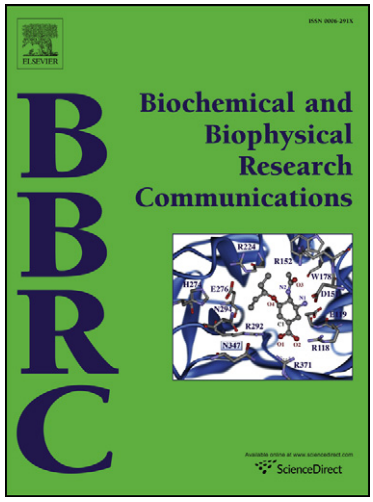

Identification of microRNAs with a role in glucose stimulated insulin secretion by expression profiling of MIN6 cells

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Title: Identification of microRNAs with a role in glucose stimulated insulin secretion by expression profiling of MIN6 cells

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Abstract

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs which regulate mRNAs at the post-transcriptional level. MiRNAs have been identified in both normal physiological and pathological conditions. To date, a limited number of miRNAs have been shown to be involved in the regulation of insulin secretion. We have identified a panel of 10 miRNAs down-regulated in glucose non-responsive MIN6 cells compared to glucose responsive cells using TaqMan Low Density human microRNA arrays. Of these 10 miRNA targets, subsequent functional investigations involving knockdown of mir-200a, mir-130a and mir-410 levels suggested that they may decrease the capability of MIN6 cells to secrete insulin in response to stimulatory levels of glucose. Conversely, experiment with over-expression of mir-410 suggest that it may enhance levels of glucose stimulated insulin secretion. In this study, we have also identified 21 miRNAs not previously known to have a potential murine homolog.

Keywords: microRNA, GSIS, insulin secretion, MIN6

Introduction

In recent years, miRNAs have been implicated to play a role in regulation of insulin secretion [1-4], pancreatic islet development and β cell differentiation [5-11], insulin resistance [12-15], and have been associated with secondary complications of diabetes, such as diabetic nephropathy [16, 17] and cardiovascular disease [18, 19].

A number of miRNAs have been shown to be directly involved in glucose stimulated insulin secretion (GSIS); indicating potential therapeutic targets for enhancement of GSIS and β cell function in Type 2 diabetes.

Mir-375 is, apparently, an important miRNA which negatively regulates GSIS in the murine insulinoma cell line, MIN6, and is thought to act at the late stages of exocytosis through its target myotrophin [1]. Glucose is a negative modulator of mir-375 expression. Mir-375 also negatively regulates insulin gene expression in INS-1E cells by directly targeting PDK1, thereby glucose induced reduction of mir-375 ultimately leads to increased *insulin* gene expression [20]. Mir-30d expression is also glucose modulated in MIN6 cells and mouse pancreatic islets; with up-regulation of mir-30d levels induced by high glucose concentrations. Over-expression of mir-30d in MIN6 cells leads to increased *insulin* gene expression, while knockdown leads to reduced expression [21], although it is unknown whether the effect of glucose-induced mir-30d on *insulin* expression is also mediated through PDK1.

Another miRNA mir-124a, targets transcription factor foxa2, which in turn regulates PDX-1 (which regulates *insulin* transcription) and potassium channel subunits Kir6.2 and sur1. Overexpression of mir-124a leads to increased Ca^{2+} levels within MIN6 cells, possibly due to dysregulation of Kir6.2 and sur1 subunits [2]. Overexpression of mir-124a in MIN6 B1 cells led to increased insulin secretion in response to basal

glucose concentrations and reduced secretion in response to stimulatory glucose concentrations [4]. Mir-124a affects the expression of a number of other exocytosis related proteins in MIN6 B1 cells including SNAP25, Rab3A, synapsin-1A, Rab27A and Noc2; although only Rab27A is a direct target of mir-124a [4]. These exocytosis-related proteins also have predicted binding sites for regulation by other miRNAs, but these still remain to be experimentally validated [22].

Mir-9 negatively regulates GSIS in INS-1E cells by directly targeting transcription factor onecut2, which in turn regulates granuphilin, a negative modulator of exocytosis [3]. Mir-96 mediated reduction of GSIS in MIN6 B1 cells is also associated with reduced expression of granuphilin and Noc2 exocytosis related proteins.

High levels of mir-34a are observed in islets from diabetic db/db mice. Over-expression of mir-34a in MIN6 B1 cells leads to reduced GSIS, possibly through its target protein VAMP2 which plays a role in β-cell exocytosis [23]. Since each miRNA can target multiple mRNAs, regulation of insulin secretion may be controlled by a small network of miRNAs co-ordinately targeting the extensive range of proteins involved in regulation of exocytosis.

This study aimed to identify other potential key miRNAs involved in the network of regulation of insulin secretion, while investigating the feasibility of more globally profiling miRNA expression in glucose responsive compared to glucose non-responsive MIN6 cells.

Materials and Methods

Cell Culture: MIN6 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum and were cultured at 37 °C with 5% CO_2.

GSIS analysis: The GSIS profiles of MIN6 cells were examined at glucose concentrations of 3.3 and 16.7 mmol/L. The GSIS response of MIN6 cells were measured according to previously published protocol [24]. Conditioned medium was used for analysis by (pro)insulin ELISA (Mercodia, AB, Sylveniusgatan, Uppsala, Sweden 10-1124-10;), following the manufacturer's instructions. D_c protein assay (Biorad) was used to determine mg of protein/well following manufacturer's instructions. ELISA results were then normalised to insulin secretion in pmol/L/mg protein.

RNA extraction and TaqMan Low Density miRNA Array: TaqMan Low Density miRNA arrays (TLDA) were performed on RNA isolated from glucose responsive and glucose non-responsive MIN6 cells, in three biological repeat experiments. Total RNA was isolated using MirVana miRNA isolation kit (Applied Biosystems), following the manufacturer's instructions. RNA quantity and purity were assessed spectrophotometrically (Nanodrop ND-1000, Labtech International, Ringmer, East Sussex, UK). cDNA was synthesised using TaqMan microRNA reverse transcription kit (Applied Biosystems) and Multiplex RT Human Primer Pool Sets. Resulting cDNA was then loaded onto TLDA cards according to manufacturer's instructions. TLDA cards were run on ABI 7900HT Real Time PCR system (Applied Biosystems).

Criteria for selecting induced/suppressed miRNAs: Controls included on the TLDA– and described as endogenous controls *i.e.* RNU44, RNU48 and RNU6B were undetected in MIN6 cells. Let-7b miRNA, expression of which was unchanged between glucose responsive and glucose non-responsive MIN6 cells, was thus assigned as endogenous control and all TLDA data was subsequently normalised to Let-7b. Following data normalisation, gene lists were generated using a *t*-test to identify genes that were significantly ($P < 0.05$) differentially expressed between glucose non-responsive MIN6 arrays and the glucose responsive MIN6 arrays (used as the calibrator/'control').

Functional validation studies: over-expression and knockdown of target miRNAs in MIN6 cells: MIN6 cells were transfected with pre-mir (miRNA precursors) and anti-mir (miRNA inhibitors) (Applied Biosystems) for over-expression and knockdown of miRNA levels, respectively. Pre-mir negative was used as a negative control for pre-mir experiments. Anti-mir negative was used as a negative control for anti-mir experiments. Transfection of miRNAs was carried out using Lipofectamine 2000 in accordance with the manufacturer's instructions (Invitrogen). To assay the effects of pre/anti-mirs on GSIS, MIN6 cells were seeded in 24-well plates at a concentration of $2x10^5$cells/well and were transfected with 50nM of pre/anti-mir. Medium was changed after 24hrs and GSIS assay was performed after 72hrs (see protocol above).

MiRNA target prediction: The analysis of miRNA-predicted targets was performed using the algorithm miRanda (http://www.microrna.org/microrna/getMirnaForm.do).

Results

Comparison of human versus murine miRNA sequences

No murine TLDAs were available at the time of conducting this study; however, human TLDAs were assessed for suitability to be used in murine experiments. TLDAs are 384 well cards, including 19 control wells. The 365 remaining wells represent different human miRNAs. The sequence of each was retrieved from the miRNA database - miRBase (www.mirbase.org/) [25-27] and compared against the murine equivalent sequence to assess homology. 242 of 365 sequences were found to be conserved between human and mouse. For a further 121 sequences there was no murine equivalent registered in miRBase. The remaining 2 miRNA sequences represent what are now described as "dead" miRNAs *i.e.* sequences which were initially thought to be miRNAs but have more recently been described as potential tRNA fragments.

Novel murine miRNAs

121 of 365 human miRNAs have no murine equivalent registered in miRBase. However, 21 of these 121 miRNAs were reproducibly detected in all 6 TLDA cards run with the murine MIN6 RNA samples, indicating that these human miRNA sequences may have a conserved murine homolog (Table 1).

MiRNA profiling of glucose responsive compared to glucose non-responsive MIN6 cells

The responsiveness of pancreatic β cells to glucose is measured as a fold change of insulin secretion in response to glucose stimulation (16.7mM glucose) compared to basal insulin secretion (3.3mM glucose). With increasing time in culture, the GSIS fold change of MIN6 cells decreases [24].

In order to identify miRNAs which may be responsible for this loss of GSIS, miRNA profiling was performed on glucose responsive MIN6 cells and glucose non-responsive MIN6 cells using TLDAs. Endogenous controls RNU44, RNU48 and RNU6B were incorporated onto each plate; however none of these transcripts were detected in the samples used. Therefore let-7b was chosen as endogenous control for normalisation, as levels of this miRNA were unchanged in all samples. Following normalisation and application of t-test, differentially expressed targets were identified as miRNAs with P-value < 0.05. Using this criterion 10 differentially expressed miRNAs were identified (Table 2).

Putative miRNA targets

Microarray and proteomic studies performed in house identified mRNAs [28] and proteins [29] differentially expressed in glucose responsive compared to glucose non-responsive MIN6 and MIN6 B1 cells. Here, bioinformatic analysis was performed on these mRNA and protein targets using microRNA.org: A resource for predicted microRNA targets and expression, based on the miRanda algorithm (http://www.microrna.org/microrna/home.do) [30, 31] to determine if they could potentially be regulated by the miRNAs identified as of interest in this study. A large number of these mRNAs were found to contain putative binding sites for the target

miRNAs, including several mRNAs of which could potentially be targeted by more than one of the identified miRNAs (Table 3).

Functional validation of miRNAs

Functional validation of miRNA targets was performed in order to evaluate miRNA effect on GSIS. Pre-mir and anti-mir technology was used to increase and knockdown miRNA levels, respectively. Anti-mir negative, pre-mir negative and untreated controls were performed with each experiment to show that effect on GSIS was due to manipulation of specific miRNA, rather than a more general or random event.

According to our TLDA analysis, each of the 10 miRNAs had reduced expression in glucose non-responsive MIN6 cells (Table 2); therefore, pre-mir over-expression of miRNA would be expected to improve GSIS function, while anti-mir reduction of miRNA would be expected to reduce GSIS function.

For miRNAs which exhibited effects on GSIS a large number of experiments were undertaken to determine if these effects were significant as we observed considerable interplate variations in GSIS, and even in basal insulin secretion (3.3mM glucose), the same pre-mir and anti-mir, and the same controls, sometimes stimulated or decreased insulin secretion. Therefore, we can only suggest functional trends rather than an absolutely certain effect. In the case of anti-mir transfection for mir 410, mir 200a and mir 130a, and pre-mir transfection for mir-410, we present in each case results based on 3 independent experiments which gave consistent results (Figure 1a, Figure 2, Figure 3 and Figure 1b respectively). These results suggest that knockdown of mir-410, mir-200a and mir-130a all decrease the magnitude of GSIS in MIN6 cells, as

would be expected from the profiling data, while overexpression of mir-410 suggests an improved GSIS in MIN6 cells.

Mir-369-5p, mir-27a, mir-320 and mir-192 also had slight effect on GSIS; however, this effect was not statistically significant. Manipulation of mir-337, mir-532 and mir-379 did not exhibit any functional effects on GSIS.

Discussion

In addition to insulin resistance in muscle, liver and adipose tissues, exhibited by Type 2 diabetes patients, this condition is also often associated with reduced functionality of pancreatic β cells. Much is known of the mechanisms of GSIS and the proteins involved therein; however, the specific regulation of exocytosis-related proteins remains less well understood. A small number of miRNAs have been shown to play a role in regulation of components of the insulin secretory pathway [1-3]. This study aimed to investigate the feasibility of identifying other miRNAs involved in the process of GSIS. In addition to increasing our basic understanding of the relevance of miRNAs in physiological events such as GSIS, miRNAs represent a potential future route of therapeutic targets which could be used to improve β cell function in Type 2 diabetes by manipulating expression of components of the exocytosis machinery and may also have relevance with regards to manipulating cells to secrete insulin in a regulated manner for use as cell replacement therapies for Type 1 diabetes.

In order to identify miRNAs involved in GSIS it was necessary to screen all possible miRNAs for differential expression in GSIS capable and incapable cells. TLDAs enable relative quantification of expression of 365 different miRNAs using TaqMan real-time PCR chemistries. These TLDAs were designed for analysis of human miRNA expression; however, the GSIS cell line model to be used in this study (as there is no human GSIS cell lines available for analysis) is of murine origin. In order to overcome this problem, homology comparisons were performed using miRBase to determine the quantity of miRNAs conserved between the two species. 67% of the 365 miRNAs were found to be conserved between human and murine; therefore; due to this high level of conservation, these human TLDAs were deemed suitable for profiling murine miRNA expression.

Of the remaining 33% of the human miRNAs represented, which were thought not to have a murine homolog according to miRBase (release 14.0), in the actual laboratory analysis, 21 of these were discovered to be reproducibly detected in all murine RNA samples evaluated in this study. Prior to this analysis, 579 miRNAs are known to be present in the murine *Mus Musculus* species (miRBase, release 14.0; as of September 2009); the results from this study potentially increases this to 602 miRNAs.

MIN6 cells secrete insulin in a glucose regulated manner; however, with increasing time in culture they lose this GSIS phenotype [24]. MiRNA profiling of glucose responsive MIN6 cells compared to glucose non-responsive MIN6 cells was performed using TLDAs to identify miRNAs involved in the GSIS mechanism. A panel of 10 miRNAs were identified as down-regulated in glucose non-responsive cells compared to glucose responsive cells.

Previous microarray and proteomic studies in our laboratory identified mRNAs [28] and proteins [29] differentially expressed in GSIS compared to non-responsive MIN6 and MIN6 B1 cells. Therefore, the miRanda algorithm was applied here to determine if any of these mRNA and proteins contained potential binding sites for regulation by the miRNAs identified in this study as potentially being involved in regulating GSIS. A number of mRNAs important for β cell function such as neuroD1, Isl1 and TGFβ1, contained potential binding sites for miRNA regulation. Thioredoxin-interacting protein (Txnip), identified as up-regulated in glucose non-responsive compared to GSIS MIN6 B1 cells, is an inhibitor of thioredoxin which plays a role in reducing oxidative stress, and it is thought that through this mechanism reduces the glucose responsiveness of MIN6 cells; conversely, down-regulation of Txnip and hence removal of the repressive effects on thioredoxin leads to improved glucose responsiveness of MIN6 cells (Rani S. et al, in prep). Here we report that Txnip

contains a potential binding site for mir-130a and mir-200a regulation. Furthermore, knockdown of mir-130a and mir-200a expression leads to reduction of GSIS in MIN6 cells (Figure 2 and Figure 3), although it has yet to be directly established if mir-130a and mir-200a effects on GSIS are mediated through Txnip.

In this study anti-mir transfections suggest that knockdown of mir-410 (Figure 1a), mir-130a (Figure 2) and mir-200a (Figure 3) may decrease the magnitude of GSIS in MIN6 cells, while pre-mir transfections suggest overexpression of mir-410 (Figure 1b) may increase the GSIS response of MIN6 cells. However, the likely involvement of multiple miRNAs in GSIS and fluctuations in the GSIS assay makes it difficult to arrive at definitive functional assignments for individual miRNAs.

Little is known of mir-410, and its role in pancreatic β cells has not been previously identified, this study suggests it may be involved in the regulation of insulin secretion, although the mechanisms by which mir-410 functions still remain to be deciphered.

Previous studies have reported mir-375, mir-9, mir-124a and mir-96 to play a role in regulation of insulin secretion in rodent pancreatic beta cell lines MIN6, INS-IE and MIN6 B1 cells [1-4]; however, in this study, mir-375, mir-9, mir-124a and mir-96 were not differentially expressed between the two populations, indicating that the loss of GSIS in these cells may potentially be *via* a myotrophin-, granuphilin- and mir-124a-independent mechanisms.

While we identified potential functional relevance in GSIS for some of the differentially expressed miRNAs identified in this study, a number of those identified here did not exhibit any functional effect on GSIS when their levels were manipulated in MIN6 cells, these miRNAs may require combined action of more than one miRNA, as miRNAs only tested individually, or these miRNAs may be involved in other changes the cells are undergoing as they lose their GSIS.

Conclusion

Keeping in mind the limitation that these TLDA cards were designed to detect human miRNAs, 66% of the 365 human miRNA targets have a murine homolog, and therefore human TLDAs are suitable for analysis of murine miRNAs. In addition, by using human TLDAs a further 21 human miRNAs not previously known to exist in mouse have been reproducibly detected in the MIN6 cell line. Mir-410 identified in this study as potentially involved in glucose induced insulin secretion represents a potential therapeutic target for manipulation to maintain/restore GSIS in insulin-producing cells. However, the mechanism by which mir-410 is involved in insulin secretion still remains to be elucidated.

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Table Legends:

Table 1. Detection of miRNAs not known to be present in mouse. Human miRNAs not previously known to have an equivalent homolog in mouse but detected by TLDA in MIN6 murine pancreatic β cells during the course of this study (n=6).

Table 2. **MiRNAs differentially expressed in glucose responsive and glucose non-responsive MIN6 cells (n=3).** Fold change of differentially expressed miRNAs indicates expression levels in glucose non-responsive MIN6 cells compared to glucose responsive cells (RQ- relative quantification).

Table 3. Potential targets of miRNA action. mRNAs and proteins previously identified in our laboratory as differentially expressed in glucose responsive compared to glucose non-responsive MIN6 and MIN6 B1 cells [28, 29] with putative binding sites for miRNAs identified here by TLDAs (x – multiple putative binding sites, N/A – non-applicable, no putative binding sites found).

Figure Legends:

Figure 1a. Effect of mir-410 knockdown on GSIS. Measurement of GSIS fold for untreated, anti-mir negative control (am-neg) and mir-410 knockdown (am-410) in MIN6 cells (numbers above bars indicate fold change of insulin secretion in response to 16.7mM glucose compared to 3.3mM glucose) (n=3). (* - statistically significant change in GSIS, error bars indicate standard error).

Figure 1b. Effect of mir-410 overexpression on GSIS. Measurement of GSIS fold for untreated, pre-mir negative control (pm-neg) and mir-410 overexpression (pm-410) in MIN6 cells (numbers above bars indicate fold change of insulin secretion in response to 16.7mM glucose compared to 3.3mM glucose) (n=3). (* - statistically significant change in GSIS, error bars indicate standard error).

Figure 2. Effect of mir-130a knockdown on GSIS. Measurement of GSIS fold for untreated, anti-mir negative control (am-neg) and mir-130a knockdown (am-130a) in MIN6 cells (numbers above bars indicate fold change of insulin secretion in response to 16.7mM glucose compared to 3.3mM glucose) (n=3). (* - statistically significant change in GSIS, error bars indicate standard error).

Figure 3. Effect of mir-200a knockdown on GSIS. Measurement of GSIS fold for untreated, anti-mir negative control (am-neg) and mir-200a knockdown (am-200a) in MIN6 cells (numbers above bars indicate fold change of insulin secretion in response to 16.7mM glucose compared to 3.3mM glucose) (n=3). (* - statistically significant change in GSIS, error bars indicate standard error).

Tables :

MiRNA	Average CT
mir-515-3p	32.33
mir-517a	33.64
mir-517b	32.68
mir-517c	33.46
mir-518c	32.61
mir-518d	32.22
mir-518e	32.88
mir-520f	34.88
mir-521	33.96
mir-518f	31.59
mir-519c	32.53
mir-519e	32.09
mir-564	33.43
mir-596	29.97
mir-597	33.86
mir-617	31.94
mir-646	31.68
mir-650	31.56
mir-572	31.84
mir-512-3p	34.05
mir-659	31.96

Table 1.

Target miRNA	RQ	Fold Change	P-value
mir-369-5p	0.391	-2.56	0.014
mir-130a	0.394	-2.53	0.037
mir-27a	0.453	-2.21	0.021
mir-410	0.456	-2.19	0.018
mir-200a	0.558	-1.79	0.037
mir-337	0.557	-1.79	0.013
mir-532	0.695	-1.43	0.017
mir-320	0.705	-1.41	0.033
mir-192	0.737	-1.35	0.015
mir-379	0.809	-1.23	0.015

Table 2.

MicroRNA	mRNA [28]	Protein [29]
mir-369-5p	Syvn1	N/A
mir-130a	Egr1, neuroD1, gap 43, txnip, Rgs4, Mxi1, IVNS1abp, meox2 (x2), pde10a, dcx, kpnb1 (x2), Ppadc1A, ccna2, smn (x2), BMP6	N/A
mir-27a	Isl1, Bhlhb9, ube2g1, Pnrc1, meox2, dcx (x2), ssr1, kpnb1, FGF12, btg2, TGFβ1	DUT, Psma1
mir-410	Pld1, Rgs4, Serf1, dcx (x2), mcfd2, atp7a, eya2	Hmgb1 (x2)
mir-200a	Ceacam1, txnip, Jun, Rgs4, IVNS1abp (x2), dcx (x3), mcfd2, smn, Faf1, TGFβ1	Hmgb1
mir-337	Dcx, VCP	N/A
mir-532	Dcx, ssr1, kpnb1	N/A
mir-320	Mxi1 (x2), bri3, gcg, rab3D, uba1, ssr1, smap1, FGF12 (x2), gnas, btg2 (x2), TGFβ1, BMP6	Ppp1cb
mir-192	TGFβ1	Hmgb1 (x2)
mir-379	Dusp1	N/A

Table 3.

Figures:

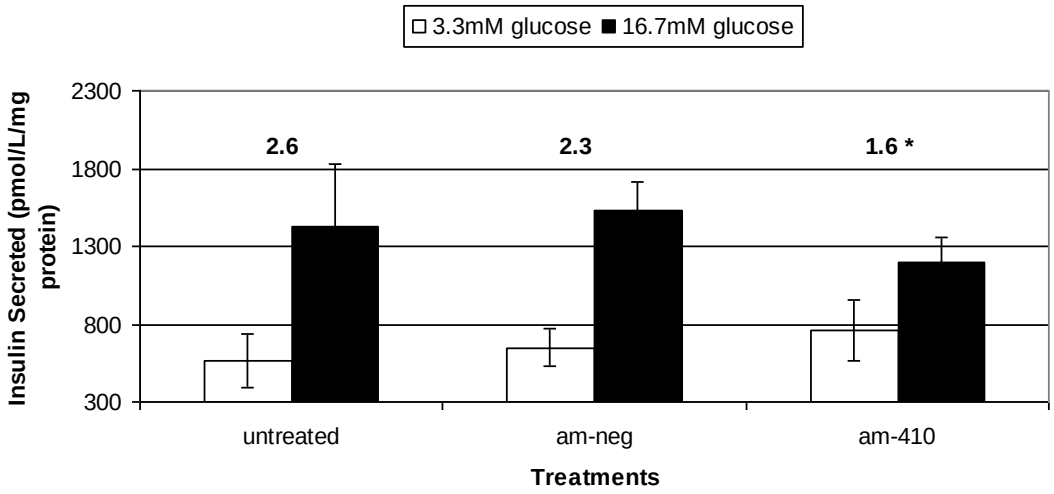

Figure 1a.

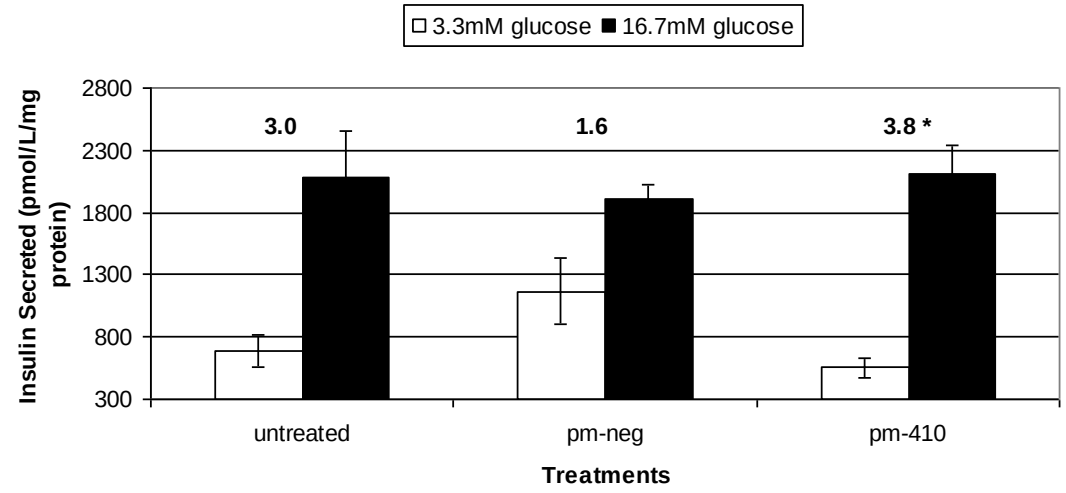

Figure 1b.

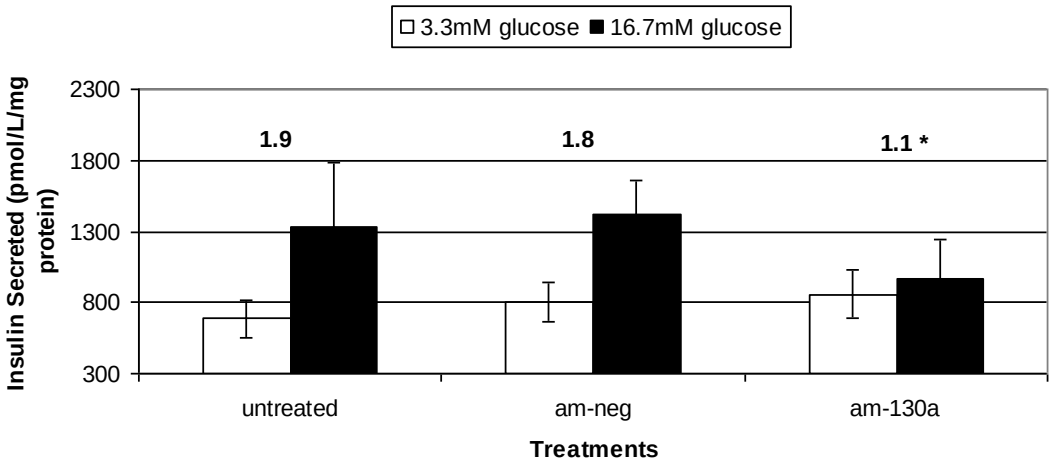

Figure 2.

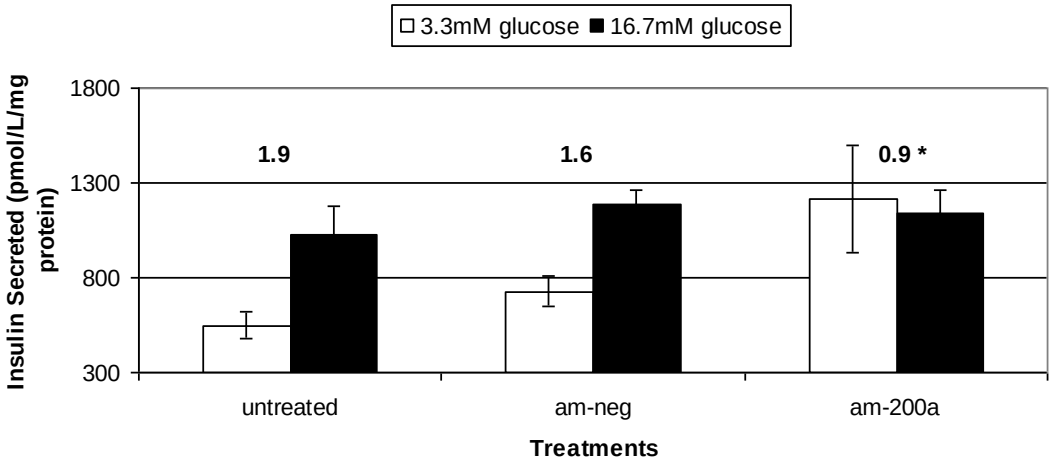

Figure 3.