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Pancreatic Reg I in Chronic Pancreatitis and Aging: Implications for New Therapeutic Approaches to Diabetes

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Abstract

Objectives—We investigated the relationship of pancreatic regeneration protein (reg) in models of acinar cell atrophy and aging, and the effect reg I protein replacement on glucose tolerance.

Methods—Rats underwent pancreatic duct ligation (PDL) and were followed through 12 months. Aging rats were studied at 12 and 20 months. Intraperitoneal glucose tolerance tests (IPGTT) were performed, pancreatic reg I, reg I receptor, insulin gene expression, and reg I protein levels measured. PDL and aged animals were treated with exogenous reg I protein and assessed for glucose metabolism.

Results—Following PDL, chronic atrophic pancreatitis developed, with a progressive loss of acinar cells and pancreatic reg I. During aging, a similar depression of reg I gene expression was also noted. Reg I levels correlated with pancreatic insulin levels. Twelve months after PDL. IPGTT was abnormal, which was significantly improved by administration of reg I protein. Aged animals demonstrated depressed IPGTT, which marginally improved after reg I administration. Anti-reg antibody administration to young rats depressed IPGTT to elderly levels.

Conclusions—Depletion of the acinar product reg I is associated with the pathogenesis of impaired glucose tolerance of pancreatic diabetes and aging, and replacement therapy could be useful in these patients.

Keywords

regeneration protein; reg; theapy; diabetes; aging; pancreas; ductal ligation

Introduction

The mass of the exocrine pancreas diminishes with time, in both chronic pancreatitis and aging (1). Both processes are associated with glucose intolerance and ultimately clinical diabetes. Pancreatic (Sandmeyer's) diabetes occurs in 40-60% of patients with chronic pancreatitis; its development correlates with progressive destruction of acinar cells. The loss of an acinar cell factor might play a role in its progression (2). Diabetes is a disease of aging; greater than 20% of patients over the age of 80 will develop it (3), and also may be linked to acinar cell loss.

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Reg I protein is a product of the acinar cells of the pancreas, and its genetic expression is linked to β-cell function. Its gene is induced during ductal proliferation, β -cell growth and islet regeneration (4,5). Reg I protein is mitogenic to ductal and β-cells (5-7), and its administration after islet failure reverses diabetes (8,9). The gene for the receptor of reg I has been isolated (10), and was shown to be involved in the differentiation of the exocrine pancreatic cells (11).

We postulated that in chronic pancreatitis and aging, pancreatic exocrine reserves of reg I are progressively depleted, and this depletion leads to islet failure, glucose intolerance and diabetes. In order to test the former hypothesis, we employed three models. For chronic pancreatitis, we used the model of pancreatic duct ligation, which induces acinar cell atrophy. Using a technique modified from Edstrom et al (12-15), only acinar cells are affected; islets remain functional until after the acinar cells atrophy and glucose intolerance develops (16, 17). We also used a model of longitudinal aging in normal rats. We finally tested a model of 'induced aging' by administering antibodies to reg I to young rats.

In all studies, we measured glucose tolerance by intraperitoneal glucose tolerance tests (IPGTT), reg I and receptor gene expression, insulin levels and serum reg I levels. We lastly determined if administration of a recombinant reg I protein could improve glucose tolerance in animals with impaired IPGTT.

Materials and Methods

Modified subtotal duct ligation (PDL) model of chronic pancreatitis

The major and minor pancreatic ducts of 6-8 week 150 gm female Wistar rats were ligated as follows: After 50 mg/kg Nembutal anesthesia, a midline laparotomy was performed. Modified subtotal ligation of the pancreas (13-17) was accomplished by initially dissecting, ligating and dividing the main ducts to the splenic and gastric lobes. Using microscopic dissection, the duodenal and parabiliary lobes were disconnected from the duodenum and common bile duct, thereby detaching the pancreas off these structures.

Animals were analyzed by intraperitoneal glucose tolerance (IPGTT), reg I and receptor gene expression and serum reg I protein levels (see below) at three time points after PDL - one, six and twelve months. After IPGTT, the animals were recovered, and the next morning, animals were sacrificed by asphyxiation. The protocol was approved by the Animal Care and Use Committee.

Pancreatic wet weight

Pancreatic wet weight as a marker for tissue edema was quantitated by the ratio of pancreas wet weight over the animal's total (mg/g) body weight (18).

Intraperitoneal Glucose Tolerance Testing (IPGTT)

Intraperitoneal glucose tolerance tests were performed under Nembutal anesthesia (intraperitoneal injection of 50 mgs/kg rat body weight). Glucose was measured by orbital or tail vain bleed, at 15-30 minute intervals post intraperitoneal injection of glucose (1 gm/kg), by glucose oxidase (Beckman Instruments) or by amperometry (Accu-Check Advantage, Roche Diagnostics) according to manufacturer's instructions.

Insulin and Reg 1 measurements

Serum insulin was measured at the indicated timepoints by ELISA (Crystal Chem Inc, Downers Grove, IL), according to manufacturer's instructions. Serum levels of reg I protein

concentrations were determined by direct ELISA in a manner described previously (19) using a monoclonal antibody to reg I (20).

Northern analysis

A 202 base pair probe for rat reg I was produced by reverse transcription-polymerase chain reaction from rat pancreatic RNA, using primers (up: 5′-CTGGCCTCTCTGATTAAGGAG-3′, down: 5′-TCAGATGATTTCAGGCTTTAA-3′) (21). This sequence is homologous to the mouse reg I published by Unno and colleagues (21), and is, within the rat reg I family, unique to reg I. The size of the PCR product was confirmed by electrophoresis. The PCR product was then ultrafiltered using a 30,000 MW filter (Millipore, Bedford, MA) to remove unincorporated dNTP's. Reg I receptor cDNA was prepared by double digestion of pCIneo-reg I receptor cDNA plasmid (10) with HindIII and Not I. Electrophoresis of the digestion complex was performed on an 0.8% agarose gel, after which the receptor band was cut from the gel, and the cDNA extracted using the QIAEX II Agarose Gel Extraction protocol (Qiagen, Germany). Probe DNA was labeled for chemiluminescent imaging with DIG High Prime DNA Labeling and Detection Kit (Boehringer Mannheim, Roche Diagnostics, Indianapolis, IN). A probe for rat insulin-I was a gift from Luciano Rossetti (Dept. of Medicine, Albert Einstein College of Medicine), and was similarly labeled.

Total pancreatic RNA was isolated by the TRI-REAGENT technique. Ten micrograms (ug) of total RNA was analyzed by 1% formaldehyde-agarose gel electrophoresis to document integrity. RNA was transferred to nitrocellulose filters and analyzed by standard Northern blot. To correct for loading, the blots were stripped and reprobed with digoxygenin-labeled oligo-dT, and quantitated using NIH- Image (Scion Corp, Frederick MD). Data are expressed as corrected counts (OD reg/OD oligo-dT), after background subtraction, and reported as mean ± SEM. Statistical analysis was performed by unpaired Student's t-tests, and significance defined as $p<0.05$.

Real-time quantitative RT-PCR

One-step real-time quantitative RT-PCR for reg I mRNA was performed as previously described (22), using a GeneAmp 5700 sequence-detection system (Applied Biosystems, Foster City, Calif.), with β-actin as an endogenous control to standardize the amount of sample RNA added to a reaction. Primers and probes were designed using Primer Express software (Applied Biosystems); the specific forward and reverse primers were designed based on published sequences of rat reg I (GenBank accession no. NM_012641). All primers and probes and other reagents for real-time quantitative PCR were purchased from Applied Biosystems (forward: 5′-TACAGCTGCCAATGTCTGGATT-3′, reverse: 5′-CAGTGTCCCAGGATTTGTAGAGA-3′, probe: 5′-FAM-ATCCCAAAAATAATCGCCGCTGGC-TA-3′). One hundred nanograms of total RNA was used to set up 25-μL real-time quantitative PCRs that consisted of 1 × TaqMan Universal PCR Master Mix, 500 nM forward and reverse primers, and 200 nM TaqMan probe. PCR amplification was carried out with the following temperature profile: 30 min at 48 °C; 10 min at 95 °C; and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Assays were performed in triplicate. Data were analyzed with the relative standard curve method. Standard curves of the genes of interest and β-actin were prepared with three 1:2 dilutions (four points, eight-fold range) of total RNA from one of the samples that was expected to have the highest amount of mRNA for the gene of interest. For each reaction tube, the amount of target or endogenous reference was determined from the standard curves. The mean amount of each sample was calculated from the triplicate data and was normalized by division by the mean quantity of β-actin RNA for the same sample. The mean and standard deviation of each treated group were calculated from the normalized value for each rat in that group.

Isolation/production of reg I Protein

Recombinant rat His-tagged reg I protein was produced in *E. coli* through EcoRI-Xho I directional cloning (Forward primer: 5′-AGCAGAATTCCAGGAGGCTGAAGAAGATCTAC-3′; reverse primer: 5′-CTCACTCGAGTCAGGCTTTGAACTTGCAGACAAATGATAATTGGGCATC-3′). Full length reg I was PCR amplified and digested with Xho/EcoRI restriction enzymes. Reg I containing constructs were confirmed by PCR (forward: 5′-TTGTCCAGAAGGTTCCAATG-3′, reverse: 5′-CAAACTCAGGATACAAGAAA-3′). Digested reg I PCR amplicons were inserted in-frame into the pET24a bacterial expression vector (Novagen, San Diego, CA). Positive clones were transformed into BL21 (DE3) *E.coli*, grown to a density of 2.0 OD in 500mls of TB broth with kanamycin and induced for three hours at 37° C with 2mM IPTG. The bacteria was centrifuged and resuspended in resuspension buffer (0.1M Sodium Phosphate, pH 8.0, with 1 mM PMSF and 1 mM dithiothreitol) containing protease inhibitors and sonicated on ice. Soluble bacterial proteins were disposed and the bacterial pellet was sequentially washed first with wash buffer A (1.5% triton, 0.1M Sodium Phosphate, 1mM PMSF) and centrifuged at 12,000 rpm for 10 minutes, and then with wash buffer B (0.5% triton, 0.1M Sodium Phosphate) and pelleted. Because reg I formed inclusion bodies, the bacterial pellet was resolubilized in 15 mls of resuspension buffer (6M UREA, 0.1M Sodium Phosphate, pH 8.0) for 10 minutes on ice. Solubilized proteins were collected after centrifugation at 4°C for 10 minutes at 10,800 rpm and batch bound to prepared His-Select Nickel Affinity Gel beads end over end overnight at 4°C. The next day, the beads were spun down and washed five times with Wash Buffer (0.1 M Sodium Phosphate, pH 7.0, 6M Urea) and centrifuged. 5 mls of Elution Buffer (0.1M Sodium Phosphate, pH 4.5, 6M Urea) was added to the beads and batch eluted end over end for 4 hours or overnight at 4°C three times. Samples of all washes, eluates and the beads at each step were analyzed by SDS PAGE. Eluates containing abundant reg I protein were dialyzed in dialysis buffer (0.1M Sodium Phosphate, 50 mM Acetic Acid, pH 4.5) in stepwise fashion, with decreasing amounts of Urea (from 6M Urea down to No urea) to enhance refolding and to prevent precipitation of the reg protein, over several hours. Protein concentrations were determined by Bradford protein assay and confirmatory SDS PAGE Coomassie stain.

Reg I treatment (in PDL experiment)

Two weeks prior to study, animals (n=6 per group) were injected with either with bovine serum albumin (BSA)(0.1mg/ml/day) or 1 mg/kg/day of recombinant rat reg I protein (8).

Aging Study

Twenty month (n=12) old female Wistar rats underwent baseline IPGTTs. IPGTT curves were similarly established for ten 1 month (n=10) and 12 month (n=6) old female Wistar rats for reference. Twenty month old rats were then randomly divided into two groups (n=6) and injected IP with either recombinant rat reg protein (1 mg/kg/day) (23) or vehicle (50mM Acetic Acid, 0.1M Sodium Phosphate, pH 4.5, 1mg/ml BSA) for a period of 14 days at which time IPGTT curves were again determined and compared to the baseline curves. A subset of reg I treated animals were again tested 14 days later.

Anti-reg I antibody administration

Young rats were treated with mouse anti-human reg I monoclonal antibody (20) (2.5 mg/kg) IV (internal jugular vein) via osmotic pumps for 7 days. Control antibody treatment consisted of non-specific mouse IgG. IPGTT measurements were taken before and after treatment.

Statistics

Animals were compared by unpaired Student's t-tests. Glucose kinetics (integrated areas of glucose) were compared longitudinally to reg I levels (serum protein and pancreatic mRNA) by correlation coefficient analyses. Pre- and post-treatment IPGTT curves were compared at each timepoint and statistically analyzed using the Wilcoxon signed rank test and Students paired t-tests. For all analyses, statistical significance was defined as $p<0.05$.

Results

Studies on Chronic Atrophic Pancreatitis

(a) Effect of PDL on pancreatic wet weight—Following PDL, as shown in Figure 1, chronic atrophic pancreatitis was noted, with loss of acinar cells and hyperplasia of ducts and islets. In Figure 2A, one month after PDL, there was a statistically significant increase in pancreatic wet weight when compared with controls. Six months post PDL there were no observable differences in wet weights from control animals (Figure 2B), but at 12 months, animals who underwent PDL demonstrated decreased pancreatic wet weights (Figure 2C). When PDL insulted animals were treated with exogenous recombinant reg I (see below), no observable differences in pancreatic wet weight were noted at 6 and 12 months post treatment when compared with PDL insulted control (BSA treated) animals (data not shown; one month reg treatment after PDL not tested).

(b) Effect of PDL on reg I, insulin and reg 1 receptor expression—One month after animals were insulted with PDL, reg I gene expression increased when compared with age-matched control animals (Figures 3A). In contrast, reg I expression was found to decrease at 6 (Figure 3B) and 12 months (Figures 3C) after PDL. Western analysis of protein levels showed similar results (data not shown). Pancreatic insulin mRNA levels paralleled reg I levels; they were high at one month and depressed at 6 and 12 months post PDL (Figure 4A-C). This correlation of expression of reg I to insulin was statistically significant ($r=0.83$, $p<0.0001$).

Serum levels of reg I did not change between groups (data not shown), and while serum insulin levels increased mildly at one month, they were not statistically different from controls at 12 months after PDL (data not shown). No significant differences in reg I receptor expression were observed at one and 12 months after PDL compared with controls (data not shown).

(c) Effect of PDL on serum glucose—We assessed glucose tolerance in the PDL-treated animals by IPGTT, and compared them to age-matched normal controls. Figure 5 demonstrates that IPGTT responses worsened with normal aging (see ‘controls’ in Figure 5A, B, and C respectively, and the integrated areas depicted in 6D). Figure 5 also shows that IPGTTs were worse at 1, 6 and 12 months after PDL insult when compared with control animals. (Figure 5 A-C). Integrated areas for all experiments are shown in Figure 5D.

(d) Reg I protein treatment—Since abnormal IPGTT correlated with depressed reg I levels, we postulated that replacement of reg I protein by intraperitoneal injections would improve IPGTT responses at 6 and 12 months when compared with PDL alone (Figure 5 B, C). Age-matched animals were treated with 1mg/kg recombinant reg I or BSA for two weeks, and subjected to IPGTT.

No effect was observed in 1 month or 6 month PDL animals. However, Figure 5C shows that treatment of 12 month PDL animals with reg I protein resulted in a statistically significant improvement in glucose tolerance. Figure 5D shows integrated glucose responses, demonstrating worsening responses with PDL and improvement with reg I treatment in the 12 month PDL animals alone.

Reg I protein treatment had no effect on pancreatic wet weight pancreatic mRNA expression of reg I, insulin or reg I receptor, or serum levels of reg I or insulin (data not shown).

Studies on Aging

(a) Effect of aging on reg I expression and its relationship to glucose metabolism—Our observation of age-related depression of reg I gene expression, age-related impaired IPGTT, and partial reversal of impaired GTT with reg I treatment in acinar cell-depleted rats, lead us to further investigate the relationship of reg I, aging and diabetes. We were particularly interested in the role of reg I as therapy for diabetes.

We first studied pancreatic reg I gene expression in aged animals by real-time PCR, then protein by Western analysis. As shown in Figure 6A&C, pancreata obtained from 1 month old rats had elevated levels of reg I mRNA and protein (Figure 6C) expression when compared with 12 month old rats (<20 fold increase in mRNA expression, $p<0.05$). This depression persisted at the same level until 20 months (data not shown). Interestingly, levels of serum reg I protein did not differ between groups (Figure 6B).

(b) Induction of 'aging' by reg I antibody—Figure 7A demonstrates that, compared with young rats, older rats have developed impaired IPGTT. Remarkably, when young rats were chronically infused with anti-reg I antibody for one week, worsening of IPGTT responses were observed, when compared with untreated animals and controls (non-specific IgG injection) (Figure 7B). The level of impairment (as measured by integrated areas under the curve) induced by reg I antibody in 1 month old rats approached the level of 20 month rats.

(c) Effect of recombinant reg I treatment and glucose metabolism—We then investigated the effect of recombinant reg I treatment, compared to vehicle, in 20 month animals. Baseline fasting glucose measurements obtained from 2 month old animals displayed lower basal glucose levels when compared with 12 or 20 month old animals (77±3mg/dl, 92 ±3 mg/dl, 91±4 mg/dl, respectively; $p<0.05$). 20 month old rats treated with recombinant reg I protein drastically improved fasting glucose levels compared with pretreated rats (79±3mg/dl vs 91±4mg/dl, $p<0.05$) and approached values obtained from 2 month old rats.

We then investigated the effect of recombinant reg I treatment, compared with vehicle, in 20 month animals, on impaired IPGTT. While there was no statistical difference between the mean +/- SEM integrated areas under the curve between recombinant reg I - or vehicle- treated animals (p=NS; Figure 8A), we found four of the six older animals who had 4 or more timepoint measurements that were within or higher than the upper 95% confidence interval above normal did show some improvement (Figure 8B, $p=0.07$).

There were no differences in serum insulin levels when 20 month old rats were treated with recombinant reg I protein when compared with untreated control animals (data not shown). But, treatment of 20 month old rats with recombinant reg I protein induced increased expression of Reg I mRNA in pancreas (n=2, data not shown), suggesting a positive feedback loop of protein production.

Discussion

The relationship between the exocrine and endocrine pancreatic mass has been separately studied for years. While integrated structurally, there is a paucity of data regarding their functional relationship. But, as the exocrine pancreas atrophies - either by disease or aging - the endocrine pancreas shows signs of failure. Our laboratory has been exploring this relationship, and believes pancreatic reg I is the link.

Insulin-dependant diabetes mellitus is a late feature of chronic pancreatitis, and has been called Sandmeyer's diabetes. While it occurs after loss of 70-80% of the islet mass, its cause has eluded investigators. To date, the only explanation given for this progressive β-cell failure is that the severe fibrotic degeneration of acinar tissue- 'acinar sclerosis'- eventually chokes the islet of local circulation and glucose diffusion. This theory has never been proven (24,25). It is also likely that the loss of other substances from the acinar pancreas is involved in this islet cell failure (26,27).

The pancreatic glandular tissue atrophies with age, in a manner which is discernable in humans on CT scan. By the age of 85 years, the gland has lost one-third its weight, and histology shows replacement of parenchyma by fatty infiltration and fibrosis (1,28). Along with pancreatic atrophy, the width of the main pancreatic duct increases at a rate of 8% per decade, with occasional ductal proliferation and metaplasia.

While functional changes in the exocrine pancreas during aging are clinically barely noted, changes in the endocrine pancreas can be noticeable. Only 3% of persons aged 18-24 have mild glucose intolerance, but the incidence is as high as 42% in persons aged 75-79. Similarly, 16% of the population over the age of 80 are clinically diabetic. (3)

Evidence that the acinar cell plays a critical role in islet β -cell development and maintenance is very strong. In experimental models of chronic atrophic pancreatitis, progressive loss of islet function parallels the loss of acinar tissue. For instance, after ligation and division of the rat main pancreatic duct, islets progressively lose their regenerative capacity and involute, paralleling the atrophy of the surrounding exocrine (acinar) tissue (15,29,30). Similarly, pancreatic duct ligation in the dog leads to progressive exocrine atrophy and islet failure (31, 32). Histologic analysis of these islets has demonstrated progressive loss of β -cell mass (33), and physiologic studies show progressively diminished insulin-secretion capacity if the pancreatic duct was ligated and not internally drained, paralleling progressive exocrine failure.

We have been interested in the role of pancreatic regenerating protein I (reg I) in islet function. It is an acinar product which has been shown to modulate islet function. Reg I mRNA is constitutively expressed in acinar cells, its expression parallels islet gene expression (6,34) and its gene is induced before and during islet regeneration (35,36). Further, reg I gene expression has been directly linked to insulin gene expression (37,38), patients who harbor antibodies to reg I have developed diabetes (39), reg knockout mice show poor beta cell recovery and regeneration after insult (40), reg administration showed amelioration of surgical induced (depancreatized) diabetes (8) and transgenic overexpression of reg in islets is linked to the development of tumors (41). We and others have shown that reg I proteins are mitogenic to pancreatic-derived cell lines ARIP (ductal) and RIN (β -cell) (4), and to isolated pancreatic ducts in culture (7), and likely exert their effect via the MAPK P38 pathway (42). The rat *reg I* receptor (10) has recently been cloned and is a transmembrane 919 amino acid protein. Cells which express the receptor proliferate in response to reg I protein (10). We have shown that the receptor gene is induced along with reg I after pancreatitis (6,43).

The potential for reg I protein as a treatment for diabetes was first proposed by Watanabe et al, who showed that exogenous administration of recombinant rat reg I protein can reverse diabetes after massive pancreatic resection, and it is mitogenic to β-cells within the islet (8).

The observations that reg I gene expression correlates with islet proliferation and gene expression (44) supports the hypothesis that this factor, from the exocrine pancreas, is involved in maintaining islet β-cell integrity. To date, reg I is the only islet growth factor known to be directly derived from the acinar cell. It could exert its effect by endocrine or paracrine actions.

A homologue of reg I, islet neogenesis associated protein (INGAP) has been isolated from regenerating pancreata (45,46), which, like reg I, promotes islet regeneration. A bioactive INGAP fragment has been identified (47) which also promotes beta cell growth, PDX gene expression and has reversed diabetes in mice. We have similarly identified a bioactive fragment in a homologous region of reg I (42), which confers mitogenesis to ductal and beta cells; but exogenous administration had no effect in any of our current models of acinar failure associated with impaired glucose tolerance (unpublished results).

We believe that reg I treatment would increase β-cell mass, as we have shown in vitro, and others in vivo (8). We did not measure the islet mass in this report, measurement of total pancreatic BrdU incorporation by Southern blot did not show an increase (data not shown). But, Watanabe and colleagues did show clear islet-specific BrdU incorporation after reg I treatment(8). Aside from beta cell expansion, other factors, such as the glucose sensitivity of islets, peripheral utilization of glucose, or insulin receptor sensitivity can be involved. But, other studies in our lab suggest this is unlikely- we have failed to show an effect of reg I insulin secretion, and sensitivity to glucagon-like peptide [GLP-1]. In fact, preliminary studies on host insulin sensitivity by intravenous insulin tolerance showed no effect by reg I (unpublished results).

We first studied whether reg I can be involved with Sandmeyer's diabetes using a model of chronic atrophic pancreatitis, as induced by modified subtotal ductal ligation in the rat. After duct ligation, animals do not appear ill, and in fact gained weight (not shown). We observed, as have others, that acinar cells alone are affected; ducts are preserved, and islets are unaffected until after the acinar cells atrophy (12-17). In this model, pancreatic wet weight, a marker of edema, was initially increased at one month and then decreased at 12 months post PDL. It is likely that the PDL model initially mimics pancreatitis, explaining the initial increase in pancreatic edema.

Gene expression patterns for both reg I and insulin correlates significantly, an observation we and others have previously observed. Reg I and insulin initially increased at one month after PDL, perhaps as a result of pancreatitis, but at six months and one year, as the acinar cells involute, both decrease. Glucose metabolism, as measured by IPGTT, gradually became more impaired over the year. Although the originally published experiments with ductal ligation of the splenic lobe alone gave inconsistent glucose intolerance, our modification of disconnecting the pancreas from the common bile duct yielded persistent glucose intolerance at six and twelve months postoperatively. The worst IPGTTs were at one year, when reg I and insulin were depressed the most compared to controls. We were able to demonstrate that administration of recombinant reg I protein improved IPGTT at 12 months.

It is therefore likely that reg I can affect glucose control in chronic pancreatitis, and may be a useful therapeutic modality. While our reg I peptide was bioactive (42), studies using it in this model failed (unpublished data)- the intact protein is critical for the effect.

Further experiments also showed in the normal aging rat, reg I levels decrease in parallel to insulin, as IPGTT gradually becomes impaired. Using this information, we developed a longitudinal study of reg I and IPGTT in aging. We noted that as animals aged to 20 months, reg I gene expression decreased, and their glucose tolerance became impaired. In concert with these findings, in normal young rats, treatment with a monoclonal antibody to reg I induced IPGTT similar to that of an old one, suggesting a direct effect of reg on glucose metabolism and may be age dependent.

Finally, we postulated that reg I was involved abnormal glucose tolerance in the aging pancreas, and replacement therapy would improve tolerance. Our hypothesis is also based on preliminary data where we demonstrated that aged rats which were treated with recombinant reg protein

had decreased levels of glycohemoglobin and hemoglobin A1C when compared with pre-treated animals where these analytes were used as a marker of glucose control (48). In our current studies we observed significant improvement in glucose tolerance in older PDL animals which were treated with reg I protein. However, we did not observe any effect of reg I treatment on IPGTT responses in older animals as group. In normal older animals with impaired baseline IPGTT an interesting trend toward improved glucose tolerance was noted after recombinant reg I treatment. It is likely that the PDL insulted animals had more significant loss of acinar cells than normal aged ones and that reg I treatment **may** serve as an ideal therapy in the setting only of severe glucose intolerance specifically associated with severe acinar cell loss. It is also possible that the dose, number of animals utilized, or duration of reg I therapy utilized in our studies was insufficient to demonstrate the effect we wished to measure.

So, in rats with severe acinar depletion (PDL), reg I treatment yields a partial reversal of impaired IPGTT. In aged rats with less acinar cell loss but depressed reg I levels and impaired IPGTT, some improvement may occur. Our data of 'partial responses' to reg I therapy may be due to the fact that the dose used was sub-optimal. More frequent administration of recombinant reg I or increased concentration per dose might demonstrate significant improvement of glucose tolerance in both models. High scale production of recombinant reg I protein would be necessary to produce sufficient quantities of reg I for studies in rats and higher order vertebrates to determine appropriate route and dosing protocols for maximal therapeutic potential.

In conclusion, our data show that progressive loss of the pancreatic acinar cell is directly related to the development of glucose intolerance, and that reduced pancreatic reg I may be responsible for this effect. Progressive islet failure in chronic pancreatitis is likely not the result of 'islet sclerosis', but secondary to the loss of functional acinar cells and pancreatic reg I. Progressive islet failure of aging is the result of a progressive loss of exocrine cells, which harbor the reg I protein. Pancreatic reg I is therefore an acinar product which appears to directly affect glucose tolerance, possibly through an effect on the islet (β-cell).

Future studies are needed to fully demonstrate if replacement therapy with reg I may prove useful in patients with impaired glucose tolerance secondary to chronic pancreatitis, and maybe even with diabetes associated with aging.

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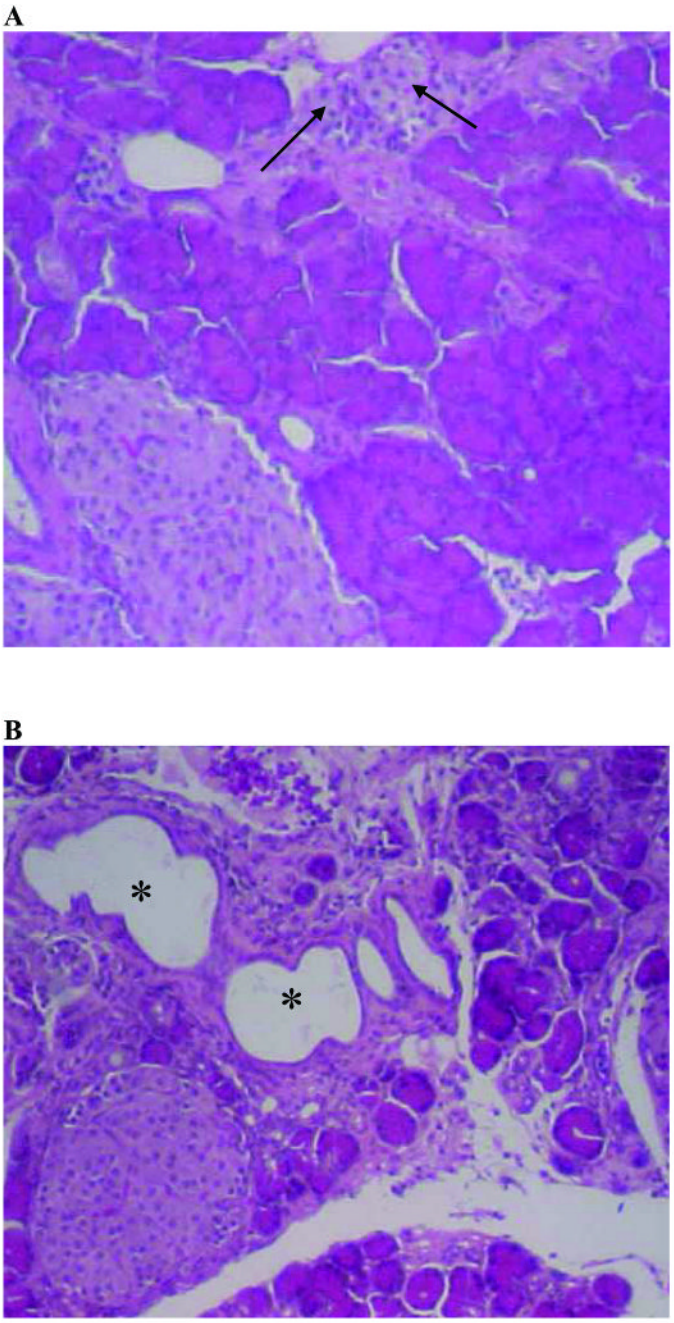


Figure 1.
H&E staining of pancreas from (A) one month and (B) six months after PDL. Photmicrographs demonstrate neo-islet proliferation associated with ductal proliferation (arrow) in (A) and chronic atrophic pancreatitis with loss of acinar cells and ultimate dilation of ducts (*) (B). Magnification = 200×.

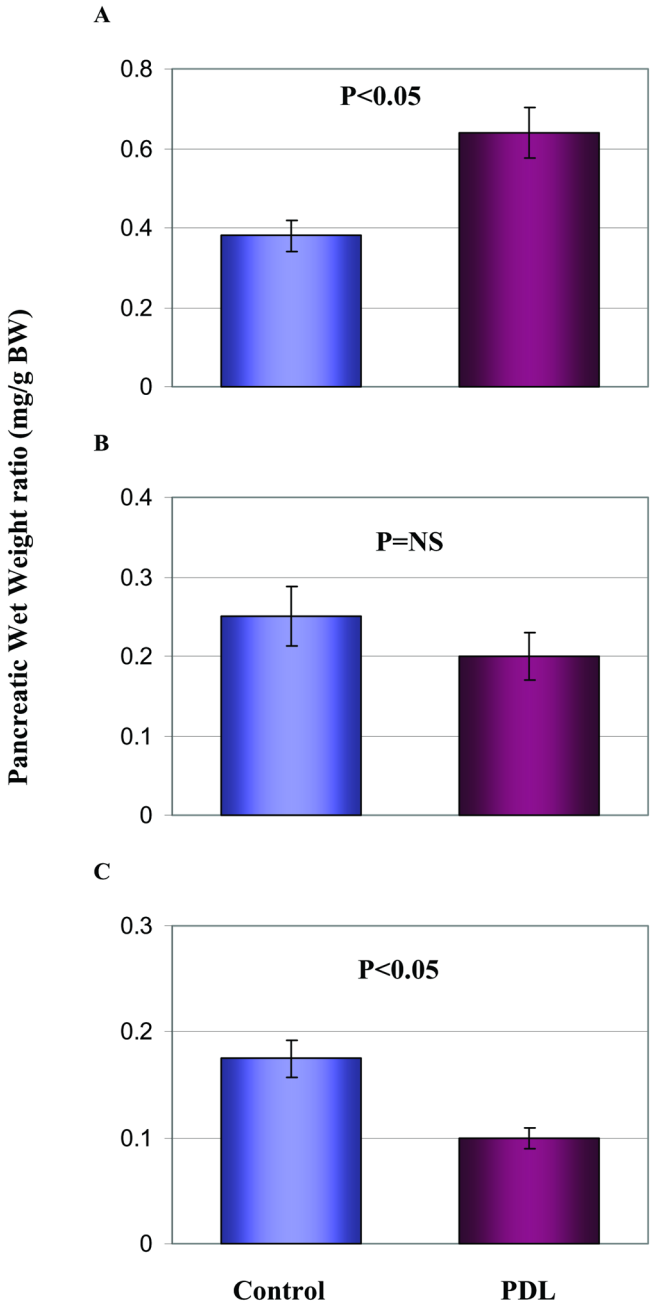


Figure 2.
Pancreatic wet weight in animals (A) one month (B) 6 months and (C) 12 months after PDL insult compared with aged matched normal controls. Data are expressed as a ratio of pancreas wet weight over animal's total body weight (mg/g).

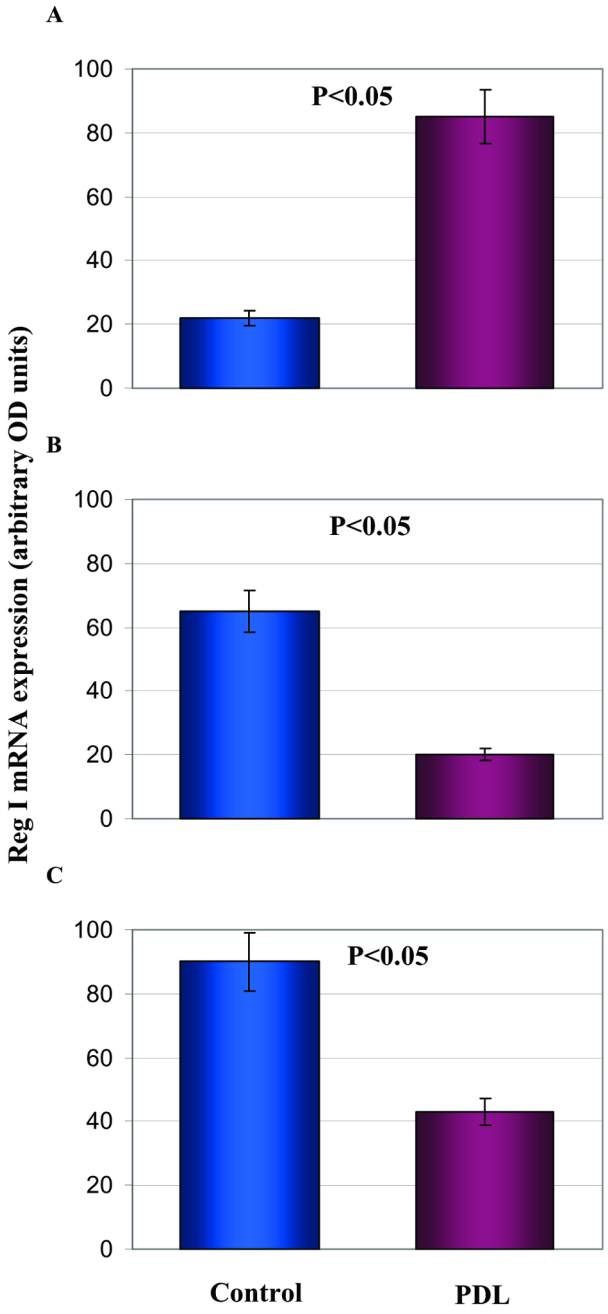


Figure 3.
Reg I mRNA quantitation (by Northern blot) in animals (A) one month (B) 6 months and (C) 12 months after PDL insult compared with aged matched normal controls. Data are expressed as arbitrary O.D. units obtained from densitometry of the blots.

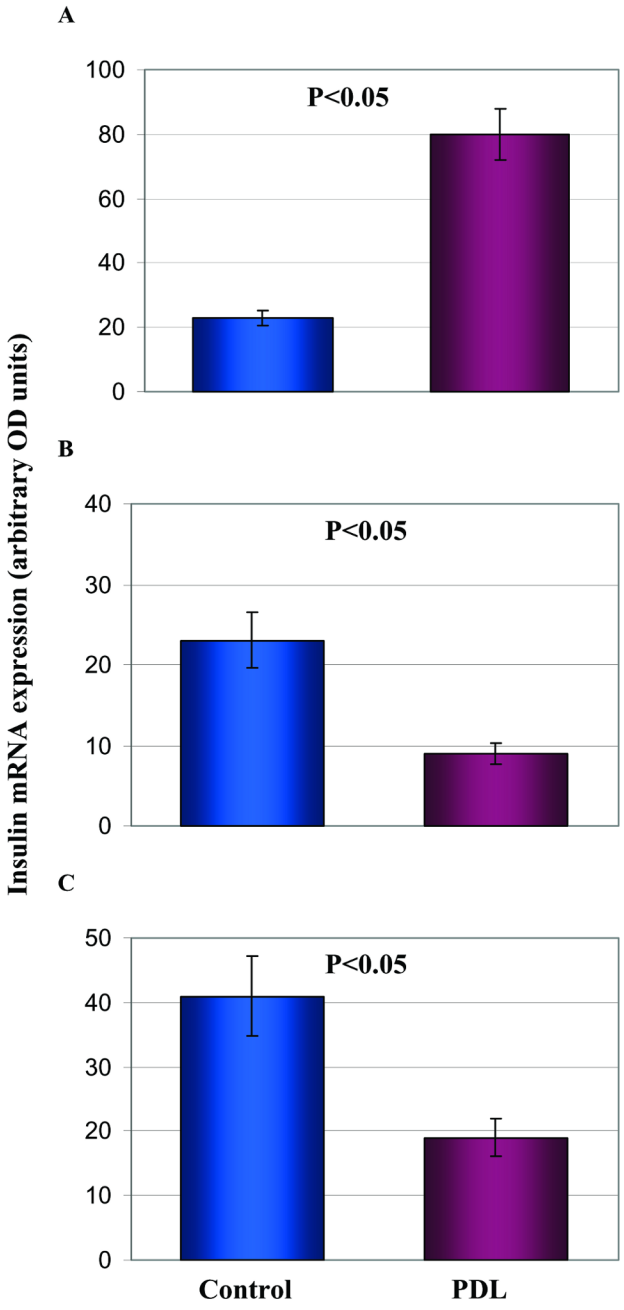
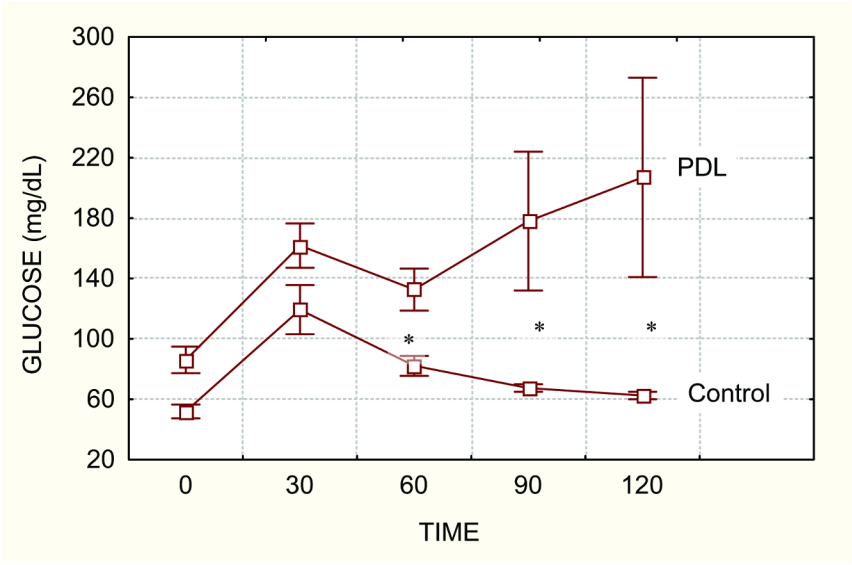
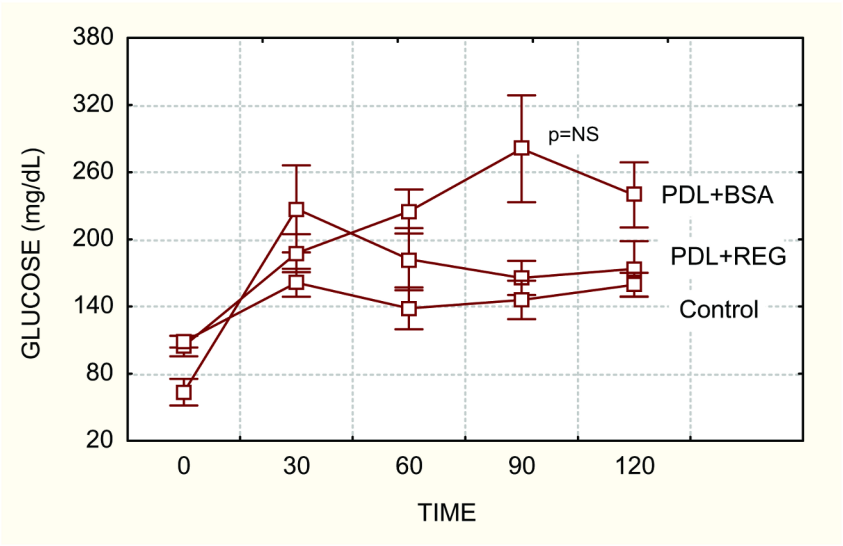


Figure 4.
Insulin mRNA quantitation (by Northern blot) in animals (A) one month (B) 6 months and (C) 12 months after PDL insult compared with aged matched normal controls. Data are expressed as arbitrary O.D. units obtained from densitometry of the blots.

A



B



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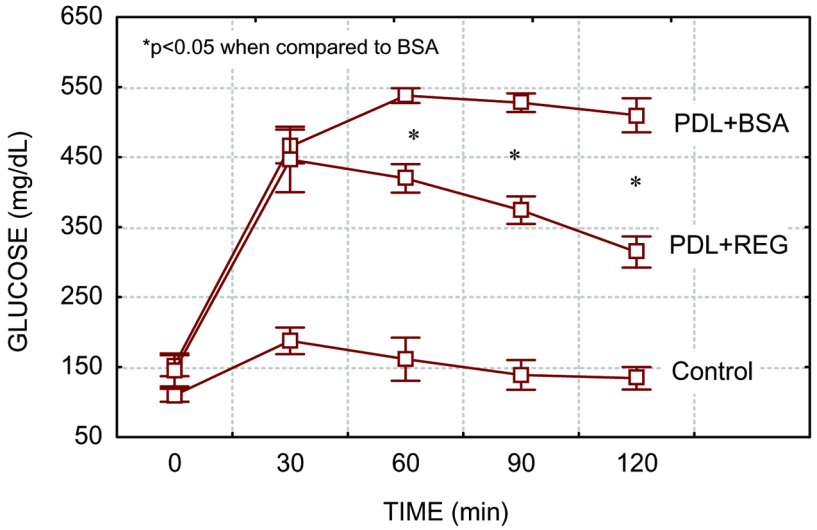

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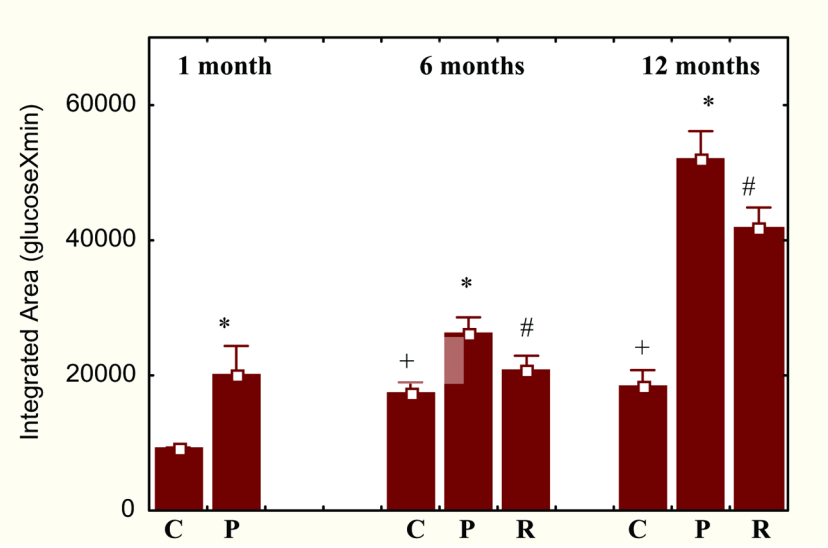
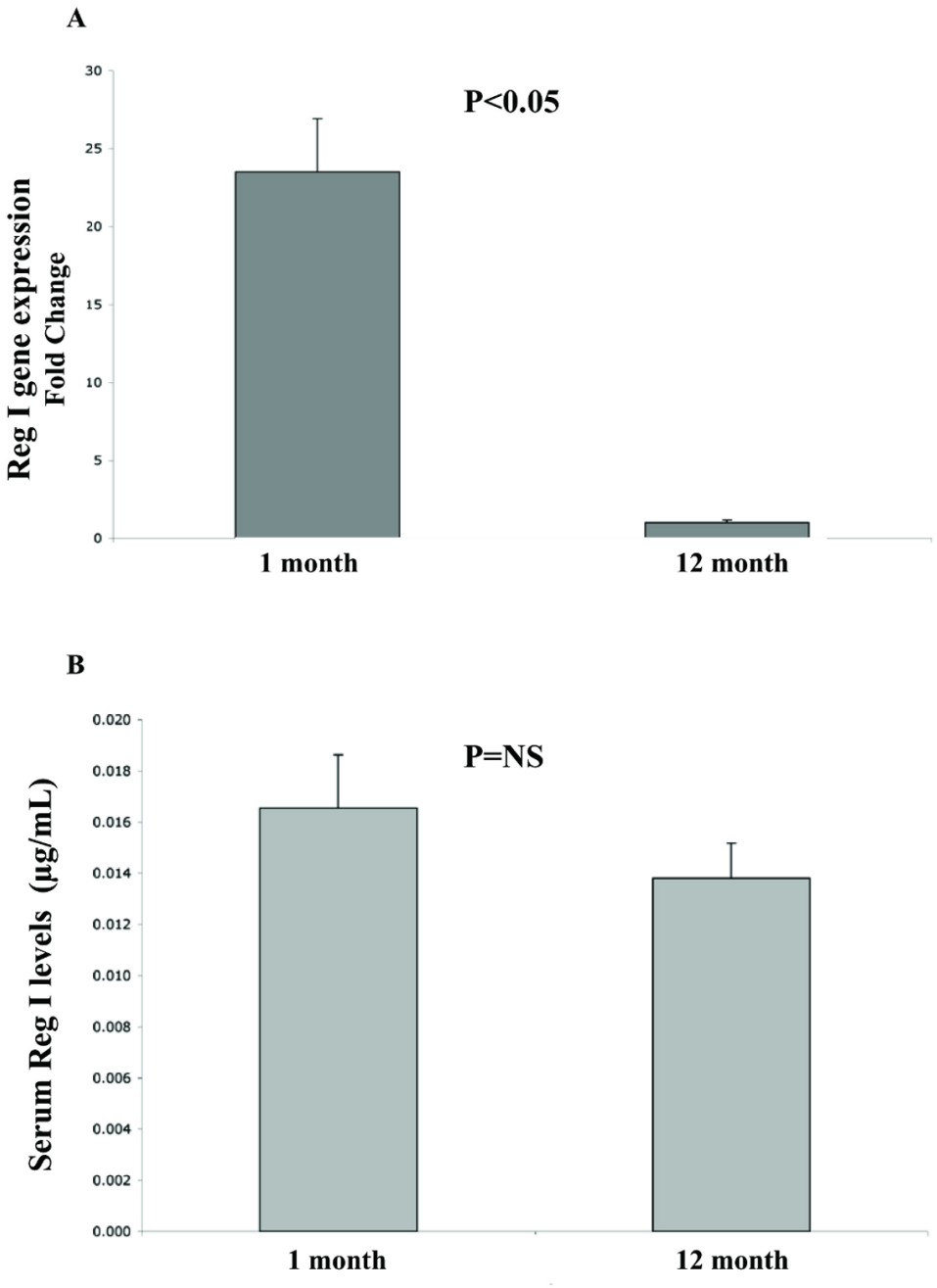


Figure 5.

Glucose tolerance tests in animals (A) one month (B) 6 months and (C) 12 months after PDL insult +/- recombinant reg I treatment compared with aged matched BSA treated controls. Data are expressed as mg/dL serum glucose. PDL-pancreatic duct ligation, BSA – bovine serum albumin, Reg – reg I protein treatment. (D) Representative integrated area under the curve glucose responses for each time point. (*) $p<0.05$ of PDL animals compared to age-matched controls, (+) $p<0.05$ of untreated animals compared to one month controls, (#) $p<0.05$ of glucoses in reg I treated animals compared to age-matched untreated PDL animals. Key: C-control, P-PDL, R- recombinant reg I treatment.



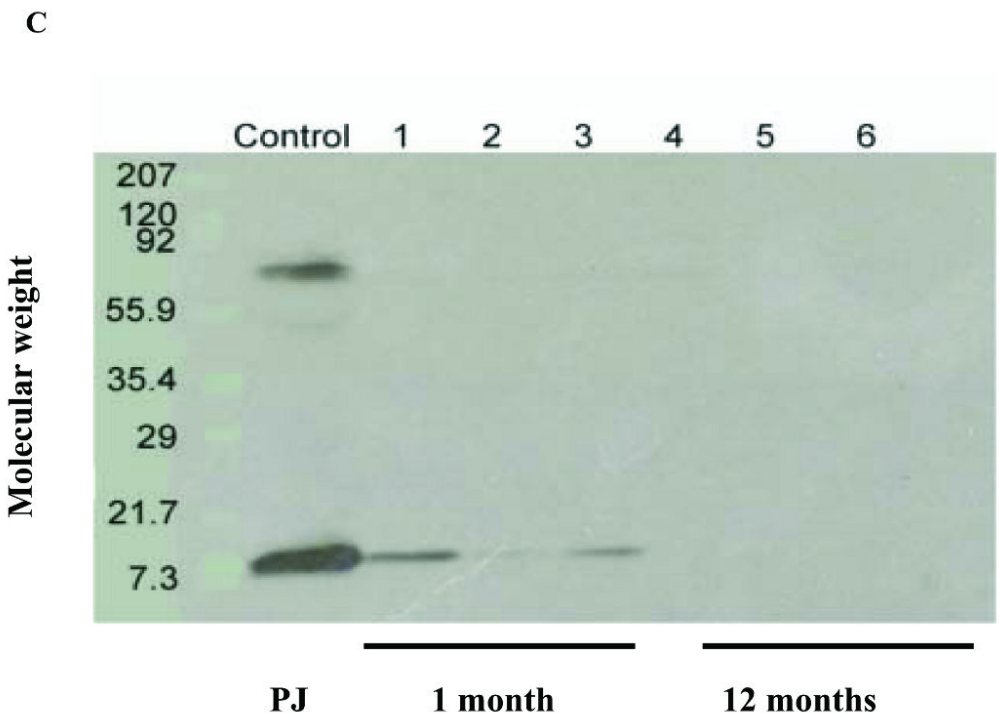


Figure 6.

(A) Real-time PCR analysis of reg I expression levels in pancreata obtained from one month and 12 month old rats. (B) serum reg I levels when assessed in one month vs 12 month old rats. Data are expressed as fold change and μg/mL respectively. (C) Western blot analysis of reg I protein in pancreas obtained from normal 1 month and 12 month old animals. Data represent 3 of 6 experiments with similar results. PJ – pancreatic juice. Pancreatic tissue lysates were processed blotted with monoclonal anti-reg I antibody as described in Materials and Methods.

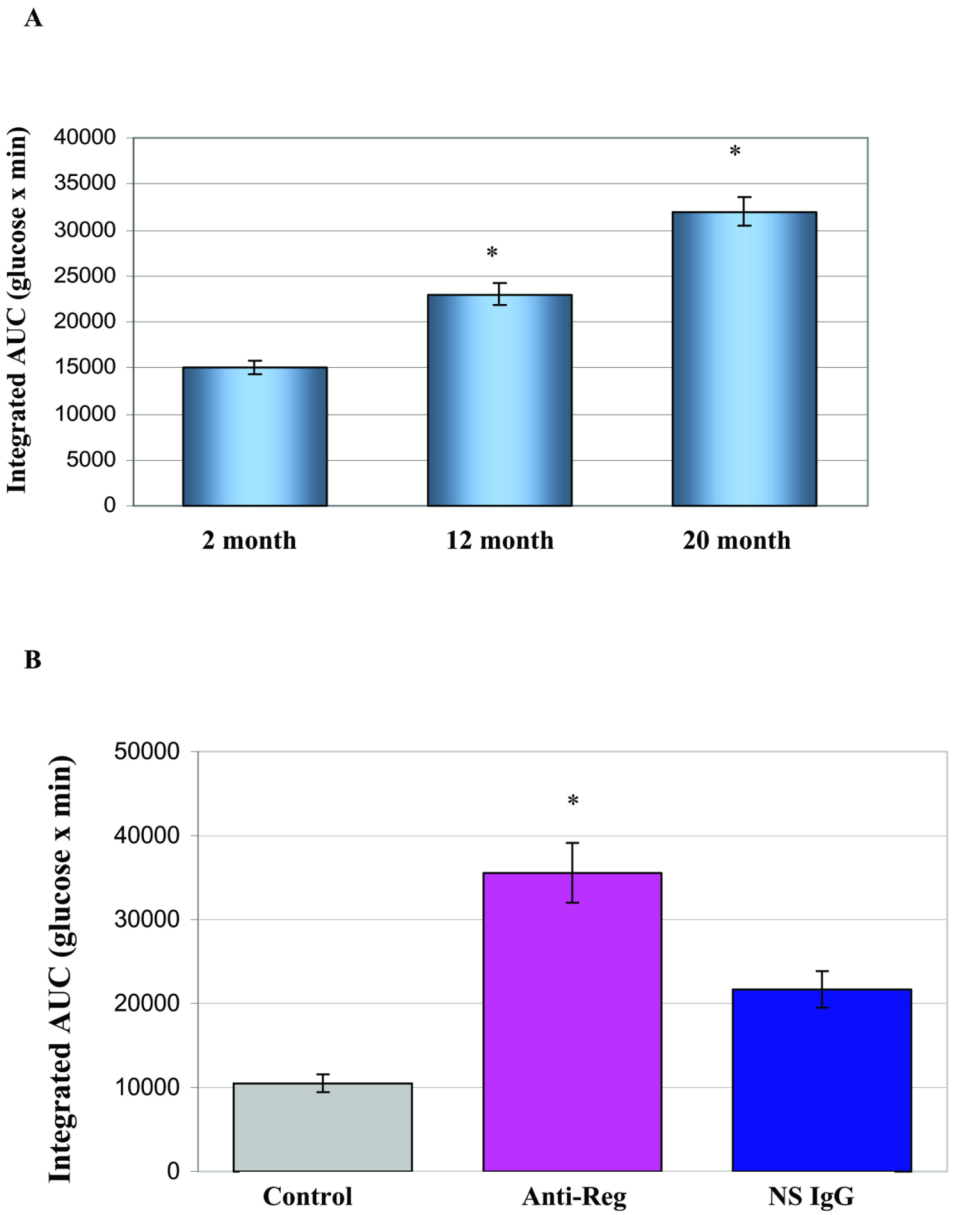


Figure 7.

(A) Glucose tolerance tests (GTT) in 2, 12 and 20month old rats. (B) GTT responses in one month old rats in response to anti-Reg I (anti-Reg) and non-specific IgG (NS IgG) antibody treatment. Data are expressed as integrated area under the curve glucose responses (glucose × minutes). (*) $p<0.05$ for anti-reg I antibody treatment compared with untreated control animals.

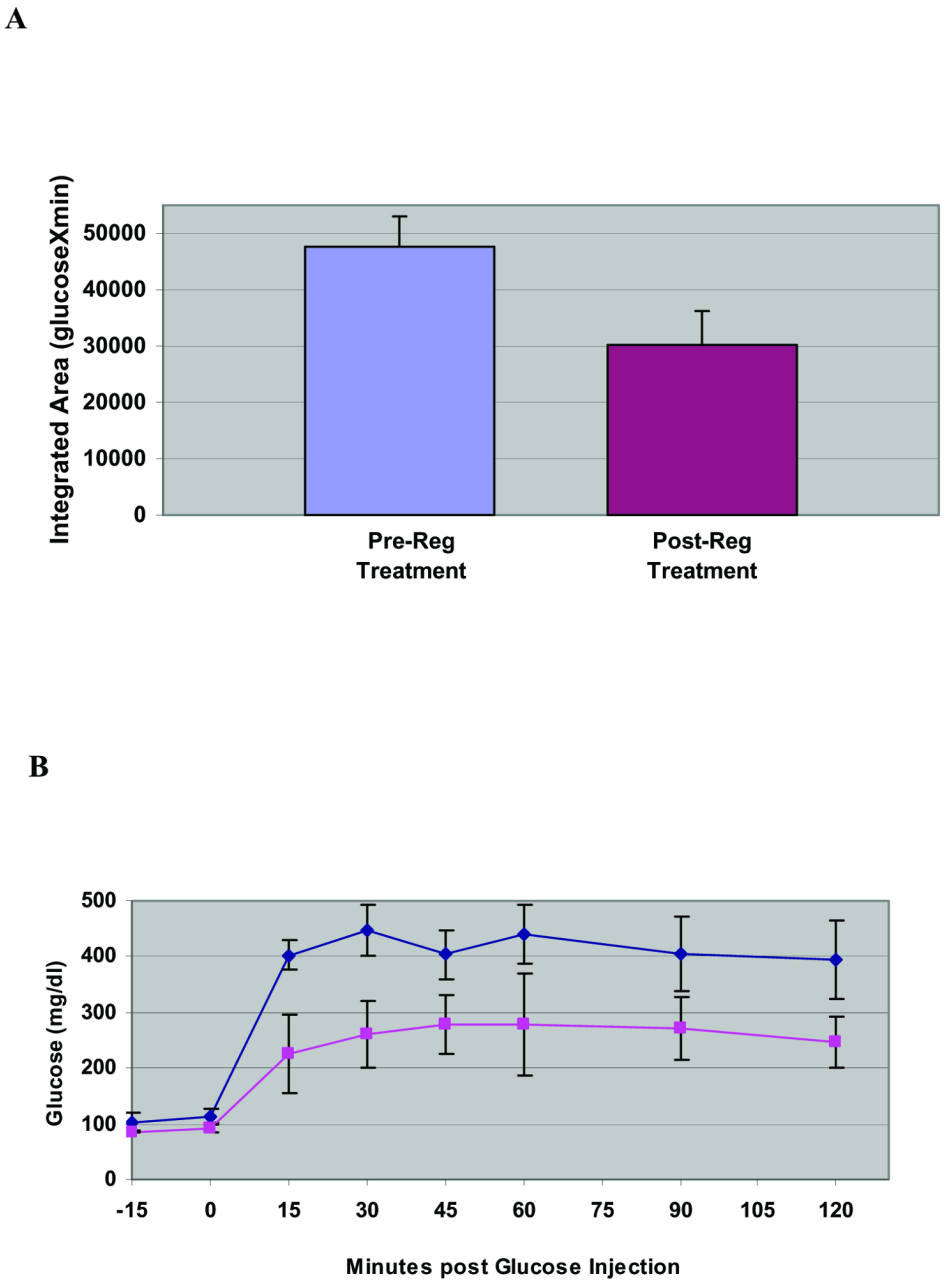


Figure 8.

(A) Representative integrated area under the curve glucose responses (glucose × minutes) for pre and post reg I treated old (20 month old) rats (n=6/group); P=NS. (B) Representative IPGTT responses in old rats with elevated baseline IPGTT responses (>400m/dL, black line) in response to reg I treatment (pink line); data represent 1 of 4 animals with similar results.