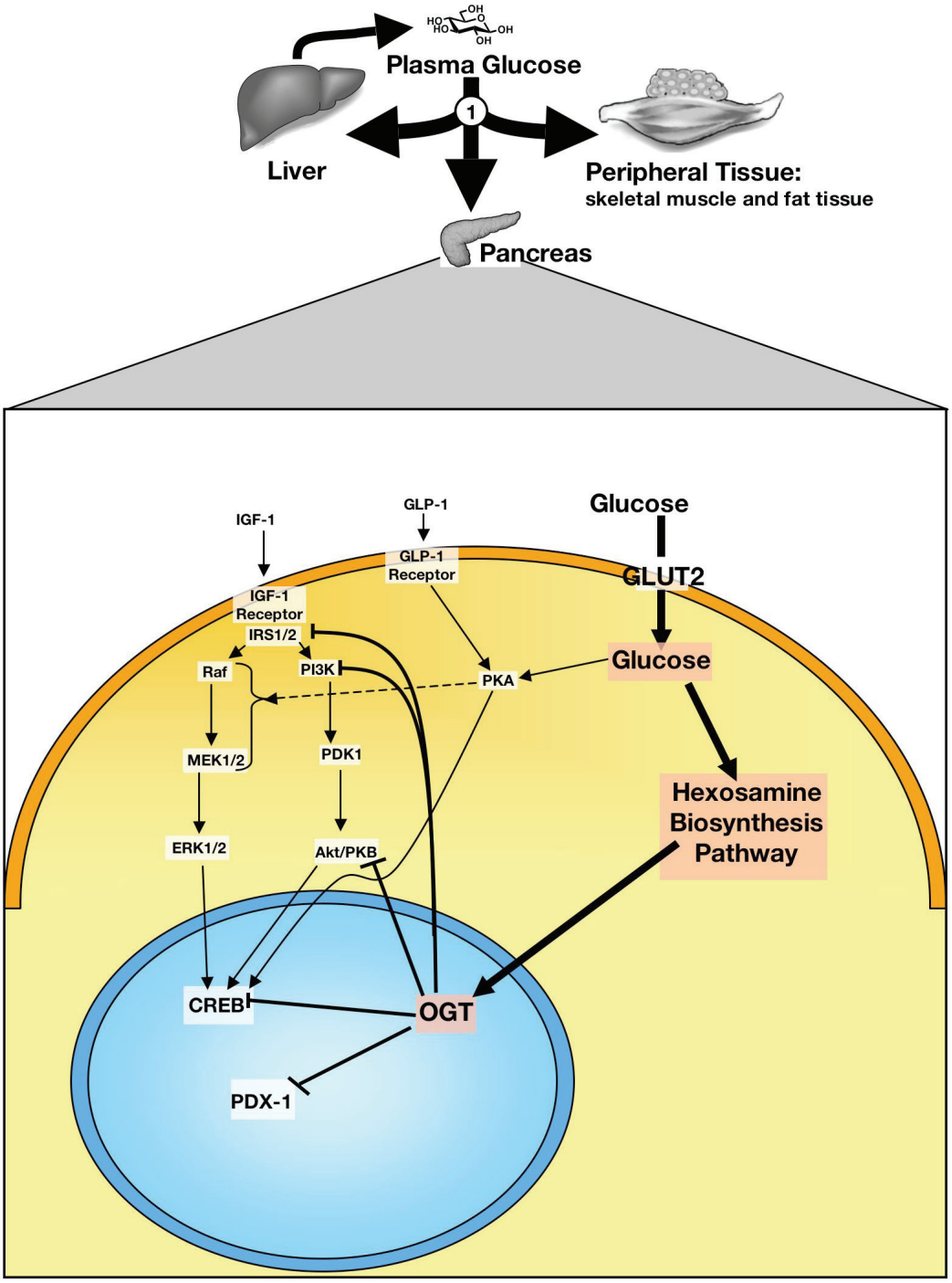

Figure 7-2. Diabetes, β-Cell Dysfunction and *O*-GlcNAc Glycosylation. Studies have shown that chronic high glucose treatment of β-cells causes an increase in global *O*-GlcNAc levels concomitant with glucose toxicity. A number of models of diabetes, including the genetic models diabetic Goto-Kakizaki rats, Zucker fatty rats and the pharmacological models streptozotocin and alloxan treatment exhibit increased *O*-GlcNAc glycosylation, suggesting that the increased glycosylation may contribute to the disease state [9, 16, 20-25]. Several of the proteins within the insulin/IGF-1 signaling pathway are *O*-GlcNAc glycosylated and de-regulation of this pathway as a result of hyper-glycosylation may affect β-cell dysfunction in diabetes [40].

Effect of High Glucose on CREB Activity in HIT-T15 Cells. Acute hyperglycemia (< 24 h) induces CREB phosphorylation via PKA, which is thought to contribute to glucose-stimulated insulin production in pancreatic β-cells [14-16]. Chronic hyperglycemia has been linked to increased *O*-GlcNAc glycosylation and β-cell failure [7, 10, 11, 17]. To determine whether chronic hyperglycemia affects CREB activity adversely, we transfected HIT-T15 cells with the CRE-luciferase reporter and monitored the relative strength of Fsk-induced activation. HIT-T15 cells cultured in low (physiological) glucose (5.5 mM) and high glucose (25 mM) media.

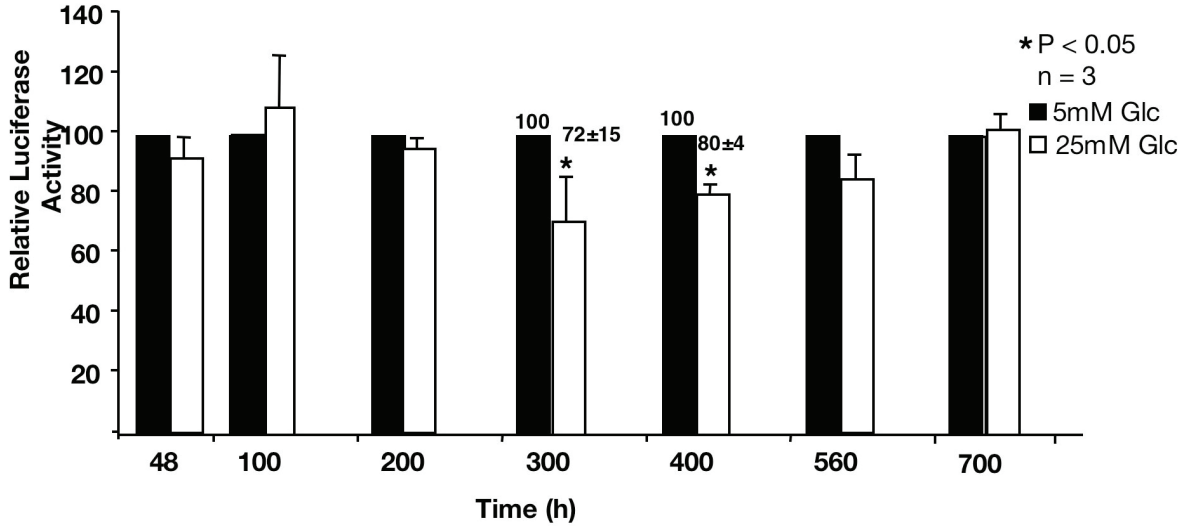

Figure 7-3. High glucose treatment reduces CREB activity in a time-dependent manner, but CREB activity recovers after 700h. HIT-T15 cells were transfected with a CRE-luciferase reporter and β-galactosidase expression plasmid 24 h prior to analysis. Cells were incubated in the presence of low glucose (5.5 mM) or high glucose (25 mM) for the indicated amount of time. Fsk (10 μM) was added to the medium for 6 h and cells were harvested, lysed and luciferase and β-galactosidase activities were quantified. Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± SEM. (Student's T-test,*P = 0.05; n = 3)

After having been cultured in high glucose media for approximately 12 days, HIT-T15 cells exhibited a decreased level of Fsk-induced transactivation. Interestingly, the reduction in CREB-mediated transcription recovered with time, such that after an additional 6 days there was no longer a statistically significant difference in the inducible activity of CREB (Fig. 7-3). The reduction in CREB activity is consistent with our studies using GlcN, which

suggests that CREB glycosylation inhibits its transcriptional activity. The recovery observed may due to compensatory effects. Furthermore, it should be noted that high glucose treatment did not seem to reduce the health of the cells, as they continued to grow at a rate more rapid than cells grown at the low glucose levels, suggesting the these *in vitro* conditions may not mimic a diabetic state.

High Glucose Elevates Cellular *O*-GlcNAc Glycosylation, But Has No Observed Effect on CREB Glycosylation. To determine if the observed decrease in CREB activity in HIT-T15 cells could be caused by a change in CREB glycosylation, HIT-T15 cells were grown for 300 to 400 h and then cellular O-GlcNAc, α–tubulin and CREB levels were measured by immunoblotting. Coincident with the change in CREB activity, an increase in cellular glycosylation was observed (Fig. 7-4A). The level of CREB glycosylation was then measured using the ketone labeling /immunoprecipitation approach previously described. No change in CREB glycosylation was observed (Fig. 7-5A).

In addition to experiments with the HIT-T15 pancreatic cell line, the effects of growth in physiological/low glucose (5.5 mM) and high glucose (25 mM) were tested in MIN6 cells and in cultured rat and human pancreatic islets. Studies of these cell types showed that after 24 h in culture, cells grown in high glucose media had increased levels of cellular *O*-GlcNAc as measured by immunoblotting (Fig. 7-4B-D).

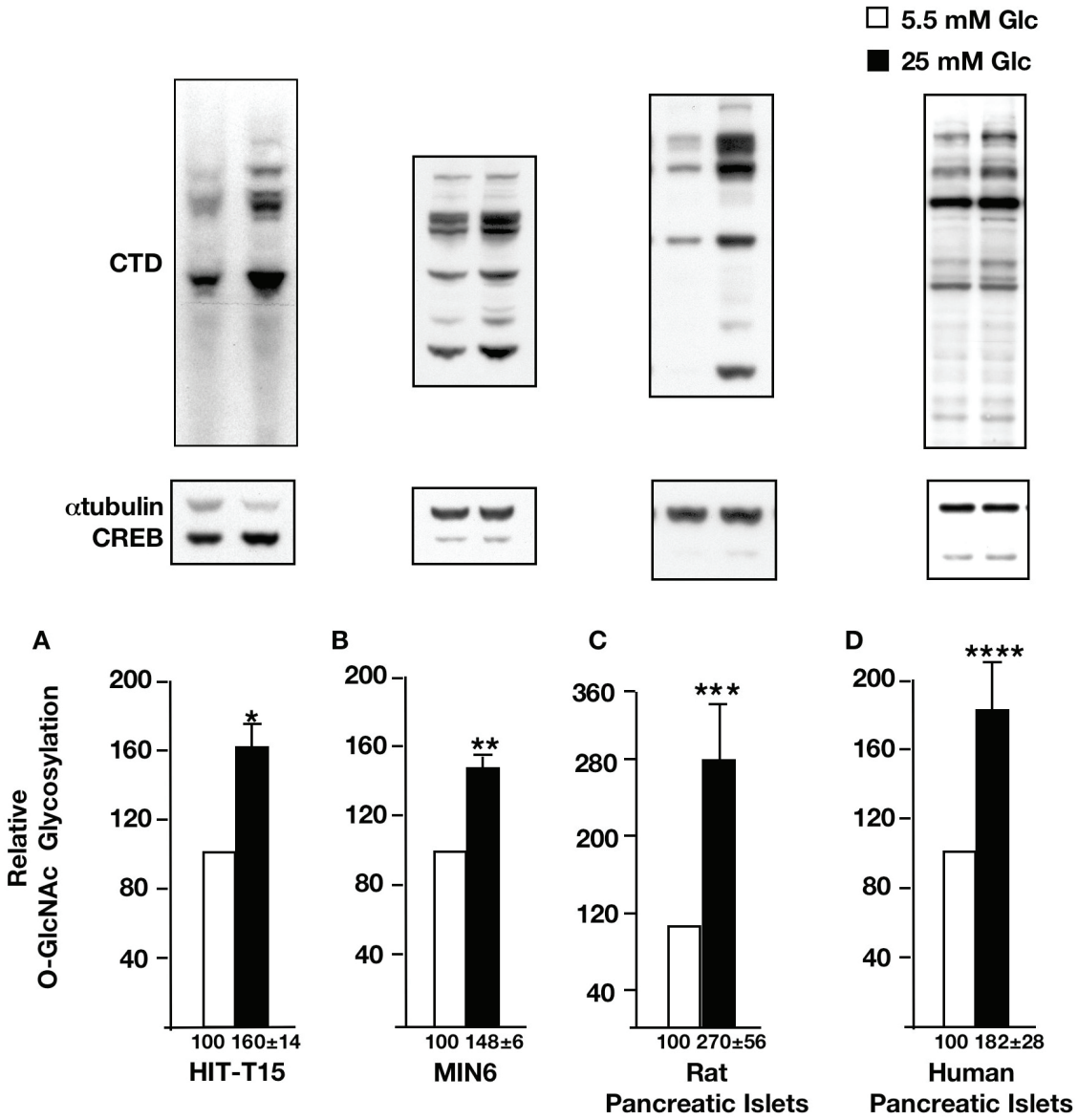

Figure 7-4. High glucose treatment increases cellular *O*-GlcNAc glycosylation. **(A)** HIT-T15 were incubated in the presence of low glucose (5.5 mM) or high glucose (25 mM) for ~300 h prior to being harvested and lysed. Cellular *O*-GlcNAc levels were measured by immunoblotting with an anti-*O*-GlcNAc antibody. **(B)** MIN6 cells were incubated in the presence of low glucose (5.5 mM) or high glucose (25 mM) for 24 h prior to analysis as in (A). **(C,D)** Human and rat pancreatic islets were isolated and incubated in low glucose (5.5 mM) or high glucose (25 mM) media for 24 h prior to analysis as described in (A). Values were normalized to total protein levels by immunoblotting with an anti-α–tubulin antibody (Sigma-Aldrich, 1:5,000) and represent the mean ± SEM (Student's T-test, *$P < 0.02$, n = 6; **$P < 0.001$, n = 5; *** $P < 0.03$, n = 7; ****$P < 0.05$, n = 5).

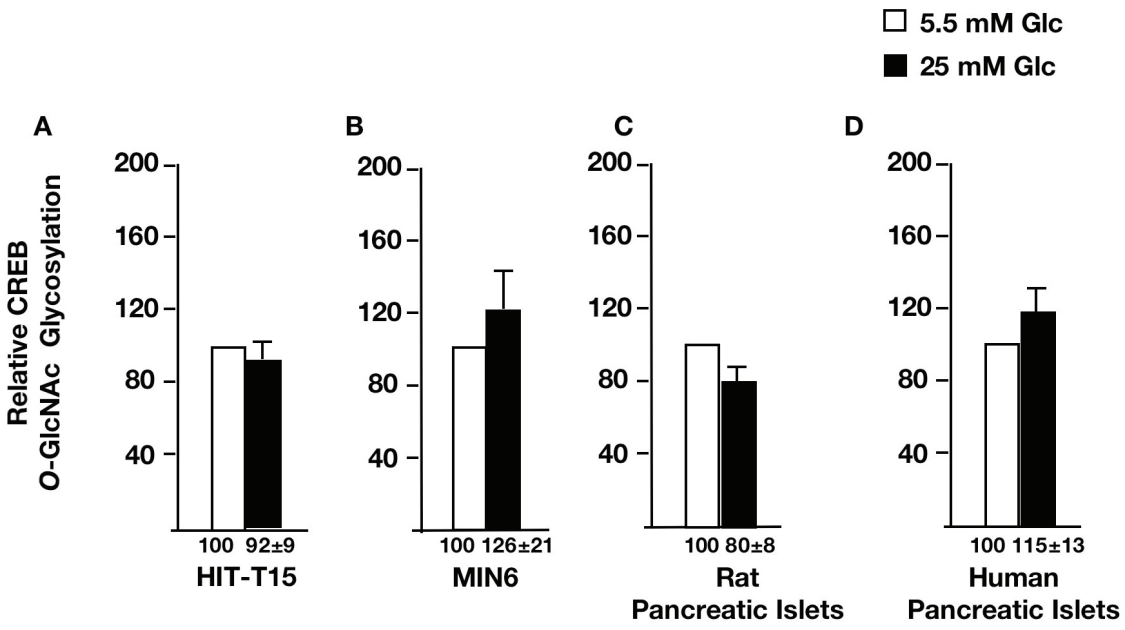

Figure 7-5. No change in CREB glycosylation was observed in response to high glucose treatment. Relative CREB glycosylation was measured using the ketone labeling approach. *O*-GlcNAc glycosylated proteins were labeled by incubating cell extracts with a non-natural UDP-galactose substrate containing a ketone functionality (UDP analogue 1) and an engineered β-1,4-galactosyltransferase enzyme (mutant Y289L GalT). Labeled proteins were then modified with an aminooxy biotin derivative (Dojindo). CREB was isolated by immunoprecipitation (IP) with a anti-CREB antibody (Upstate). The success of the IP was measured by immunoblotting with an anti-CREB antibody (Chemicon) and relative glycosylation was detected by immunoblotting with streptavidin-horse radish peroxidase (Pierce). **(A)** HIT-T15 cells were treated as in Figure 7-4A. **(B)** MIN6 cells were treated as in Figure 7-4B. **(C,D)** Pancreatic islets were treated as in Figure 7-4C. Values represent the mean ± SEM. (Student's T-test, HIT-T15 cells P = 0.43, n = 6; P = 0.43, n = 2; P = 0.08, n = 7; human islets P = 0.32, n = 5)

However, consistent with the results from the HIT-T15 cells, there was no observed change in the levels of CREB glycosylation (Fig. 7-5B-D). It is possible that, in the cases described above, the measurement of CREB glycosylation is confounded by deglycosylation during the processing of the samples, as previous studies with GlcN treatment had found changes in CREB glycosylation nearly identical to the observed changes in cellular *O*-GlcNAc (Fig. 5-3 and 5-4). On the other hand, it is also possible that alternative pathways are activated by increased flux through the hexosamine biosynthesis pathway induced by high glucose and GlcN treatment, and the failure to

increase CREB glycosylation is a result of these differences. Additional studies are being conducted with MIN6 cells and human pancreatic islets to determine if the slight increase in CREB glycosylation observed may turn out to be statistically significant.

Cellular and CREB *O*-GlcNAc Glycosylation in Animal Models of Diabetes.

An alternative to the study of cultured cells is to study the effects of hyperglycemia *in vivo* using animal models of hyperglycemia. Animal models have the advantage of allowing observation of all the effects, direct and indirect, that hyperglycemia may have on pancreatic β-cells. We looked at a number of systems where hyperglycemia was induced in rats both chemically and by genetic mutation (Fig. 7-6).

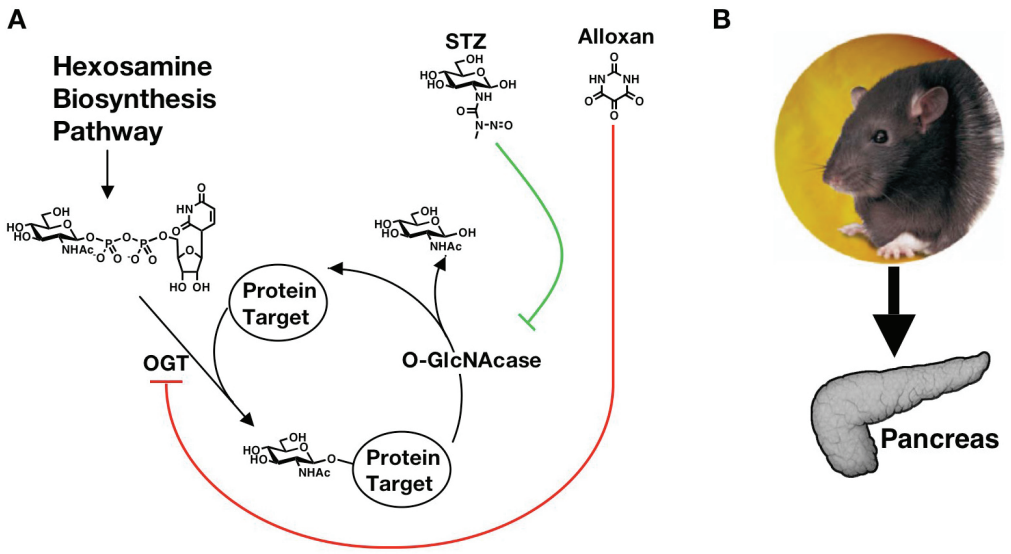

Figure 7-6. Animal models of diabetes: alloxan treatment, streptozotocin treatment, Zucker diabetic fatty rats and Goto-Kakizaki rats. **(A)** Alloxan and streptozotocin treatments are known to produce a diabetic phenotype in rats by targeted destruction of pancreatic endocrine cells. In addition, alloxan inhibits OGT activity and streptozotocin inhibits *O*-GlcNAcase activity, suggesting that a loss of the proper regulation of *O*-GlcNAc glycosylation leads to cell death. Alloxan treatment has been shown to increase *O*-GlcNAc glycosylation in the penile tissue of rats and streptozotocin treatment elevates *O*-GlcNAc glycosylation in pancreatic islets. **(B)** The Zucker diabetic fatty rats and Goto-Kakizaki rats contain genetic abnormalities that lead to obesity, hyperglycemia and elevated *O*-GlcNAc glycosylation. These models were used to determine CREB glycosylation levels in pancreatic islets as a result of chronic hyperglycemia.

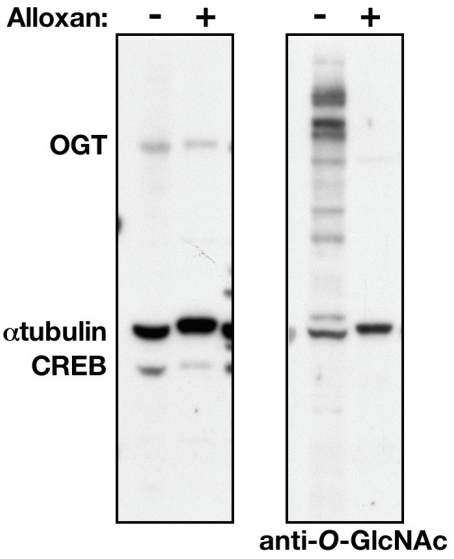

Figure 7-7. Alloxan treatment of rats causes ablation of endocrine cells in the pancreas. Male Sprague-Dawley rats were injected with 140 mg/kg alloxan or vehicle. 72 h post-injection rats treated with alloxan exhibited hyperglycemia (30 mM). Pancreatic islets were isolated, lysed and OGT, CREB, αtubulin and *O*-GlcNAc levels were measured by immunoblotting.

Alloxan Treatment Causes Ablation of Endocrine Cells in the Pancreas. Alloxan-induced hyperglycemia causes increased *O*-GlcNAc glycosylation of eNOS and disrupts erectile function of rat penile tissue, and alloxan treatment of rats is an established model of diabetes. As such, we looked at the possibility that alloxan-induced hyperglycemia may increase CREB glycosylation in pancreatic β-cells [18]. Further study revealed that it is believed that alloxan causes hyperglycemia through causing necrosis of pancreatic β-cells and also inhibits OGT activity [19-21]. 72 h after intra-peritoneal injection of alloxan (140 mg/kg), rats were severely hyperglycemic (≥542 mg/dL). Severe declines in CREB and *O*-GlcNAc levels were observed. Analysis of pancreatic islets from control and alloxan-treated rats confirmed that alloxan treatment had resulted in the near ablation of pancreatic islet endocrine cells (Fig. 7-7). The direct action of alloxan upon the endocrine cells of the pancreas and its inhibition of OGT activity appear to make it unlikely that this system could be used to measure hyperglycemia-induced increases in CREB glycosylation.

Streptozotocin Treatment Leads to Pancreatic Islet Cell Death. Another well-known model for hyperglycemia is streptozotocin treatment. Streptozotocin (STZ), like

alloxan, causes hyperglycemia by causing pancreatic β-cell toxicity. STZ is known to induce DNA alkylation, release NO and inhibit *O*-GlcNAcase, and all may contribute to its toxic effects, though there is still debate as to whether the inhibition of *O*-GlcNAcase actually plays a direct role [22-27].

Rats were administered a single intra-peritoneal injection of STZ (80 mg/kg) or vehicle and their blood glucose was monitored. Within 6 h after STZ administration, rats exhibited elevated blood glucose levels and were severely hyperglycemic 24 h after treatment (535 mg/dL) (Fig. 7-8). Pancreatic islets were analyzed from STZ-treated rats euthanized 6, 12, 24 and 48 h after STZ administration by *O*-GlcNAc, α-tubulin and CREB immunoblotting (Fig. 7-8). As with alloxan treatment, increased blood glucose levels were accompanied by loss of pancreatic islet endocrine cells as measured by a loss of *O*-GlcNAc and CREB immunoreactivity. Measurement of relative CREB glycosylation from isolated pancreatic islets from control and STZ-treated rats, performed by Nelly Khidekel, found no difference in the level of glycosylation (n = 4) (Fig. 7-8). The severe reduction in CREB levels due to advanced cell death made measurements of glycosylation difficult and may have complicated the results.

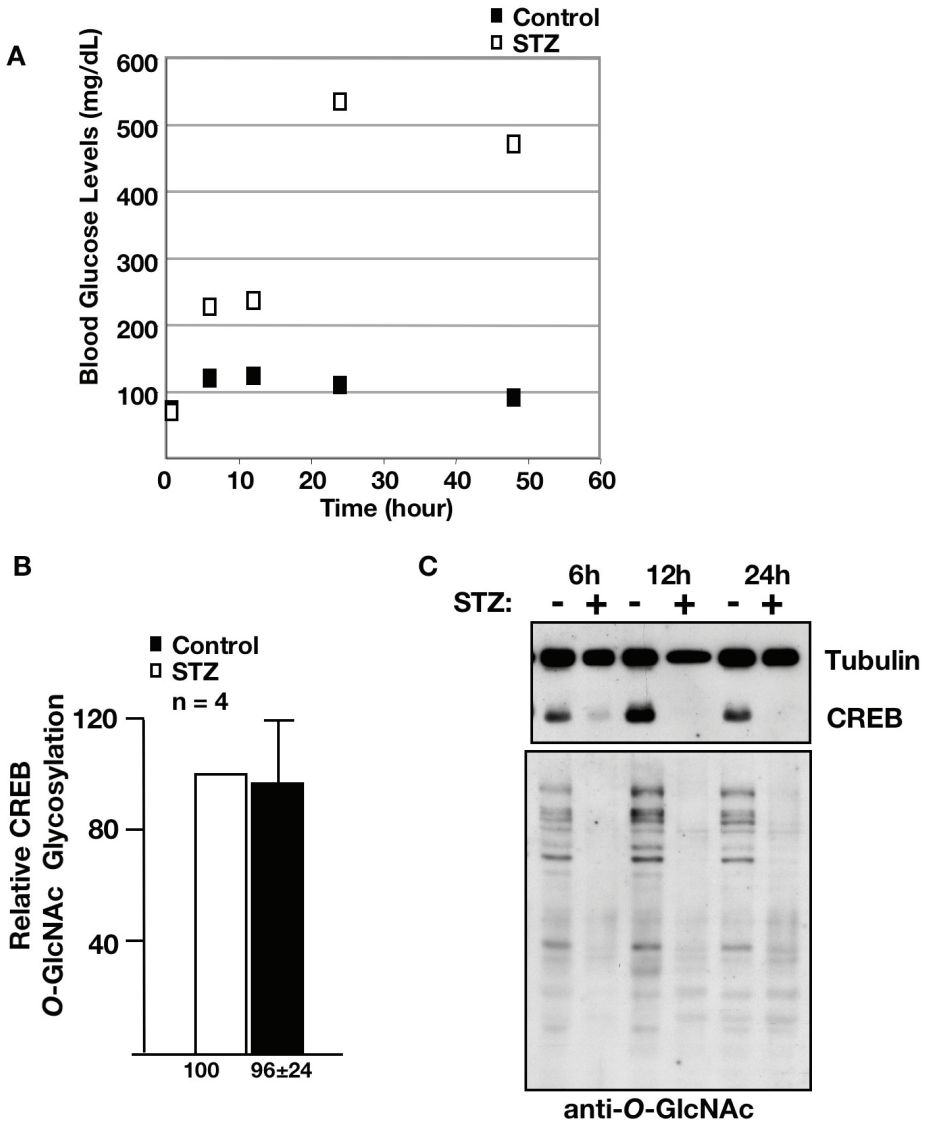

Figure 7-8. Streptozotocin (STZ) treatment leads to increases in *O*-GlcNAc glycosylation concomitant with pancreatic islet loss, but no observed change in CREB glycosylation. Male Sprague-Dawley rats were fasted overnight prior to being treated with 80 mg/kg streptozotocin or vehicle and fed a 5% glucose solution. Animals were euthanized at indicated times, pancreatic islets were isolated, lysed and analyzed by *O*-GlcNAc, CREB and αtubulin immunoblotting and relative CREB glycosylation was measured as described in Figure 7-5 at the 48 hour time point. **(A)** STZ-treatment results in hyperglycemia within 24 hours after treatment. **(B)** No difference in CREB glycosylation was observed between control and STZ-treated rats (n = 4). **(C)** The decline in measured CREB concentration is an indicator of loss of pancreatic β-cells and provided the measurement of CREB glycosylation in the majority of experiments.

_O_-GlcNAc Glycosylation Is Enhanced in Hyperglycemic Zucker Fatty Rats and Goto-Kakizaki Rats. In an effort to avoid the β-cell toxicity that compromised the alloxan and STZ experiments, we looked at two rat models of type II diabetes: Zucker diabetic fatty (ZDF) rats and Goto-Kakizaki (GK) rats.

	BGL (mg/dL)		Relative CREB Glycosylation	
	Control	ZDF	Control	ZDF
69 day old mice	85	338	100	70.8
80 day old mice	86	380	100	76.3

Figure 7-9. There is no observable increase in CREB glycosylation in hyperglycemic Zucker diabetic fatty (ZDF) rats. ZDF rats express a truncated form of the leptin receptor incapable of proper signaling, which results in the development of a diabetic phenotype, including hyperglycemia and insulin resistance. 69- and 80-day old control and ZDF male rats were euthanized, pancreatic islets were isolated and relative CREB glycosylation was measured as described in Figure 7-5.

ZDF rats have a deletion mutation in the leptin receptor that results in improper signaling and manifests itself as severe obesity and hyperglycemia [28]. The blood glucose of 69- and 80-day-old ZDF rats and heterozygous littermates was measured. Both ZDF rat age groups exhibited hyperglycemia and increased pancreatic islet cellular *O*-GlcNAc levels (data not shown). Pancreatic islets were isolated from ZDF and heterozygous litter mates, and relative CREB glycosylation levels were measured by Nelly Khidekel. Surprisingly, the ZDF rats exhibited a potential decrease in CREB glycosylation (Fig. 7-9). Only one set of data was collected using this model system, so we cannot address yet whether the observed decrease is significant.

GK rats, like ZDF rats, develop hyperglycemia, but GK rats do not become obese. The hyperglycemia in GK rats has been linked to defects with pancreatic β-cell function and proliferation [29, 30]. It was also shown that OGT and cellular *O*-GlcNAc are increased in a number of tissues in GK rats [9]. Adult GK rats exhibiting hyperglycemia were euthanized, pancreatic islets were isolated and cellular *O*-GlcNAc levels were

measured by immunoblotting. Levels of *O*-GlcNAc glycosylation were increased in the GK rats relative to paired wild-type Wistar rats (Peter Clark, personal communication). Studies are underway to determine whether the observed increase in cellular *O*-GlcNAc levels in the GK rats results in enhanced CREB glycosylation.

Possible Alternative Avenues for Regulation of CREB Glycosylation. Studies have shown that pathways other than nutrient sensing via the hexosamine biosynthesis pathway (Scheme 2-1) are capable of effecting *O*-GlcNAc glycosylation. Heat shock causes an increase in *O*-GlcNAc glycosylation in lymphocytes [31]. In neurons treatment with kinase inhibitors lead to increases in *O*-GlcNAc glycosylation [32]. Kainic acid-induced seizure resulted in elevated levels of *O*-GlcNAc glycosylation on a number of neuronal proteins [33].

Prior to looking at alternatives to hyperglycemia, I returned to look at the effect of hyperglycemia on IRS2 expression. We had observed that chronic hyperglycemia (24-29 h) was sufficient to elevate *O*-GlcNAc levels in cultured rat pancreatic islets (Fig. 7-4). Assuming that CREB glycosylation was enhanced as well, we should observe a decline in the protein levels of IRS2, as a result of inhibitory effect of CREB glycosylation. In addition, the effects of low glucose (5.5 mM, physiological) and high glucose (25 mM) treatment on IRS2 levels were measured in the absence or presence of GlcN, which has been shown to reduce IRS2 expression (Fig. 6-2). Interestingly, IRS2 levels were increased nearly 3-fold higher in islets grown in high glucose media for 29 h, which suggests that CREB is relatively more active (Fig. 7-10).

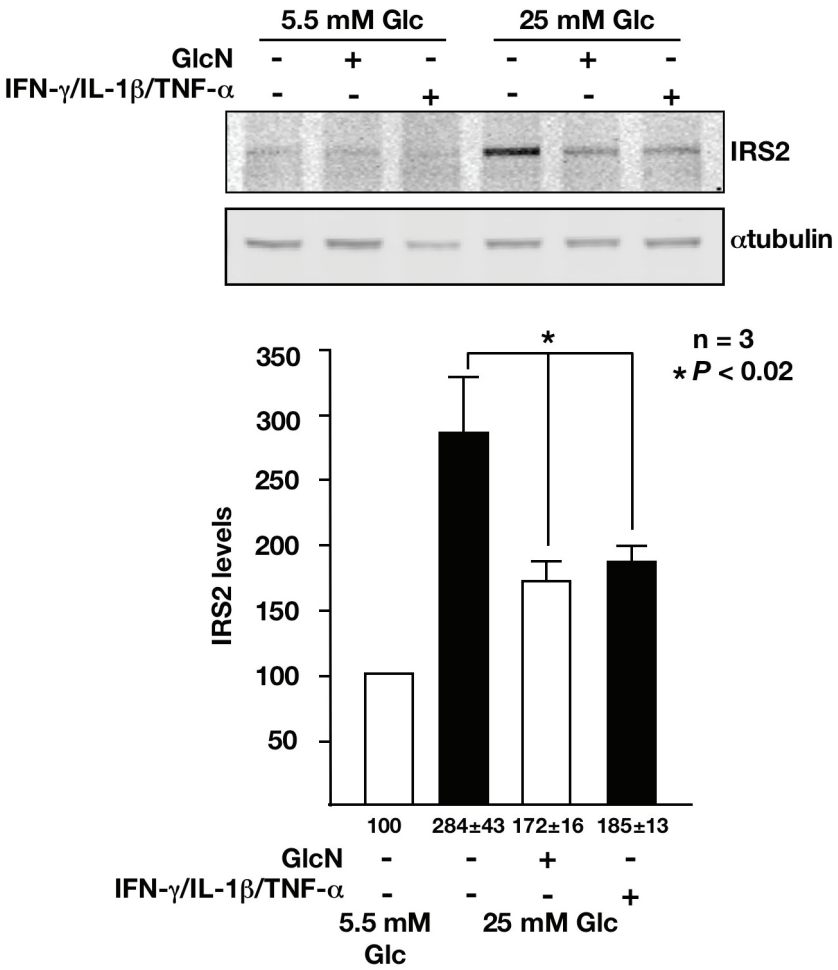

Figure 7-10. Effect of glucose, cytokines and GlcN on IRS2 expression. Isolated pancreatic islets were grown for 29 h in either low glucose (5.5 mM) or high glucose (25 mM) media. At 20 h into the incubation selected plates were administered GlcN (10 mM) or a combination of interferon-γ (IFN-γ, 1kU/ml), interleukin-1β (IL-1β, 100 U/ml), and tumor necrosis factor-α (TNF-α, 1 kU/ml). Cells were harvested after 29 h, lysed in boiling 1% SDS + protease inhibitors + phosphatase inhibitors and lysate was resolved by SDS-PAGE. IRS2 levels were measured by anti-IRS2 (Upstate, Charlottesville, VA, 1:1000) immunoblotting and corrected to protein concentration as measured by anti-αtubulin (Sigma-Aldrich, St. Louis, MO; 1:5000). Values were normalized relative to the untreated cells and represent the mean ± SEM. (Student's T-test * $P < 0.02$; n = 3).

GlcN treatment resulted in a 40% decrease in IRS2 expression in islets, similar to previous results from HIT-T15 and MIN6 cells.

These results suggest that hyperglycemia and GlcN treatment have different effects on pancreatic β-cells. Similar results have been observed in adipocytes and skeletal muscle, where both GlcN and hyperglycemia cause insulin resistance [34-37]. The hyperglycemia-induced insulin resistance can be blocked by inhibitors of protein

synthesis [35, 37]. While GlcN treatment was resistant to protein synthesis inhibitors, it was also noted that GlcN treatment can lead to the accumulation of GlcN-6-phosphate and the reciprocal depletion of ATP, which may have deleterious effects [35-38]. The accumulation of GlcN-6-phosphate after GlcN treatment is also an indicator of the fact that UDP-GlcNAc production has reached a maximum rate of production, reflected in a 5-fold increase in UDP-GlcNAc levels with GlcN treatment, while hyperglycemia elicits only a 2-fold increase in the concentration of UDP-GlcNAc [35, 39]. The 2-fold difference between the change in the UDP-GlcNAc concentration after GlcN and high glucose treatments may contribute to the failure to observe an increase in CREB glycosylation after hyperglycemia.

Finally, it should be noted that regulation of glutamine:fructose-6-phosphate amidotransferase (GFAT) may also contribute to the difference in the observed effects of high glucose and GlcN treatment. GFAT is the enzyme responsible for the conversion of glucose to glucosamine, the rate-limiting step in the production of UDP-GlcNAc (Scheme 2-1). Two forms of GFAT have been identified, GFAT1 and GFAT2. Phosphorylation by cAMP-dependent kinase (PKA) regulates both GFAT1 and GFAT2 activity [40-42]. Ser205 phosphorylation of GFAT1 by PKA inhibits enzymatic activity [42]. Within the pancreas GFAT1 is the dominant form [43]. It is possible that activation of PKA by glucose-induced cAMP production reduces GFAT1 activity, and as a result reduces flux through the hexosamine biosynthesis pathway and increased OGT activity [44].

In addition to studying the effect of hyperglycemia on *O*-GlcNAc glycosylation, I initiated investigations into alternative pathways that may be associated with *O*-GlcNAc

dynamics. The link between CREB glycosylation and the balance of cell survival and apoptosis lead me to explore the possibility that pro-survival signaling molecules, such as glucagon-like peptide-1 or insulin-like growth factor-1, or apoptotic signaling molecules, such as Interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) may modify glycosylation levels. Treatment of pancreatic islet β-cells with pro-survival signaling peptides glucagon-like peptide-1 (GLP-1) and insulin-like growth factor-1 (IGF-1) have been shown to induce CREB phosphorylation [45-50]. However, no increase in CREB phosphorylation was observed in initial experiments with 30 min to 1 h long treatments GLP-1 or IGF-1 in islets (data not shown). In the future, further investigation with varied concentrations and time treatments of GLP-1 and IGF-1 to measure their effect on CREB phosphorylation and GlcN-induced CREB repression would be warranted. IGF-1 has pro-survival effects on nerve cells, as well as pancreatic β-cells, and future studies could be extended to measure the effects of IGF-1 and GlcN in nerve cells [47, 51].

An alternative mechanism to glucotoxicity by which β-cell apoptosis is induced in both type I and type II diabetes is cytokine-induced activation of caspase signaling pathways [4, 52-57]. A cocktail of cytokines (IFN-γ, IL-1β and TNF-α) have been shown to induce apoptosis in pancreatic β-cells [26, 56-58]. IRS2 levels were used as a proxy for CREB activity, as previous experiments have shown that IRS2 protein levels are sensitive to CREB transactivation (Fig. 6-2 and 6-3). Treatment with a combination of IFN-γ/IL-1β/TNF-α reduced IRS2 expression (Fig. 7-10). These cytokines have been found to stimulate the degradation of IRS2 by activation of c-Jun terminal kinase (JNK) (Fig. 6-1) [59]. Phosphorylation of IRS2 by JNK leads to ubiquitination and proteasomal

degradation [59-61]. These results support findings that cytokines reduce IRS2 levels. Measurement of cellular *O*-GlcNAc levels was hampered by problems with the anti-*O*-GlcNAc antibody, and as a result I could not determine whether the cytokines elicit an increase in *O*-GlcNAc glycosylation. It would be interesting in future studies to determine whether the cytokines are capable of inhibiting CREB-dependent transcription, with the possibility that increasing CREB glycosylation may contribute to this repression. Such a discovery would provide a link between CREB glycosylation and the pathology of both type I and type II diabetes. These studies suggest that there is still a great deal that we do not understand about the regulatory pathways responsible for the activation of *O*-GlcNAc glycosylation: what differentiates the effects of glucose and GlcN in pancreatic cells, why glucose fails to stimulate CREB glycosylation and what are the mechanisms by which OGT activity and CREB glycosylation are regulated. It is left to future investigators to address these questions.

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