Beneficial effects of lipids and prolactin on insulin secretion and β -cell proliferation: a role for lipids in the adaptation of islets to pregnancy

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Abstract

To meet the increased demand for insulin during pregnancy, the pancreatic islets undergo adaptive changes including enhanced insulin secretion and β -cell proliferation. These changes peak in mid-pregnancy and return to control levels by parturition. Because lactogens (placental lactogen and/or prolactin) induce this up-regulation and remain elevated throughout gestation, we examined whether lipids alter the effects of prolactin on islets. In response to prolactin, there was a 2.5-fold increase in insulin secretion when compared with control islets. There was also a 2.5-fold increase in insulin secretion in response to palmitate and a fivefold increase when islets were cultured with a combination of prolactin and palmitate. After culture with prolactin and palmitate, acute stimulation with 10 mM glucose for 1 h showed a suppression of insulin release. However, including palmitate in the stimulation media (a condition similar to late pregnancy *in vivo*) restored a higher rate of insulin release. This suggests that elevated lipids in late pregnancy lead to enhanced insulin secretion that is increasingly dependent on

lipids and less sensitive to glucose. β -Cell proliferation was also increased sixfold by prolactin and threefold with palmitate. The combination of both was slightly more than additive (11-fold). Similar experiments with oleate had no effect on insulin secretion. However, oleate stimulated β -cell division by threefold and was synergistic with prolactin (21-fold). These results were repeated in experiments including normal serum. Interestingly, prolactin also blocked the reduction of glucokinase levels observed with fatty acids. Overall, these results suggest that increased lipids during pregnancy likely contribute to the adaptation of islets to pregnancy by further enhancing β -cell division. In addition, the increase in lipids leads to enhanced insulin secretion that is less sensitive to glucose and more dependent on lipids. This provides a potential mechanism for maintaining elevated insulin secretion until parturition while preparing islets for normal glucose sensitivity *post partum*. *Journal of Endocrinology* (2008) **197**, 265–276

Introduction

During pregnancy, there is an increased need for insulin to accommodate the developing insulin resistance and growing fetal compartment. The pancreatic islets of Langerhans meet this demand by increasing both insulin secretion and islet mass (Parsons *et al.* 1992, Sorenson & Brelje 1997). Glucose-stimulated insulin secretion from isolated perfused rat pancreases during pregnancy was increased by day 10, peaked on day 15, and then declined to near control levels by parturition (Parsons *et al.* 1992). This increase in glucose-stimulated insulin secretion is accompanied by a lowering of the threshold for glucose stimulation from 5.5 mM glucose in controls to 3.3 mM glucose in mid-pregnancy. Islet mass is also increased 1.5- to 2-fold during pregnancy in rats (Parsons *et al.* 1995, Sorenson & Brelje 1997). β -Cell proliferation during pregnancy in rats was increased by day 10, peaked on day 14, and declined to control levels after day 18 (Parsons *et al.* 1992).

Through a series of *in vivo* and *in vitro* studies, we have shown that the activation of prolactin receptors by lactogenic hormones, either placental lactogen or prolactin, induces all of the known up-regulatory changes in islet function, which are known to be characteristic of pregnancy (Brelje & Sorenson 1988, Brelje *et al.* 1989, 1993). The length of time required to induce these changes is similar to that observed during pregnancy. In addition, in none of the experiments was a decrease in islet function observed with continued stimulation with prolactin. Since placental lactogen remains elevated until parturition (Parsons *et al.* 1992), additional factors must be responsible for the decline in glucose-stimulated insulin secretion and β -cell proliferation from their peaks in mid-pregnancy to control levels by parturition. However, serum lipids, triglyceride, and free fatty acids also increase several fold during pregnancy (Scow *et al.* 1964, Knopp *et al.* 1973, Chen *et al.* 1992). This elevation is first observed in rats between days 11 and 14 of pregnancy and

peaks on the last 2 days of pregnancy. As a result, peak lipid concentrations coincide with the decline of glucose-stimulated insulin secretion and β -cell proliferation to control levels by parturition. This marked increase in serum lipids is of particular interest considering the powerful interaction between glucose and fatty acids in the regulation of normal β -cell function (Nolan *et al.* 2006, Poitout *et al.* 2006, Prentki & Nolan 2006). Thus, lipids may have a role in modulating islet β -cell function in the latter half of pregnancy. Since palmitate and oleate are among the fatty acids that increase during pregnancy (Chen *et al.* 1992), we investigated their influence on the effects of prolactin in cultured islets. These conditions should mimic those islets experience during late pregnancy *in vivo* when they are exposed to high circulating levels of both lactogens and lipids.

Materials and Methods

Hormones

The rat prolactin (NIDDK-rPRL-B-8-SIAFP) was provided by the National Hormone and Pituitary Program (Dr A F Parlow, National Hormone and Pituitary Program, University of California Los Angeles Medical Center, Torrance, CA, USA).

Rat islet isolation and culture

The pancreatic islets were isolated from 3- to 5-day-old Sprague-Dawley rats (Sasco, Omaha, NE, USA) by a nonenzymatic culture method described previously (Hegre *et al.* 1983). After this initial culture, groups of 30 islets were cultured free floating in 24-well plates (Costar, Cambridge, MA, USA) in 2 ml RPMI-1640 with 10 mM glucose supplemented with 1% fatty acid-free BSA (Sigma), 10 mM HEPES, 2 mM GlutaMax (Gibco), and 1% penicillin-streptomycin-fungizone antibiotic-antimycotic (Sigma) at 37 °C in a humidified atmosphere of 5% CO₂ in air. The effects of prolactin and fatty acids on islet function were examined by culturing islets for 4 or 8 days with 500 ng/ml rat prolactin and/or 0.05–0.80 mM of either palmitate or oleate. The medium was changed each day and the amount of insulin released into the culture media determined. All procedures using animals were approved by the Animal Care and Use Committee of the University of Minnesota.

Fatty acid solutions

Stock solutions of the sodium salts of palmitic and oleic acids were prepared as follows (Briaud *et al.* 2001): the fatty acids were dissolved in 25% ethanol at 50 °C at a final concentration of 150 mM. Aliquots of stock solutions were complexed with fatty acid-free BSA (10% solution in culture media) by stirring for 1 h at 37 °C and then diluted with additional culture medium. The final BSA concentration was 1% with a molar ratio of fatty acid:BSA of 5:1 or less

depending on fatty acid concentrations. The final ethanol concentration in culture was $\leq 0.12\%$ (vol:vol). All control conditions contained the same amount of BSA and an equivalent amount of ethanol.

The concentrations of free fatty acids were calculated according to the method of Richieri *et al.* (1993). In serum-free media, 0.4 mM palmitate or oleate bound to 1% BSA should result in 16.7 and 24.9 nM free fatty acids respectively. For experiments using 10% horse serum (assuming albumin is 4.5% horse serum), 0.6 mM palmitate or oleate bound to 1% BSA in 10% horse serum should result in 17.6 and 26.6 nM free fatty acids respectively. Assay values for free fatty acids in normal horse serum were expected to be 0.5 mM (Zimmerman *et al.* 1992), thus the concentrations of 10% horse serum would be expected to be 0.05 mM.

Insulin secretion and insulin content

For the assessment of glucose-stimulated insulin secretion in static incubations, batches of 30 islets were washed thrice in Krebs-Henseleit buffer containing 1% BSA, 10 mM HEPES, and 2.8 mM glucose for 20 min. After this pre-incubation, the islets were incubated for 60 min in the presence of 5.6 mM glucose followed by 60 min in the presence of 10.0 mM glucose. In parallel trials, 0.4 mM palmitate was also included in the stimulation medium. The amount of insulin released into the media during the 1-h incubations was then determined. Intracellular insulin content was measured in each batch of 30 islets after acid-alcohol extraction. Insulin was measured using a sensitive rat insulin RIA (Linco Research, St Charles, MO, USA).

Islet cell proliferation

To estimate islet β -cell proliferation, 10 μ M 5-bromo-2'-deoxyuridine (BrdU) were added during the final 12-h culture (Brelje & Sorenson 1991). The islets were rinsed with Hank's balanced salt solution (HBSS), fixed in 4% paraformaldehyde for 30 min, and incubated in 2.0 M HCl for 20 min. The BrdU-labeled nuclei were identified by staining overnight at 4 °C with a 1:200 dilution of a mouse monoclonal anti-BrdU antibody (Caltag Laboratories, San Francisco, CA, USA) and a 1:600 dilution of a CY3-18-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Because of the large size of intact islets, glass beads of 50–100 μ m maximal diameter were included in the mounting media to support the cover slips and prevent excessive deformation of the islets. The number of BrdU-labeled nuclei per islet was counted by direct observation with a conventional epifluorescence microscope with a minimum of 75–100 islets examined per condition. To facilitate comparison of data, changes in islet cell proliferation are expressed as fold the value observed in control islets (2.6 ± 0.3 BrdU-labeled nuclei/islet for all experiments).

Islet cell viability

To estimate the rate of cell death in islet β cells, 1 μ M of the cell impermeant nucleic acid stain YoYo-1 (Molecular Probes, Eugene, OR, USA) was added during the final 3-h culture (Weinhaus *et al.* 2000). The islets were rinsed with HBSS and fixed in 4% paraformaldehyde for 30 min. The islets were mounted as described previously, and the number of YoYo-labeled nuclei per islet was counted by direct observation with a conventional epifluorescence microscope with a minimum of 50 islets examined per condition.

Western blots

Western blots were prepared from islets as described previously (Brejje *et al.* 2004). An equivalent amount of islet protein was loaded in each lane for electrophoresis. Sheep anti-glucokinase (provided by Dr Mark Magnuson, Vanderbilt University, Nashville, TN, USA) was used at a dilution of 1:6000. Bound antibodies were detected using a 1:30 000 dilution of alkaline phosphatase-conjugated donkey secondary antibodies (Jackson ImmunoResearch Laboratories) and the chemiluminescent substrate CPD-Star (Applied Biosystems, Foster City, CA, USA). A Bio-Rad GS-700 (Bio-Rad) was used for quantitative densitometry with the volume density of bands calculated as $\text{O.D.} \times \text{mm}^2$ after background correction.

Expression of data and statistics

Data are expressed as the mean $\pm$ S.E.M. of the number of observations. Statistical analysis was performed by the Student–Neuman–Keuls one-way ANOVA for multiple comparisons. Each treatment group within an experiment was replicated five times and each experiment repeated three to six times.

Results

Effects of prolactin and palmitate on insulin secretion

To investigate whether fatty acids can counteract the effects of prolactin on insulin secretion, islets were cultured for 8 days with 500 ng/ml prolactin, 0.4 mM palmitate, or the combination of both prolactin and palmitate (Fig. 1A). The insulin secretion data are presented as fold increase over that of controls. With the exception of the first 24 h where there is an approximate 30% decrease in insulin values, the daily insulin release for the rest of the experiment remained stable. Prolactin or palmitate by themselves increased insulin released on day 1 and further increased it through day 3 after which it was maintained through the 8 days ($P < 0.05$). The combination of both prolactin and palmitate was more effective than either alone ($P < 0.01$). The cumulative insulin secretion was calculated as the sum of the daily values (Fig. 1B). A 2.6- and 2.8-fold increase in insulin secretion was observed with prolactin or palmitate by themselves respectively ($P < 0.01$). A 4.8-fold increase in insulin secretion

Figure 1 Effects of prolactin and palmitate on insulin secretion. Islets were cultured for 8 days with 500 ng/ml prolactin (PRL) and/or 0.4 mM palmitate (Palm; $n=5$). (A) Insulin secretion was increased on all days with prolactin or palmitate by themselves ($P < 0.05$). The combination of prolactin and palmitate was more effective than either alone on all days ($**P < 0.01$). (B) Cumulative insulin secretion was calculated as the sum of the daily values. Prolactin and/or palmitate increased insulin secretion by themselves ($*P < 0.01$) and in combination further increased insulin secretion compared with prolactin alone ($**P < 0.01$).

was observed with the combination of both prolactin and palmitate ($P < 0.01$). Although these results suggest that palmitate does not counteract the effects of prolactin on insulin secretion, differences in glucose-stimulated insulin release cannot be inferred from these data because of the presence of palmitate in the media.

Effects of prolactin and palmitate on glucose-stimulated insulin secretion

For a better estimate of actual differences in glucose-stimulated insulin secretion, islets were stimulated with only

glucose in 1-h static incubations after culture for 4 days with 500 ng/ml prolactin, 0.4 mM palmitate, or the combination of both prolactin and palmitate. The islets were first stimulated with 5.6 mM glucose to approximate their effects on basal insulin secretion at the normal serum glucose concentrations. Insulin release with 5.6 mM glucose was increased from islets cultured with prolactin alone or in combination with palmitate (Fig. 2A, $P < 0.05$). The islets were then stimulated with 10 mM glucose to examine their response to glucose stimulation. As expected from previous studies, insulin release with 10 mM glucose was increased nearly twofold from islets cultured with only prolactin (Fig. 2A, $P < 0.05$), while it was reduced by 38% from islets cultured with only palmitate (Fig. 2A, $P < 0.05$). More importantly, insulin release with 10 mM glucose was also reduced by 31% from islets cultured with both prolactin and palmitate compared with those cultured with only prolactin (Fig. 2A, $P < 0.05$). In this case, the extent of insulin release was now identical to that from control islets at 10 mM glucose. This result demonstrates that palmitate can inhibit the effects of prolactin on insulin release at the higher glucose concentrations. This supports our hypothesis that fatty acids may counteract the effects of lactogens on insulin secretion stimulated with only glucose in late pregnancy.

However, this inhibition of glucose-stimulated insulin secretion by prior culture with palmitate is the opposite of the increase in insulin secretion during culture (10 mM glucose data of Fig. 2A versus Fig. 1). This suggested that the islets have adapted to the chronic elevation in fatty acids and are dependent on palmitate to maintain the normal responsiveness. To examine this possibility, additional trials were done to examine insulin release in 1-h static incubations in the presence of 0.4 mM palmitate. In islets cultured with only palmitate, the addition of palmitate to the stimulation media increased insulin release with 5.6 mM glucose (Fig. 2B, $P < 0.05$) and reversed the inhibition of insulin release with 10 mM glucose (Fig. 2B, $P < 0.05$). In the latter case, the extent of insulin release was now 1.5-fold higher than from control islets stimulated with only glucose. More important was the similar effects on insulin release from the islets cultured with both prolactin and palmitate. The addition of palmitate to the stimulation media further increased insulin release with 5.6 mM glucose (Fig. 2B, $P < 0.05$). Remarkably, the extent of insulin release was now similar to that from control islets stimulated with only 10 mM glucose (Fig. 2B). Similarly, the addition of palmitate to 10 mM glucose also reversed the inhibition of insulin release from islets cultured with both prolactin and palmitate (Fig. 2B, $P < 0.05$). In this case, insulin release was now twofold higher than from islets cultured with only prolactin. (Also, note that the relative relationships between insulin release with 10 mM glucose and 0.4 mM palmitate in Fig. 2B matches the cumulative insulin secretion shown in Fig. 1B). These results demonstrate that prior culture with palmitate can actually enhance the effects of prolactin on insulin secretion when stimulated with glucose in the presence of palmitate. As this is the likely condition *in vivo*,

Figure 2 Glucose-stimulated insulin release (GSIS) in response to 5.6 mM (open bars) or 10 mM (filled bars) glucose for 1 h from islets after culture for 4 days with 500 ng/ml prolactin (PRL) and/or 0.4 mM palmitate (Palm). (A) At 5.6 mM glucose, insulin release was increased from islets cultured with either prolactin or in combination with palmitate ($*P < 0.05$). At 10 mM glucose, insulin release was increased from islets cultured with prolactin ($*P < 0.05$). In contrast, prior culture with palmitate inhibited subsequent insulin release by itself ($*P < 0.05$) or in combination with prolactin ($**P < 0.05$ versus prolactin alone). (B) Insulin release was also examined with 0.4 mM palmitate in the stimulation media. At both 5.6 and 10 mM glucose, the addition of palmitate increased insulin release from islets cultured with palmitate alone ($*P < 0.05$) or in combination with prolactin ($**P < 0.05$ versus prolactin alone). The addition of palmitate to the stimulation media restores insulin release from islets cultured with palmitate alone or in combination with prolactin to levels above those from controls or cultured with prolactin alone respectively. Results are the means $\pm$ S.E.M. of six separate experiments.

it suggests that fatty acids may actually enhance rather than inhibit the effects of lactogens on insulin secretion in late pregnancy.

Effects of prolactin and palmitate on islet cell proliferation

To investigate whether fatty acids counteract the effects of prolactin on islet cell proliferation, islets were cultured for 4 or 8 days with 500 ng/ml prolactin either in the absence or presence of 0.4 mM palmitate. The dividing cells were visualized by labeling with BrdU during the last 12-h culture (Fig. 3A). As expected from previous studies (Brelje *et al.* 1994, 2004), the number of BrdU-labeled nuclei/islet was increased eightfold by prolactin ($P < 0.05$). A smaller fourfold increase was observed with 0.4 mM palmitate ($P < 0.05$). Unexpectedly, the combination of prolactin and 0.4 mM palmitate was slightly more than additive with a 17-fold increase ($P < 0.05$). Similar results were observed after 4 and 8 days. These results suggest that palmitate enhances rather than inhibits the effects of prolactin on islet cell proliferation.

To examine whether a higher concentration of palmitate inhibits the effects of prolactin on islet cell proliferation, additional trials were done with 0.8 mM palmitate (Fig. 3A). The number of BrdU-labeled nuclei/islet was similar to the lower concentration of palmitate after 4 days with 0.8 mM palmitate alone or in combination with prolactin ($P < 0.05$). However, unlike the lower concentration of palmitate, these effects were absent after 8 days for both conditions. That this higher concentration of palmitate was deleterious to islets was supported by their irregular shape, signs of disintegration, and loss of islets. (<20% of the islets were recovered after 8 days compared with 70% after 4 days regardless of the presence of prolactin). These results suggest that this higher concentration of palmitate also stimulates islet cell proliferation, but that prolonged exposure is toxic to β cells under these culture conditions.

Effects of prolactin and palmitate on islet cell viability

To investigate the effects of prolactin and palmitate on islet cell viability, islets were cultured for 4 days with 500 ng/ml prolactin either in the absence or presence of 0.4 or 0.8 mM palmitate. Dead cells were visualized by incubation with the YoYo-1, a cell impermeant nuclei acid stain, during the last 3-h culture (Fig. 3B). The low number of YoYo-labeled nuclei/islet was unchanged by either prolactin or 0.4 mM palmitate by themselves or in combination. This result indicates that 0.4 mM palmitate does not adversely affect islet cell viability after 4 days under these culture conditions. In contrast, there was a large increase in the number of YoYo-labeled nuclei with 0.8 mM palmitate ($P < 0.01$). This effect was substantially reduced by prolactin. These results indicate that the higher concentrations of palmitate decrease islet cell viability, but that its effects are partially blocked by the anti-apoptotic effects of prolactin on β cells.

Figure 3 Effects of prolactin and palmitate on islet cell proliferation and viability. (A) Islets were cultured for 4 or 8 days with 500 ng/ml prolactin (PRL) and/or 0.4/0.8 mM palmitate (Palm) and dividing cells visualized by labeling with BrdU during the last 12 h. Islet cell proliferation was increased after both 4 and 8 days with prolactin or 0.4 mM palmitate by themselves (* $P < 0.05$). The combination of prolactin and 0.4 mM palmitate was more effective than either alone (** $P < 0.05$ versus prolactin alone). Palmitate (0.8 mM) by itself or in combination with prolactin only increased islet cell proliferation after 4 days. Results are the means $\pm$ S.E.M. of 75–100 islets per condition. (B) Islets were cultured for 4 days with 500 ng/ml prolactin (PRL) and/or 0.4/0.8 mM palmitate (Palm) and dead cells identified by staining with YoYo-1 during the last 3 h. Only 0.8 mM palmitate increase the number of YoYo-labeled cells which was prevented by the addition of prolactin. Results are the means $\pm$ S.E.M. of 75–100 islets per condition. * $P < 0.01$.

Dose-dependent effects of palmitate on islets

Because the serum concentration of fatty acids gradually increase during the last half of pregnancy (Scow *et al.* 1964, Knopp *et al.* 1973, Chen *et al.* 1992), we examined whether the lower concentrations of palmitate were also effective in increasing insulin secretion and islet cell proliferation from islets cultured with prolactin. Islets were cultured for 4 days

with 0.05–0.8 mM palmitate either in the absence or presence of 500 ng/ml prolactin. Palmitate increased the cumulative insulin secreted into the media in a dose-dependent manner at the concentrations of 0.1 mM and higher (Fig. 4A, $P < 0.05$). In combination with prolactin, an increase was detected for the concentrations of 0.2 mM and

Figure 4 Dose-dependent effects of palmitate on insulin secretion and islet cell proliferation. Islets were cultured for 4 days with 500 ng/ml prolactin (PRL) and/or 0.05–0.8 mM palmitate. (A) Palmitate by itself increased insulin secretion in a dose-dependent manner at concentrations of 0.1 mM and higher (* $P < 0.05$; $n = 5$). Palmitate also increased insulin secretion with prolactin at concentrations of 0.2 mM and higher (** $P < 0.05$ versus prolactin alone, $n = 5$). (B) Palmitate by itself increased the number of BrdU-labeled nuclei/islet in a dose-dependent manner at concentrations of 0.05 mM and higher (* $P < 0.05$). Palmitate also increased islet cell proliferation with prolactin at concentrations of 0.2 mM and higher (** $P < 0.05$ versus prolactin alone). Results are the means $\pm$ S.E.M. of 75–100 islets per condition.

higher when compared with prolactin by itself (Fig. 4A, $P < 0.05$). Similarly, palmitate increased the number of BrdU-labeled nuclei/islet in a dose-dependent manner at concentrations as low as 0.05 mM and reached a maximum threefold increase at 0.8 mM (Fig. 4B, $P < 0.05$). Prolactin by itself induced a sixfold increase. The combination of both prolactin and palmitate resulted in even larger increases at the concentrations of 0.2 mM palmitate and higher compared with prolactin by itself (Fig. 4B, $P < 0.05$). These results demonstrate that the lower concentrations of palmitate can increase insulin secretion and islet cell proliferation suggesting the changes in serum levels of fatty acids may be sufficient to alter insulin secretion and islet cell proliferation during pregnancy.

Effects of prolactin and oleate on islets

Because the serum concentration of oleate also increases during the last half of pregnancy (Chen *et al.* 1992), we also examined whether oleate had similar effects on islet function. Islets were cultured for 4 days in the presence of 500 ng/ml prolactin either in the absence or presence of 0.4 mM palmitate or oleate. Although palmitate increased insulin secretion from islets cultured by itself or in combination with prolactin, oleate had little effect on the cumulative insulin secreted into the medium whether by itself or in combination with prolactin (Fig. 5A). Likewise, differences were also observed in the intracellular insulin content of these islets (Table 1). Palmitate reduced insulin content ($P < 0.05$). This reduction was prevented when in combination with prolactin. In contrast, oleate did not alter insulin content, but in combination with prolactin increased insulin content even higher than that with only prolactin ($P < 0.05$). Unlike their differences on insulin secretion, both palmitate and oleate modestly increased the number of BrdU-labeled nuclei/islet by about threefold (Fig. 5B, $P < 0.05$). Prolactin by itself induced a sixfold increase that was further increased when in combination with either palmitate or oleate. Although the effects of prolactin and palmitate were slightly more than additive, the 21-fold increase with the combination of prolactin and oleate (Fig. 5B, $P < 0.01$ versus prolactin by itself) was more than twice the tenfold increase expected from adding together the effects of prolactin and oleate by themselves (note that the combinations of both palmitate and oleate gave similar results as expected from adding their individual responses, data not shown). Although these results suggest that oleate does not counteract the effects of prolactin on insulin secretion, oleate stimulates even larger increases in islet cell proliferation than palmitate when in combination with prolactin.

Dose-response of the effects of oleate on islets

To determine the dose-response effect of oleate on insulin secretion and islet cell proliferation, islets were cultured for

Figure 5 Effects of prolactin and oleate on insulin secretion and islet cell proliferation. Islets were cultured for 4 days with 500 ng/ml prolactin (PRL) and/or 0.4 mM palmitate (Palm)/oleate (Ole). (A) Although insulin secretion was increased by prolactin and palmitate, oleate by itself or in combination with prolactin did not further increase cumulative insulin secretion ($n=5$). (B) Oleate by itself modestly increased the number of BrdU-labeled nuclei/islet ($*P<0.05$). Although the effects of prolactin and palmitate are slightly more than additive, the combination of prolactin and oleate was more than twice the expected increase from adding together the effects of prolactin and oleate by themselves ($**P<0.05$ versus prolactin alone). Results are the means $\pm$ S.E.M. of 75–100 islets per condition.

4 days with 500 ng/ml prolactin either in the absence or presence of 0.05–0.8 mM oleate. As previously observed, oleate at the lower concentrations had no effect on the cumulative insulin secreted into the media whether by itself or in the presence of prolactin (Fig. 6A). In contrast, oleate increased the number of BrdU-labeled nuclei/islet in a dose-dependent manner at concentrations as low as 0.1 mM (Fig. 6B, $P<0.01$). In combination with prolactin, an increase was detected with concentrations as low as 0.2 mM oleate compared with prolactin by itself (Fig. 6B, $P<0.05$). These results demonstrate that the lower concentrations of oleate also increase islet cell proliferation.

Table 1 Effects of palmitate and oleate on the intracellular insulin content of islets after culture with prolactin. The insulin content of islets was determined after culture for 4 days in the presence of 500 ng/ml prolactin either in the absence or presence of 0.4 mM palmitate or oleate. The reduction in insulin content with palmitate was prevented by prolactin. In contrast, insulin content was unchanged by oleate but further increased the effects of prolactin

	Insulin content (mU/islet)	
	Control	500 ng/ml prolactin
Fatty acid		
None	4.35 $\pm$ 0.40	5.47 $\pm$ 0.58
0.4 mM palmitate	2.87 $\pm$ 0.18*	4.33 $\pm$ 0.33
0.4 mM oleate	4.85 $\pm$ 0.74	6.93 $\pm$ 0.69*

* $P<0.05$; $n=5$.

Effect of prolactin, palmitate, and oleate on glucokinase

Since fatty acid treatment has previously been shown to reduce the islet glucokinase levels (Gremlich *et al.* 1997) it was of interest to determine whether prolactin could prevent this from occurring. To examine this possibility, islets were cultured for 4 days with 500 ng/ml prolactin, 0.4 mM palmitate or oleate, or a combination of prolactin with palmitate or oleate. When glucokinase expression was examined by western analysis, prolactin resulted in a 30% increase in glucokinase whereas palmitate or oleate by themselves resulted in a 25–35% reduction in glucokinase (Fig. 7, $P<0.05$). When prolactin was combined with either palmitate or oleate, glucokinase levels were no longer inhibited by the presence of the fatty acids.

Effects of prolactin and fatty acids in serum on islets

Because all of our experiments were done in serum-free media with 1% BSA, we examined whether these fatty acids had similar effects on insulin secretion and β -cell proliferation in the presence of serum. Because the additional albumin in the horse serum will bind the small amount of free fatty acids, the concentration of fatty acids was increased to give comparable amounts with those in the serum-free media (see Materials and Methods).

Islets were cultured for 4 days with 500 ng/ml prolactin either in the absence or presence of 0.6 mM palmitate or oleate in 10% horse serum. Although the addition of 1% fatty acid-free BSA to 10% horse serum did not affect insulin secretion (Fig. 8A), it surprisingly reduced the number of BrdU-labeled nuclei/islet by two-thirds in both control islets and those cultured with prolactin (Fig. 8B, $P<0.05$). The dramatic decrease in cell division by the addition of fatty acid-free BSA suggests β cells may require a basal level of fatty acids for the normal function. Nevertheless, the effects of palmitate or oleate by themselves or in combination with prolactin were similar to those observed in serum-free media. Insulin secretion was increased by palmitate by itself (Fig. 8A, $P<0.01$) or in combination with prolactin (Fig. 8A, $P<0.05$ versus prolactin by itself). Similarly, palmitate and oleate increased islet cell proliferation by themselves in serum

Figure 6 Dose-dependent effects of oleate on insulin secretion and islet cell proliferation. Islets were cultured for 4 days with 500 ng/ml prolactin (PRL) and/or 0.05–0.4 mM oleate. (A) Although insulin secretion was increased by prior culture with prolactin, oleate by itself or in combination with prolactin did not increase cumulative insulin secretion at any concentration ($n=5$). (B) Oleate by itself increased the number of BrdU-labeled nuclei/islet in a dose-dependent manner at concentrations of 0.1 mM and higher ($*P<0.01$). Oleate also increased islet cell proliferation with prolactin at concentrations of 0.2 mM and higher ($**P<0.05$ versus prolactin alone). Results are the means $\pm$ S.E.M. of 75–100 islets per condition.

(Fig. 8B, $P<0.05$). In combination with prolactin and palmitate was still nearly additive while oleate was synergistic (Fig. 8B, $P<0.05$ versus prolactin by itself). These observations show that the effects of prolactin in combination with palmitate or oleate on β -cell proliferation also occur in the presence of serum.

Discussion

This study examined whether the elevation in serum lipids during pregnancy could counteract the effects of lactogens on islets during late pregnancy. To examine this possibility, we

Figure 7 Effects of prolactin and fatty acids on glucokinase expression. Islets were cultured for 48 h with 500 ng/ml prolactin (PRL) and either 0.4 mM palmitate (Pal) or oleate (Ole). The islets were examined by western blot analysis. Prolactin resulted in a 30% increase in glucokinase, whereas both fatty acids resulted in a 25–30% decrease in glucokinase. The combination of prolactin with either fatty acid prevented the inhibition of glucokinase by fatty acids. ($*P<0.05$, $n=4$).

investigated the influence of fatty acids in the form of palmitate or oleate on the effects of prolactin in cultured islets. These conditions should mimic those islets experience during late pregnancy *in vivo* when they are exposed to high circulating levels of both lactogens and lipids. Insulin secretion was increased 2.5-fold from islets cultured with prolactin and/or palmitate (but not oleate) with the combination being additive. After culture with prolactin and palmitate, acute stimulation with 10 mM glucose for 1 h showed suppression of insulin release. However, including palmitate in the stimulation media restored insulin release to even higher levels. These results provide new insight into the changes in insulin secretion that occur in late pregnancy. Prolactin also increased islet cell proliferation by sixfold, while palmitate and oleate stimulated smaller threefold increase. However, in combination with prolactin, palmitate had a slightly more than additive effect on islet cell proliferation (11-fold), while the effect of oleate was synergistic (21-fold). These observations suggest serum lipids may further enhance islet cell proliferation in mid-pregnancy and that some other factor(s) are responsible for the suppression of islet cell proliferation in late pregnancy.

Although our proposed model for regulation of β cells by lactogens can account for the elevation of insulin secretion in mid-pregnancy, it cannot explain the return of glucose-stimulated insulin secretion to the control levels as observed in

Figure 8 Effects of prolactin and fatty acids in serum on insulin secretion and islet cell proliferation. Islets were cultured for 4 days with 500 ng/ml prolactin (PRL) and either 0.4 mM palmitate (Palm) or oleate (Ole) in 10% horse serum. (A) Insulin secretion was unaffected by the addition of 1% fatty acid-free BSA to 10% horse serum. As before, insulin secretion was increased by palmitate, but not oleate, by itself ($*P < 0.01$, $n = 5$) or in combination with prolactin ($**P < 0.05$ versus prolactin alone, $n = 5$). (B) In contrast, the addition of 1% fatty acid-free BSA to 10% horse serum reduced the number of BrdU-labeled nuclei/islet by two-thirds in both control islets and those cultured with prolactin. However, similar to the results in the absence of horse serum, both palmitate and oleate increased islet cell proliferation by themselves ($*P < 0.05$) or in combination with prolactin ($**P < 0.05$ versus prolactin alone). Results are the means $\pm$ S.E.M. of 75–100 islets per condition.

late pregnancy. In our previous study, glucose-stimulated insulin secretion was examined *ex vivo* from pancreases of pregnant rats (Parsons *et al.* 1995). Insulin secretion peaked on day 15 of pregnancy after exposure *in vivo* to high circulating levels of lactogen for several days while serum lipids were still in a normal range. Insulin secretion then returned to control levels in late pregnancy after continued exposure *in vivo* to high circulating levels of lactogens and several days of elevated lipids. However, glucose tolerance tests have shown that insulin secretion in late pregnancy, although less than

mid-pregnancy, is still higher than in non-pregnant individuals (Spellacy & Goetz 1962, Leturque *et al.* 1980) and that the serum insulin levels remain elevated until parturition (Kawai & Kishi 1999). This discrepancy between these *ex vivo* and *in vivo* observations has been puzzling. However, our experiments examining insulin release from islets cultured with prolactin and/or palmitate provides a possible explanation.

First, insulin release in response to only high glucose (i.e., 10 mM) suggests an explanation for the *ex vivo* observations. Insulin release was almost doubled in response to high glucose from islets cultured with only prolactin – a condition similar to mid-pregnancy with high circulating levels of lactogen. However, insulin release returned to the control levels in response to high glucose from islets cultured with prolactin and palmitate – a condition similar to late pregnancy with high circulating levels of lactogen and lipids. Thus, chronic exposure to palmitate suppressed the effects of prolactin on insulin release when stimulated with only high glucose in our model system. This observation suggests that elevated lipids *in vivo* may be responsible for the reduction in glucose-stimulated insulin secretion to the control levels in late pregnancy from pancreases examined *ex vivo* (Parsons *et al.* 1995). In both cases, the islets have adapted to elevated fatty acids but are being stimulated with only glucose.

Second, insulin release in response to high glucose (i.e., 10 mM) in the presence of palmitate suggests an explanation for the *in vivo* observations. For islets cultured with prolactin and palmitate, insulin release in response to high glucose in the presence of palmitate was now almost double that from islets cultured with only prolactin and stimulated with only high glucose. Thus, chronic exposure to palmitate further enhances the effects of prolactin on insulin release when stimulated with high glucose in the presence of palmitate in our model system. This suggests that the elevation in lipids during pregnancy leads to enhanced rather than reduced insulin secretion in late pregnancy *in vivo*. This would explain why glucose tolerance tests appear better throughout pregnancy than in non-pregnant individuals (Spellacy & Goetz 1962, Leturque *et al.* 1980).

Third, insulin release was dramatically elevated in response to glucose in the range of normal serum concentrations (i.e., 5.6 mM) from islets cultured with prolactin and palmitate. Remarkably, the extent of insulin release was now similar to that from control islets stimulated with only 10 mM glucose. Thus, lipids should induce an increase in basal insulin secretion during pregnancy. This is consistent with the maintenance of elevated serum insulin levels until parturition (Kawai & Kishi 1999).

Overall, these results suggest that the elevated insulin secretion in mid-pregnancy is primarily due to the effects of lactogen on islets. As pregnancy progresses, the increase in circulating levels of lipids induces a further adaptation in the islets. By late pregnancy, insulin secretion is less sensitive to glucose stimulation and becomes more dependent on the presence of fatty acids. This situation allows the maintenance

of elevated insulin secretion until parturition and provides a mechanism for the rapid return to normal glucose sensitivity and insulin secretion in the early postpartum period when lipids return to non-pregnant levels.

It is noteworthy that our results are similar to the studies investigating insulin secretion from fasting animals both *in vivo* and *ex vivo* (Stein *et al.* 1996, Dobbins *et al.* 1998). Insulin secretion was reduced by 80% from perfused pancreases *ex vivo* after fasting. However, insulin secretion *in vivo* was not similarly decreased. This suggested another factor was responsible for maintaining insulin secretion *in vivo* but was absent *ex vivo*. This circulating factor was free fatty acids. The simple addition of palmitate to the perfusion media boosted insulin secretion from the fasted pancreas, so that it was 80% greater than that from fed pancreases challenged only with glucose. Similarly, in our studies, insulin release from islets cultured with palmitate is reduced by 40% when stimulated with glucose alone (Fig. 2A) and increased 1.5-fold when stimulated with glucose and palmitate (Fig. 2B).

Fatty acids have been shown to inhibit the expression of glucokinase (Gremlich *et al.* 1997). However, in late pregnancy, glucokinase is not suppressed (Weinhaus *et al.* 1996). Since fatty acids are at their highest levels at the end of pregnancy (Scow *et al.* 1964, Knopp *et al.* 1973, Chen *et al.* 1992), it was unclear why islet glucokinase was not suppressed at this time. Therefore, it was of interest to determine if prolactin could prevent the inhibitory effects of fatty acids on glucokinase. Our results confirm that fatty acids are capable of reducing islet glucokinase and importantly their effects can be prevented by prolactin. Thus, prolactin may be preventing the suppression of glucokinase in late pregnancy that should otherwise occur with the elevated serum levels of fatty acids.

Although the increase in lipids during pregnancy may account for the changes in insulin secretion in late pregnancy, the results from our experiments examining islet cell proliferation in islets cultured with prolactin and/or palmitate were unexpected. In our previous study, β -cell division peaked in mid-pregnancy and returned to the control levels by day 18 of pregnancy in rats (Parsons *et al.* 1995). Although insulin secretion remains elevated during an artificial prolongation of pregnancy, β -cell division still remains at the control levels after day 18 (Kawai & Kishi 1999). Our original hypothesis was that fatty acids would counteract the effects of prolactin on β -cell division. This would provide a mechanism for the return to control levels in late pregnancy. However, palmitate by itself had a small stimulatory effect on islet cell proliferation which was slightly more than additive with the effect of prolactin. Thus, it is unlikely that elevated lipids in late pregnancy are responsible for the reduction in β -cell division to the control levels. Rather, it may contribute to the increase in β -cell division in mid-pregnancy because serum lipids start to increase as early as days 12–14 of pregnancy in rats (Scow *et al.* 1964, Knopp *et al.* 1973). In a large number of *in vitro* studies, we have consistently observed a sixfold increase in β -cell division with prolactin. During pregnancy in rats, the increase in β -cell division has been

reported to peak at eight- to tenfold in mid-pregnancy by several groups (Parsons *et al.* 1992, Nieuwenhuizen *et al.* 1998, Kawai & Kishi 1999). This is comparable with what was observed in this study with lower concentrations of palmitate or oleate in combination with prolactin. This suggests that fatty acid concentrations characteristic of pregnancy may enhance rather than inhibit β -cell division.

The combination of prolactin and higher concentrations of oleate was unusual in that it had a synergistic effect on islet cell proliferation. In previous experiments, we have examined a large number of growth factors on islets by themselves and in combination with prolactin. Prolactin has always been the most potent growth factor examined and in combination with other growth factors have, at best, been additive with prolactin (Brelje & Sorenson 1997). In this study, islet cell proliferation was increased more than twice that expected from adding together the effects of prolactin and 0.4 mM oleate by themselves. In this case, islet cell division was increased 6-fold with prolactin, 3-fold with oleate by itself, and 21-fold with the combination of both prolactin and oleate. This is the largest increase in growth promotion of islets in culture that we have observed. Although the reasons are unknown, synergy between IGF-1 and oleate on cell division in smooth muscle cells has been reported (Askari *et al.* 2002).

Although fatty acids can induce a large expansion of β -cell mass *in vivo* (Prentki & Nolan 2006), most studies have focused on the ability of saturated fatty acids, such as palmitate, to repress insulin gene expression, glucose-stimulated insulin secretion, and induce apoptosis in β cells (Nolan *et al.* 2006, Poirout *et al.* 2006). Their effect on β -cell replication has been a minor aspect of these studies except for those with β -cell lines. In general, palmitate appears to stimulate apoptosis and either reduce (Cousin *et al.* 2001, Xiao *et al.* 2002), or less frequently have no affect (Roche *et al.* 1999) on cell division in β -cell lines. Palmitate also prevents the stimulation of cell division by glucose or IGF-1 in INS-1 cells (Cousin *et al.* 2001). In contrast, oleate either stimulates (Roche *et al.* 1999) or has no affect (Higa *et al.* 2006) on cell proliferation in β -cell lines. However, studies with Zucker diabetic fatty rats noted that culturing islets from non-diabetic rats with a 2:1 mixture of palmitate:oleate for 7 days increases β -cell replication by threefold (Milburn *et al.* 1995, Hirose *et al.* 1996). Similarly, the infusion of rats with Intralipid for 4 days also increased β -cell mass by 54% and β -cell proliferation by 2.2-fold (Steil *et al.* 2001). Finally, palmitate was shown to slightly decrease and oleate to slightly increase β -cell proliferation in cultured human islets (Maedler *et al.* 2003). Overall, our observations provide further evidence that under certain conditions palmitate or oleate can enhance cell division in the normal β cells. This suggests that non-toxic concentrations of fatty acids may have beneficial effects on β -cell division in cultured islets. Further studies are needed to clarify their effects on the regulation of β -cell proliferation and their interactions with other β -cell growth factors.

It remains to be determined which factor(s) is responsible for the decline in β -cell proliferation from the peak in

mid-pregnancy to the control levels by parturition (Parsons *et al.* 1992, Nieuwenhuizen *et al.* 1998, Kawai & Kishi 1999). In previous studies, we have shown that progesterone (Sorenson *et al.* 1993) or glucocorticoids (Weinhaus *et al.* 2000) can counteract the stimulation of insulin secretion and β -cell proliferation by lactogens in cultured islets. Female mice lacking the progesterone receptor have an increase in islet mass and β -cell proliferation (Picard *et al.* 2002). In another study, progesterone given to pregnant rats increased β -cell replication in mid-pregnancy, but did not prevent the decline to the control levels in late pregnancy (Nieuwenhuizen *et al.* 1998). Although this supports a stimulatory role for progesterone on β -cell division, it is also compatible with the proposal that prolonged treatment with progesterone may lead to counteraction of the proliferation effect of lactogens (Sorenson *et al.* 1993). The effect of glucocorticoids on β cells is most likely restricted to the last 1–2 days as this would correlate with their peak serum levels.

A recent paper from the laboratory of Seung K Kim provides further insights into the regulation of β -cell proliferation during pregnancy (Kamik *et al.* 2007). Prolactin stimulates the JAK2/STAT5 pathway and induces the expression of targets like Bcl6. Bcl6 is a transcriptional repressor of *Men1* and reduces the expression of the protein menin. Menin functions as a complex that maintains expression of p27^{Kip1} and p18^{INK4C} which encode cyclin-dependent kinase inhibitors that prevent proliferation. They also showed that progesterone and dexamethasone partially reversed the effects of prolactin on menin expression. These data are particularly interesting because progesterone has been shown to increase the expression of p21^{cip} and p27^{Kip1} and inhibit the growth of breast cancer cells (Owen *et al.* 1998, Gizard *et al.* 2005). It is also interesting that prolactin increases the expression of p21^{cip} in β cells (Cozar-Castellano *et al.* 2006a,b). Although progesterone remains an interesting candidate for the suppression of β -cell replication in late pregnancy, further investigation is required.

In summary, this study provides evidence in support of the hypothesis that increasing concentrations of fatty acids contribute to the adaptation of islets to pregnancy. At mid-pregnancy, the elevated insulin secretion is largely accounted for by the effects of lactogens on islets. As circulating levels of lipids increase during pregnancy, the islets adapt to their continued presence and become less sensitive to glucose stimulation and more dependent on fatty acids. Although lactogens are primarily responsible for the increased β -cell division during pregnancy, fatty acids may also contribute to the large increase in β -cell division.

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