Reduction of islet pyruvate carboxylase activity might be related to the development of type 2 diabetes mellitus in Agouti-K mice

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Abstract
Pyruvate carboxylase (PC) activity is enhanced in the islets of obese rats, but it is reduced in the islets of type 2 diabetic rats, suggesting the importance of PC in β-cell adaptation to insulin resistance as well as the possibility that PC reduction might lead to hyperglycemia. However, the causality is currently unknown. We used obese Agouti mice (AyL) as a model to show enhanced β-cell adaptation, and type 2 diabetic db/db mice as a model to show severe β-cell failure. After comparison of the two models, a less severe type 2 diabetic Agouti-K (AyK) mouse model was used to show the changes in islet PC activity during the development of type 2 diabetes mellitus (T2DM). AyK mice were separated into two groups: mildly (AyK-M, blood glucose <250 mg/dl) and severely (AyK-S, blood glucose >250 mg/dl) hyperglycemic. Islet PC activity, but not protein level, was increased 1.7-fold in AyK-M mice; in AyK-S mice, islet PC activity and protein level were reduced. All other changes including insulin secretion and islet morphology in AyK–M mice were similar to those observed in AyL mice, but they were worse in AyK–S mice where these parameters closely matched those in db/db mice. In 2-day treated islets, PC activity was inhibited by high glucose but not by palmitate. Our findings suggest that islet PC might play a role in the development of T2DM where reduction of PC activity might be a consequence of mild hyperglycemia and a cause for severe hyperglycemia.

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Introduction
Pyruvate carboxylase (PC) is located in the mitochondria and converts carbons from pyruvate derived from glucose into the Krebs cycle intermediate oxaloacetate (MacDonald et al. 1996a,b). The major roles of PC are related to β-cell adaptation and insulin secretion. Inhibition of PC with its inhibitor phenylacetic acid (PAA) prevents glucose-stimulated insulin secretion (GSIS; Farfari et al. 2000, Liu et al. 2002, 2005). We found that PAA also reduces the β-cell proliferation response, a portion of adaptation of β-cell, in 60% pancreatectomized rats (Liu et al. 2005) and Zucker fatty rats (Liu et al. 2002). We reduced and increased PC activity in primary β-cells or β-cell line INS-1 cells and found that PC down- and up-regulated GSIS in both cell types and cell proliferation in INS-1 cells (Xu et al. 2008). Hasan et al. (2008) used PC siRNA to inhibit PC activity and subsequently inhibit GSIS. 13C-NMR isotopomer analysis (Lu et al. 2002, Cline et al. 2004) has shown a close correlation between flux through PC and the capacity of glucose to stimulate insulin secretion. In addition, Palmer et al. (2006) found that a single nucleotide polymorphism in the PC gene of African–Americans is significantly associated with the magnitude of the acute insulin response.

Oxaloacetate, a product of PC, enters pyruvate–malate shuttle (MacDonald 1995a, Lu et al. 2002) and pyruvate–citrate shuttle (Khan et al. 1996). These two shuttles play important roles in NADPH production and insulin secretion in the β-cells (MacDonald 1995a, Khan et al. 1996, Lu et al. 2002). In addition, oxaloacetate can be used for aspartate synthesis (Menendez et al. 1998), and NADPH for lipids and fatty acids synthesis (Infante & Huszagh 1998, Dmitriev 2001) and anti-apoptosis (Brune et al. 1992, Sheline & Choi 1998), and importantly for insulin secretion (MacDonald 1995a, 2003).

Pyruvate is also catalyzed into acetyl-CoA (Wallace 1985) in the mitochondria by pyruvate dehydrogenase (PDH; Zhou et al. 1995, 1996). In most cell types, the PDH pathway predominates. However, pancreatic β-cells express unusually high levels of PC (MacDonald 1995b). As a result, the β-cell is unique in that approximately equal amounts of pyruvate enter into Krebs cycle via PC and PDH (Khan et al. 1996). A primary role of PC in tissues such as liver and kidney is to provide substrate for gluconeogenesis (Bahl et al. 1997, Baverel et al. 2003). However, as pointed out by MacDonald (1995a), β-cells lack the essential gluconeogenic enzyme phosphoenolpyruvate carboxykinase, therefore high levels of PC are required for a different function in the β-cells such as
insulin secretion. On the other hand, entry of pyruvate into the Krebs cycle via PDH does not appear to be important since activation of PDH had only a minor effect on insulin release (Nicholls et al. 2002). We have demonstrated that inhibition of PDH activity by overexpressing pyruvate dehydrogenase kinase 4 in INS-1 cells could not significantly reduce insulin secretion (Xu et al. 2008).

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance and β-cell failure (DeFronzo & Prato 1996). Once insulin resistance occurs, pancreatic β-cells must secrete more insulin to maintain normal glucose levels. Increased insulin secretion requires β-cell adaptation, a process that includes both enhanced insulin secretion and β-cell proliferation. During compensated obesity, islet PDH activity is reduced while PC activity is unaffected (Liu et al. 2002, 2005), indicating that normal PDH activity is not important for β-cell adaptation. In T2DM, the failure of β-cell adaptation coincides with reduced mRNA levels of PC (Jonas et al. 1999, Kjorholt et al. 2002, 2005) and activities of PC (MacDonald et al. 1996a,b, 2009) and PDH (Zhou et al. 1995, 1996). These reports suggest that PC, not PDH, plays a key role in the development of T2DM.

However, the cause and effect of reduction of PC activity in the islets of type 2 diabetic mice on hyperglycemia are still unknown. In the current study, we used an obese mouse model (obese Agouti mice, AyL) and two type 2 diabetic mouse models (Agouti–K (AyK) and db/db) to observe the correlation between islet PC activity and hyperglycemia. During compensated obesity, islet PDH activity is reduced while PC activity is unaffected (Liu et al. 2002, 2005), indicating that normal PDH activity is not important for β-cell adaptation. In T2DM, the failure of β-cell adaptation coincides with reduced mRNA levels of PC (Jonas et al. 1999, Kjorholt et al. 2002, 2005) and activities of PC (MacDonald et al. 1996a,b, 2009) and PDH (Zhou et al. 1995, 1996). These reports suggest that PC, not PDH, plays a key role in the development of T2DM.

Materials and Methods

Animals and blood glucose and plasma insulin assay

Male type 2 diabetic Agouti–KK (AyK, KK background) and control KK mice, AyL (C57BL/6 background), and control C57BL/6 mice as well as type 2 diabetic db/db and control db/+ mice (all were obtained from Jackson Laboratory, Bar Harbor, ME, USA) aged 10–20 weeks were used for this research. The principles of animal laboratory care under the guidelines of both NIH and the University of Louisville and Research Institute for Children’s Institutional Animal Care and Use Committees were followed strictly. The mice were maintained at 25°C with a 12 h light:12 h darkness cycle. Body weight was determined and blood was collected after tail snipping. Blood glucose was measured with a glucose analyzer (Analox Instruments, Lunenburg, MA, USA). For measuring blood insulin, blood samples were collected in heparinized capillary tubes, and plasma insulin levels were determined with the Ultrasensitive Mouse Insulin-ELISA Test Kit (Mercodia, Uppsala, Sweden).

Glucose tolerance test

After 3 h of fasting, mice were administered i.p. 1.0 g glucose per kg body weight. Blood glucose levels were determined at 0, 15, 30, 60, and 120 min, and blood samples were collected (at 0, 15, and 30 min only) to measure plasma insulin levels.

Islet isolation, culture, and high glucose and palmitate treatment

Islets were isolated from the mice by an adaptation of the Gotoh method (Gotoh et al. 1987). Islets were cultured using previous method (Han et al. 2005). For high glucose or palmitate treatment, the islets that were isolated from 10-week-old KK mice were divided into seven groups the next morning, and different concentrations of glucose or palmitate were added to each group. After 48 h of cultivation, the islets were collected for PC activity assay.

Islet DNA, protein, and insulin contents

DNA was measured by the Labarca method (Labarca & Paigen 1980) and protein was measured by a commercial kit that used BSA as a standard (Bio-Rad). Islets were homogenized in acid ethanol and stored at −20°C until assay for insulin contents.

Insulin secretion

Ten islets were cultured in each 5-ml vial with 1 ml Krebs–Ringer bicarbonate buffer (KRB supplemented with 10 mmol/l Hepes, pH 7.4, and 0.1% BSA, bubbled with 5% CO2 and 95% O2) containing 2.8, 5.5, and 16.7 mmol/l glucose in a 37°C shaking water bath as we have described previously (Liu et al. 1998). After incubation for 60 min, vials were moved from water bath to ice to stop the reaction followed by a brief centrifugation at 4°C, and 0.5 ml KRB was moved into a glass tube and stored at −20°C until assay for insulin as described above.

PC activity assay and PC protein detection

PC activity was measured according to the method of MacDonald et al. (1996b). PC protein was detected using the method of MacDonald (1995b) and Xu et al. (2008).

Active PDH activity assay

Active PDH (aPDH) assay was measured as described previously (Zhou et al. 1995, Liu et al. 1999).

Islet morphology, immunohistochemistry, and β-cell mass

Immunohistochemistry was carried out as described previously (Jetton et al. 2001, Liu et al. 2002).
Table 1 General characteristics of 10- and 20-week-old KK, Agouti-K (AyK), C57BL/6, obese Agouti mice (AyL), db/+ , and db/db mice. Data are mean±S.E.M.

<table>
<thead>
<tr>
<th>Strains (n)</th>
<th>10 weeks</th>
<th>20 weeks</th>
<th>10 weeks</th>
<th>20 weeks</th>
<th>10 weeks</th>
<th>20 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK (30)</td>
<td>22.3±1.2</td>
<td>32.4±2.3</td>
<td>163±17</td>
<td>171±15</td>
<td>2.8±0.6</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>AyK-M (32–40)</td>
<td>24.2±1.1*</td>
<td>37.8±2.8*</td>
<td>214±22*</td>
<td>232±15*</td>
<td>6.5±1.9*</td>
<td>9.9±1.2*</td>
</tr>
<tr>
<td>AyK-S (10–15)</td>
<td>25.4±1.6*</td>
<td>39.4±2.5*</td>
<td>315±32†</td>
<td>357±56†</td>
<td>22.3±2.3*</td>
<td>26.0±2.6*</td>
</tr>
<tr>
<td>AyL (30)</td>
<td>22.4±1.3</td>
<td>33.6±2.5</td>
<td>165±15</td>
<td>174±18</td>
<td>2.3±0.7</td>
<td>2.8±0.8</td>
</tr>
<tr>
<td>C57BL/6 (30)</td>
<td>24.3±1.4</td>
<td>38.6±2.1*</td>
<td>152±12</td>
<td>165±13</td>
<td>3.2±1.2</td>
<td>3.5±1.4*</td>
</tr>
<tr>
<td>db/+ (30)</td>
<td>23.5±1.6</td>
<td>32.4±3.3</td>
<td>174±18</td>
<td>176±16</td>
<td>2.1±0.8</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>db/db (30)</td>
<td>26.6±1.7*</td>
<td>44.6±2.8†</td>
<td>365±42†</td>
<td>436±67†</td>
<td>4.5±1.3*</td>
<td>5.3±1.5†</td>
</tr>
</tbody>
</table>

KK means KK mice for control of AyK mice, AyK-M indicates mildly hyperglycemic AyK mice; AyK-S indicates severely hyperglycemic AyK mice, AyL indicates obese mice (A⁻⁻ gene mutation on C57BL/6 background), db/+ means littermates without leptin gene mutation and used for control of db/db mice, db/db indicates db/db mice with leptin gene mutation. Weeks indicate age. *P<0.05, †P<0.01 versus parallel controls; ‡P<0.01 versus AyK-M mice.

Data presentation and statistical methods
All data are expressed as mean±S.E.M. The listed n values represent the number of individual experiments performed, and every experiment was duplicated. Comparisons between two groups were performed by Student’s t-test. Comparisons between multiple groups were performed by one-way ANOVA (Tukey post hoc test). A value of P<0.05 was considered significant.

Results

Mildly and severely hyperglycemic AyK mice
Hyperglycemia in AyK mice was less severe than that in db/db mice; we simply designed a blood glucose value of 250 mg/dl as a standard point to separate the AyK mice into mildly (<250 mg/dl, AyK-M) and severely (>250 mg/dl, AyK-S) hyperglycemic groups. We used this value (250 mg/dl) to separate the mice because we found that most parameters including insulin secretion and PC activity in isolated islets were significantly changed if blood glucose levels were over 250 mg/dl and were consistently maintained for over 2 weeks. About 17% of AyK mice at an age of 10 weeks and 39% at an age of 20 weeks became severely hyperglycemic respectively. Because of the slow developmental process of severe diabetes, the AyK mice we kept provided an excellent model for investigating the pathogenesis of T2DM. db/db Mice were not ideal for this purpose because they became severely diabetic in a very short period of time: 57% of db/db mice at an age of 6 weeks and 100% of db/db mice at an age of 10 weeks became severely hyperglycemic respectively. However, db/db mice would be a good control model for showing severe diabetes.

General characteristics of 10- and 20-week-old AyK, AyL, and db/db mice
Body weights were measured periodically, and 3-h fasting blood glucose and insulin levels in 10- and 20-week-old AyL, AyK, and db/db mice were compared with their parallel normal controls. As shown in Table 1, AyL, AyK-M, AyK-S, and db/db mice were obese and had a similar body weight, but db/db mice were the heaviest. Compared with control KK mice, glucose and insulin levels of 10- and 20-week-old

Table 2 General characteristics of the islets isolated from 20-week-old KK, Agouti-K (AyK), C57BL/6, obese Agouti mice (AyL), db/+ , and db/db mice. Data are mean±S.E.M.

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Protein content (µg/islet)</th>
<th>DNA content (ng/islet)</th>
<th>Insulin content (ng/islet)</th>
<th>Insulin content (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK</td>
<td>0.72±0.15</td>
<td>20.6±5.3</td>
<td>56±12</td>
<td>78±17</td>
</tr>
<tr>
<td>AyK-M</td>
<td>1.35±0.24*</td>
<td>48.7±9.6*</td>
<td>115±38*</td>
<td>85±28</td>
</tr>
<tr>
<td>AyK-S</td>
<td>1.87±0.32†</td>
<td>52.4±12.4*</td>
<td>73±18*</td>
<td>39±8*</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0.67±0.18</td>
<td>18.9±4.5</td>
<td>47±15</td>
<td>70±19</td>
</tr>
<tr>
<td>AyL</td>
<td>1.28±0.25†</td>
<td>43.6±9.7†</td>
<td>152±32†</td>
<td>117±26*</td>
</tr>
<tr>
<td>db/+</td>
<td>0.75±0.17</td>
<td>22.4±5.7</td>
<td>64±17</td>
<td>85±16</td>
</tr>
<tr>
<td>db/db</td>
<td>1.66±0.38†</td>
<td>46.8±12.3†</td>
<td>35±9†</td>
<td>21±5†</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01 versus parallel controls; ‡P<0.05, †P<0.01 versus AyK-M mice.
AyK mice significantly increased, especially in AyK-S mice. Blood glucose levels in db/db mice were higher and blood insulin levels were lower than those in AyK-S mice. Islet protein and DNA contents were significantly increased in 20-week-old AyL, AyK-M, AyK-S, and db/db mice (Table 2); however, islet insulin contents in AyK-S and db/db mice were markedly reduced, especially in db/db mice. In contrast, islet insulin content in AyK-M mice was significantly increased, similar to those in AyL mice. The following results were obtained from 20-week-old mice except the data presented in Fig. 5.

**Glucose tolerance tests and plasma insulin levels**

As shown in Fig. 1A, during the course of each time point, the blood glucose levels of AyK-M mice elevated slightly but significantly compared with those of the control KK mice, suggesting the predominance of insulin resistance, but not β-cell failure. In AyK-S mice, however, blood glucose levels were strikingly elevated at every time point compared to those of either AyK-M or KK mice, indicating that severe insulin resistance and β-cell failure developed. Figure 3B indicates AUCglucose for Fig. 1A; the values are consistent with curve changes shown in Fig. 1A. Fasting blood glucose levels in AyK-S mice were lower than those in db/db mice (Table 1), suggesting that diabetes in AyK-S mice was less severe than that in db/db mice. We measured plasma insulin levels at 0-, 15-, and 30-min time points during glucose tolerance tests. As shown in Fig. 1C, the highest levels of fasting plasma insulin (0 min) were observed in AyK-S mice; fasting plasma insulin levels in AyK-M mice were also higher than those in the control KK mice. During glucose tolerance tests, similar to KK mice, plasma insulin levels in AyK-M mice at 15-min time point were significantly increased, indicating that partial β-cell function in response to high glucose stimulation was preserved. In contrast, AyK-S mice showed no significant change in insulin plasma level at the 15-min time point, suggesting that AyK-S mouse β-cells lost the responsive capability to glucose stimulation.

**Insulin secretion from islets isolated from AyK mice**

To confirm the results of in vivo insulin secretion, we isolated the islets from AyK-M and AyK-S mice and performed in vitro GSIS in the presence of 2.8, 5.5, and 16.7 mmol/l glucose. As shown in Fig. 1D, compared to control group, islet GSIS was significantly increased in AyK-M mice but it was...
reduced in AyK-S mice at each glucose concentration, indicating that β-cell insulin secretory function was significantly enhanced in AyK-M mice but definitely impaired in AyK-S mice. These data are consistent with the in vivo observation (Fig. 1C).

**PC and PDH activities in the islets of AyK-M and AyK-S mice**

Because of the importance of PC activity described in the Introduction section, we measured PC activity in AyK islets. As shown in Fig. 2A, islet PC activity but not protein expression level significantly increased (1.7-fold) in AyK-M mice; quantified PC protein contents increased by 1.3 ± 0.2-fold after normalizing to actin (P < 0.05 versus KK islets; n = 6), which is not consistent with increased PC activity (1.7-fold increase) but this change may account for the increase in PC activity. In contrast, a reduction in PC activity and protein level was observed in the AyK-S mice. Quantified PC protein contents in AyK-S islets were 45 ± 8% of control KK mice after normalizing to actin (P < 0.01 versus KK islets; n = 6); this change accounts for the reduction in PC activity. The changes in PC activity in AyK-M islets are consistent with those in an adaptive 60% pancreatectomized model, where islet PC activity is increased while protein concentration is not (Liu et al. 2005). However, the changes in PC activity in AyK-S islets are similar to those in human severe type 2 diabetic islets, and both Pc mRNA (significantly) and PC activity (slightly) levels are reduced (MacDonald et al. 2009). We measured αPDH activity in AyK islets to know whether the changes in PDH activity were consistent with those in obese rat islets (Liu et al. 2002). Our data shown in Fig. 2B demonstrate that islet αPDH activity was significantly reduced in both AyK-M and AyK-S mice. These data suggest that islet PC might play a compensatory role in β-cell adaptation to insulin resistance when PDH is reduced in AyK-M islets, and reduced islet PC activity in AyK-S mice might be associated with β-cell failure.

**PC and PDH activities and insulin secretion in the islets of AyL and db/db mice**

AyL and db/db mice were used as control models as described above, thus we measured islet PC and αPDH activities and insulin secretion in these mice. As shown in Fig. 3A, a 2.8-fold increase was observed in PC activity of AyL islets compared to that of C57BL/6 mice. This elevation was higher than that observed in AyK mice (1.7-fold increase, Fig. 2A). Similar to AyK-M islets, PC protein levels in AyL islets were slightly increased; quantified PC protein contents were 1.4 ± 0.2-fold increased after normalizing to actin (P < 0.05 versus C57BL/6 islets; n = 6), which is not consistent with increased PC activity (2.8 ± 0.4-fold increase). Similar to AyK-S mice, both PC activity and protein levels in db/db mouse islets were significantly reduced (Fig. 3A). Quantified PC protein contents in db/db islets were 35 ± 6% of control db/+ mice after normalizing to actin (P < 0.01 versus db/+; n = 6); this change accounts for the reduction in PC activity. Islet αPDH activity (Fig. 3B) was reduced in both AyL and db/db mice, which was similar to that in AyK-M and AyK-S mice (Fig. 2B). Islet GSIS was significantly increased in AyL mice (Fig. 3C), but it was reduced in db/db mice (Fig. 3D), and these results were also similar to those observed in AyK-M and AyK-S mice respectively (Fig. 1D). These data demonstrate that the changes in AyK-M islets were similar to
those in AyL mice, and that the changes in AyK-S mice were similar to those in db/db mice. In other words, β-cell adaptation was enhanced in both AyL and AyK-M mice; β-cell failure occurred in both AyK-S and db/db mice.

Comparison of physiological and biochemical parameters and islet characteristics in six strains of 20-week-old mice

To clearly highlight the importance of PC during the development of T2DM, we have summarized the dynamic changes in insulin resistance, blood glucose levels, PC activity, β-cell secretory function, and β-cell mass in Fig. 4, and these data are compared with islet morphological alterations. We also used C57BL/6 (control of AyL mice) and db/+ (control of db/db mice) mice as normal controls, followed by obese AyL, diabetic AyK-M, and AyK-S mice as well as db/db mice. The progressive process of T2DM is demonstrated clearly in all groups from normal, obese, mildly diabetic to severely diabetic. Insulin resistance was gradually enhanced starting at obese AyL mice; blood glucose levels were slightly increased in AyK-M mice and further increased in AyK-S mice and db/db mice; the latter had the highest blood glucose levels. Interestingly, PC activity was paralleled with β-cell function and mass, and these three parameters were increased in AyL mice, indicating that increased PC activity was associated with enhanced β-cell adaptation. In AyK-M mice, β-cell function and mass were also enhanced, but these parameters were partially suppressed because of a slight reduction in PC activity compared to that in AyL mice. β-Cell function and mass were significantly reduced in AyK-S and db/db mice, while PC activity was significantly reduced. Importantly, significant reduction in PC activity was also paralleled with the aberrations in islet morphology. All diabetic mice had large islet size. However, islet insulin staining was gradually reduced starting at AyK-M mice, and this change was paralleled with the reduction in PC activity. Islet insulin staining in these mice was consistent with the changes in insulin contents in isolated islets presented in Table 2. In db/db mice, they are supposed to be at an ending stage of T2DM, and most islet insulin staining was lost; in contrast, glucagon staining in the islets was greatly enhanced and aberrant glucagon distribution occurred. Figure 4 demonstrates a strong association between PC activity and β-cell function/mass and islet morphological aberrations during the pathogenesis of T2DM.

Figure 3 PC activity and protein expression (A), aPDH activity (B) and GSIS (C and D) in isolated islets of 20-week-old C57BL/6, AyL, db/+ and db/db mice. C57BL/6 mice were for control of AyL mice; AyL indicates obese (not diabetic) mice on C57BL/6 background; db/+ indicates littermates without leptin gene mutation and used for control of db/db mice; and db/db indicates db/db mice. Western blot on the top of (A) shows PC protein (∼130 kDa) contents and actin; each lane was loaded with 20 µg protein extract obtained from islets isolated from one animal; similar results were obtained in other two independent experiments (total six mice). Quantified PC protein contents are given in the Results section. Data are mean±S.E.M.*P<0.05,**P<0.01 versus parallel controls. In (A and B), n=6; in (C and D), n=4.
PC activity in high glucose and palmitate 2-day treated islets

Although Fig. 4 demonstrates an association between PC activity level and blood glucose level, the causality remains unknown. We hypothesized that slightly increased blood glucose level might suppress PC activity, because in mildly hyperglycemic AyK-M mice islet PC activity seems to be reduced to 1.7-fold if compared to AyL mice islet PC activity (2.8-fold increased). To test this hypothesis and test what level of hyperglycemia can initially inhibit PC activity, we isolated the islets from 10-week-old KK mice, and treated these islets with different concentrations of glucose for 2 days, and then measured PC activity. As shown in Fig. 5A, 11.1 mmol/l (equal to 200 mg/dl) glucose had no effect on islet PC activity; PC activity was initially suppressed by 12.5 mmol/l (225 mg/dl) glucose, and it was significantly reduced by 13.9 mmol/l (250 mg/dl) glucose. Interestingly, fasting blood glucose levels (Table 1) in AyK-M mice were consistent with these glucose concentrations. This result clearly demonstrates that mild hyperglycemia inhibits PC activity in KK mouse islets. To test if high fatty acids contribute to islet PC reduction, we treated the same islets with palmitate (up to 0.8 mmol/l, bound with BSA) for 2 days. Our data shown in Fig. 5B indicate that 2-day palmitate treatment did not significantly inhibit islet PC activity.

Discussion

Most data regarding the importance of PC were obtained from rat pancreatic islets (MacDonald et al. 1996a,b, Liu et al. 2002) or rat β-cell lines (Lu et al. 2002, Cline et al. 2004), and few studies have tested islet PC activity in type 2 diabetic mice. In the current study, we tried to observe whether islet PC activity is reduced in type 2 diabetic mice. As expected, islet PC activity and protein level were significantly reduced in severely diabetic AyK-S and db/db mice, and these results are consistent with those in type 2 diabetic rats (MacDonald et al. 1996a,b, 2009). Surprisingly, islet PC activity and protein concentration in mildly diabetic AyK-M mice were significantly increased (1.7-fold and 1.3-fold respectively). As a distinct compensatory model of obesity, AyL mice have shown a higher level of islet PC activity (2.8-fold, Fig. 3A) compared with C57BL/6 mouse islets. Thus, the elevation of islet PC activity in AyK-M mice may be a portion of enhanced β-cell adaptation to insulin resistance; reduced islet PC activity and protein level may be related to β-cell failure in AyK-S and db/db mice (Figs 2 and 3), and this observation is consistent with previous reports (MacDonald et al. 1996a,b, 2009). Our recent results (Xu et al. 2008) in PC up- and down-regulated β-cells support this hypothesis. Although a strong correlation between islet PC activity and hyperglycemia or β-cell failure ...
was observed, this correlation, however, did not uncover the causality. In addition, whether PC regulates GSIS is still controversial; Jensen et al. (2006) have observed that Pc siRNA did not inhibit GSIS in β-cell line INS-1 cells, and they have suggested an acetyl carnitine mechanism that compensates for PC inhibition in these cells.

We have known that obesity enhances insulin resistance (White 2003, Shoelson et al. 2006), and the latter can slightly elevate blood glucose levels after a meal challenge compared to noninsulin-resistant individuals (Ahren et al. 1999, Bajaj et al. 2002). Once significant insulin resistance develops, elevated blood glucose is expected to be higher and last for a longer time (Bruning et al. 1997, Gabriely et al. 2002). Compared to frank diabetes, however, this elevation in insulin-resistant individuals is mild (Zhou et al. 1999, Kido et al. 2000). Based on this recognition, we tested whether mild hyperglycemia inhibits islet PC activity. We isolated the islets from KK mice and treated the islets with different concentrations of glucose, from physiological to diabetic levels. This in vitro study confirmed that mild high glucose (12.5 mmol/l, equal to 225 mg/dl) initially reduced islet PC activity (Fig. 5A). This result helps us to interpret islet PC activity in AyK-M, AyK-S, and db/db mice. First, in AyK-M mice, islet PC activity was preserved because blood glucose levels were lower than 250 mg/dl (Table 1; mean values were 214 mg/dl at 10 weeks of age and 232 mg/dl at 20 weeks of age). Second, if 2.8-fold increase in islet PC activity in AyL mice is assumed as a 'normal level' for β-cell adaptation, then 1.7-fold increase in AyK-M islets would be a 'reduced level'. Because 12.5 mmol/l (equal to 225 mg/dl) glucose initially inhibited islet PC activity, a portion of elevated islet PC activity (the values from 1.7-fold to 2.8-fold) in AyK-M mice may be suppressed by mild hyperglycemia (232 ± 15 mg/dl, Table 1), thus only 1.7-fold increase rather than 2.8-fold increase in PC activity was observed in AyK-M islets. Third, based on the results shown in Fig. 5A, if the levels of glycemia are elevated to or over 13.9 mmol/l (equal to 250 mg/dl), islet PC activity may be significantly inhibited. Because the average value of blood glucose in AyK-S mice was 315 mg/dl at 10 weeks of age or 357 mg/dl at 20 weeks of age (Table 1), islet PC activity may be significantly suppressed (Fig. 2A).

Figure 5 PC activity in high glucose (A) and palmitate (B) 2-day treated islets isolated from 10-week-old KK mice. In (A), glucose was added to each group of the islets to yield 5-5, 8-3, 11-1, 12-5, 13-9, 16-7, and 19-4 mmol/l (final concentration) respectively, and 5-5 mmol/l glucose-treated group was used as control. In (B), palmitate was added to each group of the islets to yield 0-2, 0-3, 0-4, 0-5, 0-6, and 0-8 mmol/l (final concentration); a control group of islets were not treated with palmitate (0 mmol/l). All groups of islets in (B) were supplied with 5-5 mmol/l glucose. Palmitate was dissolved in 10% fatty acid free-BSA as a stock solution (8 mmol/l); the latter was diluted with 10% fatty acid free-BSA to yield different concentrations (10× of final concentration) just before use, and the final concentration of fatty acid free-BSA in the culture medium in all groups including control was 1%. Data are mean ± S.E.M., n = 4. In (A), *P < 0.05, **P < 0.01 versus control (5-5 mmol/l glucose).

Figure 6 Hypothetic scheme for islet PC activity as a consequence and cause of hyperglycemia during the pathogenesis of T2DM. 1) indicates that mild hyperglycemia leads to an inhibition in PC activity in the islets; 2) indicates that the inhibition of islet PC activity works as a cause of severe hyperglycemia; and 3) indicates that severe hyperglycemia in turn inhibits islet PC activity.
by higher levels of glycemia. Low islet PC activity in db/db mice (Fig. 3A) may also be explained by the same mechanism described above. Fourth, because PC has been demonstrated to be important in β-cell adaptation to insulin resistance and in insulin secretion (Liu et al. 2002, 2005, Xu et al. 2008), inhibited islet PC activity might contribute to β-cell failure; thus, severe hyperglycemia caused by β-cell failure would in turn suppress islet PC activity. This hypothesis is supported by the result shown in Fig. 5A, where high glucose (16.7 or 19.4 mmol/l, equal to 300 or 350 mg/dl respectively) significantly reduced islet PC activity; this may explain PC reduction in AyK-S and db/db mice (Table 1, Figs 2A and 3A).

Although our data can partially explain the inhibition of PC activity by hyperglycemia in diabetic mouse islets, and this is consistent with previous report in rat islet research (Liu et al. 2004), the ‘real’ mechanisms by which hyperglycemia inhibits PC activity are entirely unknown, thus further efforts are needed to reveal the ‘real’ mechanisms.

Because high levels of free fatty acids (FFAs) are also present in obese individuals (Unger 1995, Liu et al. 2002), they may be a factor to inhibit islet PC activity. Actually, Busch et al. (2002) have found that treating mouse cancer cell line MIN6 cells with palmitate and oleate for 48h resulted in a 2-2- and 1.8-fold reduction in Pc mRNA respectively. Our data, however, demonstrated that palmitate (up to 0.8 mmol/l) did not inhibit PC activity in 2-day treated KK mouse primary islets (Fig. 5B), and these results were consistent with our previous observation in rat islets (Liu et al. 1999, Xu et al. 2006). Compared with high glucose, FFA may not be a key factor to inhibit PC activity in 2-day treated mouse primary islets.

If AyL and db/db mice are considered as pre-diabetic and severely diabetic respectively, diabetic states in AyK-M and AyK-S mice would be in the early-middle and late-middle stages of the pathogenesis of T2DM. The islet micrographs shown in Fig. 4 clearly demonstrate that AyK-M and AyK-S mice stay on the way between pre-diabetes and severe diabetes: islet structure and staining density and distributions of insulin and glucagon in AyK-M mice are similar to those in AyL mice but are slightly worse. In contrast, all these changes in AyK-S mice are similar to those in db/db mice but are less severe. Importantly, PC activity levels were paralleled with β-cell function and mass during the pathogenesis of T2DM, strongly suggesting that PC is associated with insulin secretion and β-cell proliferation.

In summary, we found that islet PC activity and insulin secretion were increased in AyL and AyK-M mice and were reduced in AyK-S and db/db mice; additionally, islet morphological aberrations and a clear association between PC activity and hyperglycemia/β-cell function/mass have been demonstrated. An in vitro study has shown that islet PC activity was inhibited by high glucose but not by fatty acid palmitate. Our results suggest that increased islet PC activity might play an important role in β-cell adaptation to insulin resistance, and the reduction of islet PC activity in type 2 diabetic mice might be a consequence of mild hyperglycemia and a cause of severe hyperglycemia. We have summarized our hypothesis in Fig. 6. Having said this, the results in our current study would not be strong enough to draw a precise conclusion; β-cell Pc transgenic or knockout mouse models would be excellent tools for future studies. Because PC activity is sensitive to mild hyperglycemia indicated in this study, strict control of blood glucose levels would be very important to prevent the onset of frank diabetes in the patients with pre-diabetes or mild T2DM.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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