Research Paper

Decreased β-cell insulin secretory function in aged rats due to impaired Ca^{2+} handling

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Ageing is associated with an increased impairment in glucose homeostasis and an increased incidence of type 2 diabetes. In this study, we evaluated β-cell function and its implications for glucose homeostasis in 24-month-old female Wistar rats. Aged rats showed lower plasma glucose levels in the fed and fasting states compared with control rats. In addition, insulinaemia in the fed state was reduced in the older rats. Insulin receptor β (IRβ) expression was lower in the livers of the aged animals, whereas IRβ and $Akt_{1/2/3}$ protein expressions were higher in the muscles. These effects may contribute to the normal glucose tolerance observed in older rodents. Isolated islets from aged rats secreted less insulin in response to 8.3 and 16.7 mM glucose. Accordingly, this group presented a lower $[Ca^{2+}]_i$ in the presence of glucose and a depolarizing stimulus (30 mM K^+). In addition, islets from aged rats showed reduced insulin secretion in response to 100 μM carbachol (CCh), 10 nM phorbol 12-myristate 13-acetate and 10 μM forskolin. The expressions of protein kinase C, protein kinase A and exocytotic proteins, such as syntaxin 1 and synaptosomal-associated protein 25 kDa (SNAP-25), were similar in islets from aged and control rats. In conclusion, our evidence suggests that the increased incidence of type 2 diabetes with age may be due to a progressive decline in β-cell secretory capacity due to disruption of Ca^{2+} handling. Furthermore, the expression of proteins of the insulin transduction cascade showed an adaptive profile, with a compensatory increase in IRβ and $Akt_{1/2/3}$ in gastrocnemius muscles, which may maintain normal glucose homeostasis in 24-month-old rats.

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Elderly humans are more glucose intolerant and insulin resistant than young people, showing a higher prevalence of type 2 diabetes. Controversy remains as to whether this decrease in glucose homeostasis and insulin action is an inevitable consequence of biological ageing or due to environmental or lifestyle variables (Coon *et al.* 1992; Scheen, 2005; Cowie *et al.* 2006, 2009).

Age-related insulin secretory dysfunction may have a central role in alterations in glucose metabolism with age and contribute to the high rates of glucose intolerance in the older population (Scheen, 2005; Gunasekaran & Gannon, 2011). Pancreatic β-cells show a decline in secretory function with age (Ammon *et al.* 1984, 1987; Chiu *et al.* 2000; Muzumdar *et al.* 2004; Scheen, 2005; Ihm *et al.* 2007). Islets isolated from aged rodents have demonstrated a disruption in K^+ and Ca^{2+} handling (Ammon *et al.* 1987), lower insulin gene expression and content (Ammon *et al.* 1984; Ihm *et al.* 2007), decreased glucose transporter 2 mRNA (Ihm *et al.* 2007), reduced glucose oxidation via the pentose phosphate shunt, and reduced glutathione/oxidized glutathione ratio in response to increasing glucose concentrations (Ammon *et al.* 1984). In addition, β-cell proliferation demonstrates a progressive reduction in ageing humans (Chiu *et al.* 2000; Butler *et al.* 2003; Reers *et al.* 2009).

In the present study, we investigated Ca^{2+} handling and insulin secretion in response to glucose, and activation of phospholipase (PL)-C/protein kinase (PK)-C and

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adenylate cyclase (AC)/PKA pathways in pancreatic islets isolated from 24-month-old female rats. In addition, we verified some aspects of body glucose homeostasis in aged rats.

Methods

Animals

All experiments were approved by the ethics committee at Universidade Federal de São Paulo. The studies were carried out on 4-month-old (control group; CTL) and 24-month-old female Wistar rats (aged group) obtained from the breeding colony at Universidade Federal de São Paulo and maintained on a 12 h–12 h light–dark cycle (lights on 06.00–18.00 h) with controlled temperature (22 ± 1°C) and allowed free access to food (Rodent chow; Nutrilab, Colombo, Brazil) and water.

Intraperitoneal glucose tolerance test

For the intraperitoneal glucose tolerance test, after an overnight fast, blood glucose levels (time 0) were measured from the tail tip in rats using a glucose analyser (Accu-Chek Advantage; Roche Diagnostic, Basel, Switzerland). A glucose load of 2 g (kg body weight)$^{-1}$ was then administered by intraperitoneal injection, and additional blood samples were collected at 15, 30, 60 and 120 min.

Plasma levels of insulin and glucose

Fasted and fed rats were decapitated, their blood collected, and plasma was stored at −20°C for the measurement of insulin by radioimmunoassay (as previously reported by Ribeiro *et al.* 2010). Glucose was measured as described above.

Islet isolation and insulin secretion

Islets were isolated by collagenase digestion of the pancreas. For static incubations, groups of four islets from aged and CTL rats were first incubated for 30 min at 37°C in Krebs–Ringer bicarbonate (KRB) buffer with 5.6 mM glucose and 0.3% bovine serum albumin, and equilibrated with a mixture of 95% O_2–5% CO_2 to give pH 7.4. This medium was then replaced with fresh buffer and the islets incubated for 1 h in the presence of 2.8, 8.3 or 16.7 mM glucose. In another series of experiments, islets were incubated in the presence of 8.3 mM glucose without or with 100 μM carbachol (CCh), 100 nM phorbol 12-myristate 13-acetate (PMA), 10 μM forskolin or 1 mM 3-isobutyl-1-methyl-xanthine (IBMX). Aliquots of the supernatant at the end of the incubation period were kept at –20°C for subsequent insulin measurement by radioimmunoassay, as previously reported (Ribeiro *et al.* 2010).

Cytoplasmic Ca^{2+} oscillations

Fresh pancreatic islets were incubated with fura-2 AM (5 μM) for 1 h at 37°C in KRB buffer containing 5.6 mM glucose and 0.3% bovine serum albumin (pH 7.4). After this period, the islets were washed with the same medium and placed in a chamber that was thermostatically regulated at 37°C on the stage of an inverted microscope (Nikon UK, Kingston, UK). Islets were then perifused with albumin-free KRB buffer containing increasing glucose concentrations (2.8, 8.3 and 16.7 mM), as indicated in the figures. Another group of islets were perifused with 30 mM K^+ for 7 min. In addition, in order to assess Ca^{2+} release from intracellular stores, islets were perifused with a Ca^{2+}-free KRB buffer containing 8.3 mM glucose plus 250 μM of diazoxide and 10 mM EGTA without or with 100 μM of CCh (Ribeiro *et al.* 2010). A ratio image was acquired approximately every 5 s with an ORCA-100 CCD camera (Hammamatsu Photonics Iberica, Barcelona, Spain), in conjunction with a Lambda-10-CS dual filter wheel (Sutter Instrument Company, Novato, CA, USA), equipped with 340 and 380 nm, 10 nm bandpass filters and a range of neutral density filters (Omega opticals, Stanmore, UK). Data were obtained using the ImageMaster3 software (Photon Technology International, NJ, USA).

Western blot

For protein expression experiments, fragments of liver and muscle were quickly removed and solubilized in homogenization buffer [100 mM Tris (pH 7.5), 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 1% Triton X-100] at 4°C using a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Pancreatic islets were sonicated (three times, with 10 s pulses). The extracts were then centrifuged at 15300 *g* at 4°C for 40 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method (Bradford, 1976), using bovine serum albumin as a standard curve and a commercial Bradford reagent (Biotechnologia Ltda., São Paulo, SP, Brazil). For SDS gel electrophoresis and Western blot analysis, the samples were homogenized with a loading buffer containing 291 mM dithiothreitol. After heating at 95°C for 5 min, the proteins were separated by electrophoresis (70 μg protein per lane; 8 or 12% gels) and then transferred to nitrocellulose membranes.

The membranes were subsequently blotted with specific primary antibodies against IRβ (1:1000 dilution; cat no. sc-711), $Akt_{1/2/3}$ (1:1000 dilution; cat no. sc-8312), PKCα (1:1000 dilution; cat no. sc-8393), PKAα (1:1000 dilution; cat no. sc-903), syntaxin 1A (Synt 1; 1:1000 dilution; cat no. 12736) and synaptosomal-associated protein 25 kDa (SNAP-25; 1:1000 dilution; cat no. S5187). With the exception of SNAP-25 (Sigma-Aldrich, St Louis, MO, USA), all primary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Visualization of specific protein bands was carried out by incubating the membranes with appropriate secondary antibodies (1:10,000 dilution; Zymed Laboratories, Inc., San Francisco, CA, USA), followed by exposure to X-ray films. The band intensities were quantified by optical densitometry using the free software ImageJ (http://rsbweb.nih.gov/ij). After assaying the target proteins, Western blotting was repeated using a rabbit polyclonal antibody to glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH; 1:1,000 dilution; cat. no. sc-25778; Santa Cruz Biotechnologies) as an internal control.

Statistical analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. For insulin secretion experiments, 'n' refers to the number of experiments performed with groups of four islets each. For Ca^{2+} measurement, each perifused islet constitutes one 'n'. The statistical analyses were carried out using Student's unpaired t test (with significance accepted at $P \leq 0.05$) and performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

Animal features

Aged rats demonstrated an increased final body weight (247.5 ± 15 g) compared with CTL rats (208 ± 7 g; $P = 0.05$). Additionally, 24-month-old rats had lower blood glucose in both the fasting ($P < 0.05$) and the fed state ($P < 0.001$; Fig. 1*C* and *E*). Plasma insulin was also lower in aged rats in the fed state when compared

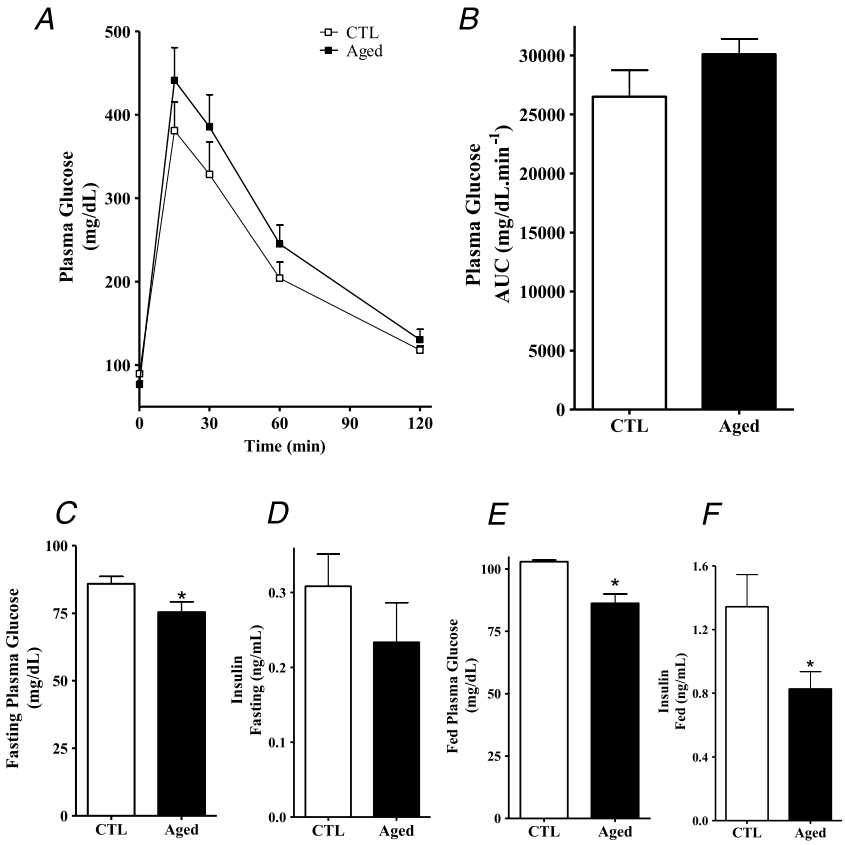

Figure 1.
(*A*) Changes in plasma glucose levels during ipGTT in aged and CTL female rats. Total glucose plasma concentration during the ipGTT, expressed by AUC (*B*). Fasting and fed plasma glucose (*C* and *E*) and insulin (*D* and *F*) concentrations. Data are means ± SEM obtained from 7–8 rats. *$P < 0.05$ indicates significant difference *versus* CTL.

with CTL rats ($P < 0.05$; Fig. 1*F*). Both groups showed similar tolerance to glucose, as indicated by the area under the curve (AUC) of the total glycaemia during the intraperitoneal glucose tolerance test (Fig. 1*A* and *B*).

Insulin-signalling protein expression in the liver and gastrocnemius muscle

Both liver and skeletal muscle are key organs for the control of glucose homeostasis through regulation of glucose production and uptake, respectively (Kahn *et al.* 2006). We next assessed the protein content of IRβ and $Akt_{1/2/3}$ in the liver and gastrocnemius muscle of aged and CTL rats. As indicated in Fig. 2*A*, aged rats presented a lower content of total IRβ in the liver ($P < 0.05$), whereas, despite a tendency towards a reduction in the aged group, the liver $Akt_{1/2/3}$ content did not differ between older and CTL rats ($P = 0.13$; Fig. 2*B*). Surprisingly, IRβ and $Akt_{1/2/3}$ protein levels were increased in the skeletal muscle of aged rats ($P < 0.05$; Fig. 2*C* and *D*, respectively), showing an opposite pattern to that observed in the liver. A global increase in peripheral glucose uptake, mediated by higher muscle insulin sensitivity, could explain the lower blood glucose observed in the older group.

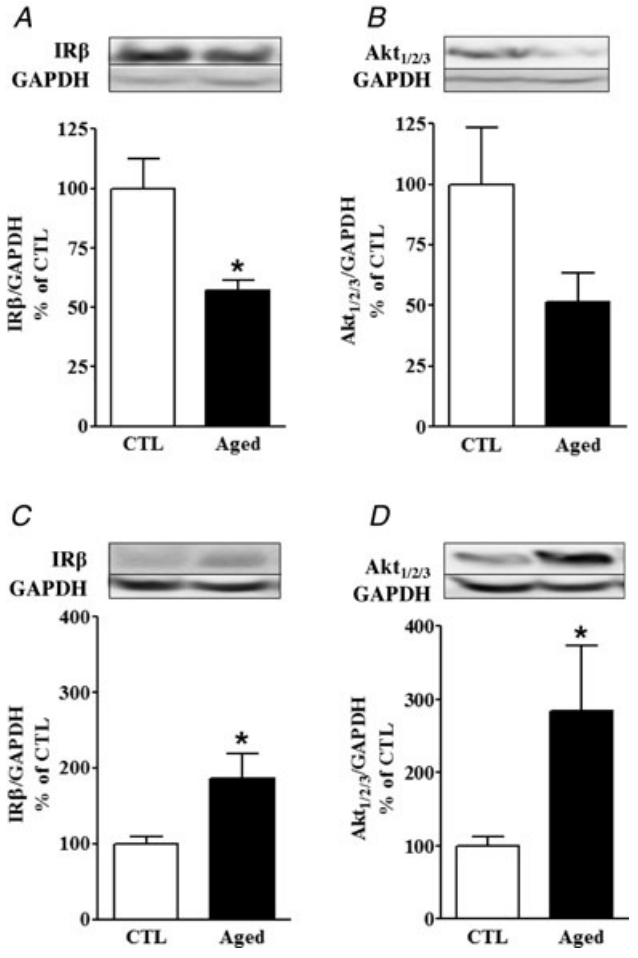

Figure 2. Protein expression of insulin receptor β (IRβ) and $Akt_{1/2/3}$ in the liver and gastrocnemius muscle from aged and CTL female rats
Fragments were obtained from the liver (*A* and *B*) and skeletal muscle (*C* and *D*) and used for immunoblotting experiments. The bars represent means + SEM of densitometric values obtained for the bands of aged ($n = 4$) and CTL rats ($n = 4$). $^*P < 0.05$ *versus* CTL.

Glucose-induced insulin secretion and cytoplasmic Ca^{2+} oscillations

To further evaluate adaptations in glucose homeostasis during ageing, we assessed β-cell pancreatic function by measuring insulin secretion and cytoplasmic Ca^{2+} oscillations in response to glucose. Figure 3 shows glucose-stimulated insulin secretion in isolated pancreatic islets. Isolated islets from aged rats secreted less insulin in the presence of 8.3 and 16.7 mM glucose, when compared with CTL islets ($P = 0.01$ and $P < 0.05$, respectively). In basal conditions of glucose concentration, insulin secretion was similar in the groups (Fig. 3).

In β-cells, membrane depolarization, opening of voltage-sensitive Ca^{2+} channels and subsequent Ca^{2+} influx are key events for granule trafficking and insulin exocytosis (Eliasson *et al.* 2008). Thus, islets were perifused with increasing glucose concentrations (2.8, 8.3 and 16.7 mM), and Ca^{2+} dynamics were registered in aged and CTL islets (Fig. 4*A* and *B*). Both islet groups demonstrated Ca^{2+} oscillations at 8.3 mM glucose, with a further increase at 16.7 mM glucose. However, aged islets presented a lower total $[Ca^{2+}]_i$ than CTL islets when perifused with

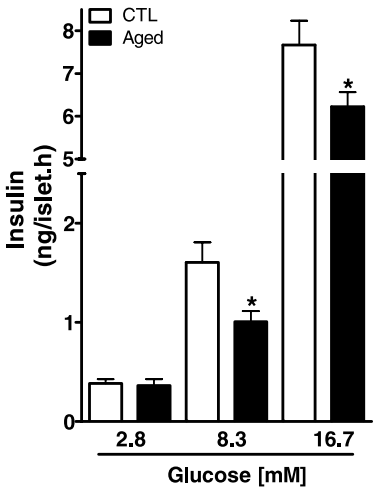

Figure 3. Glucose-induced insulin secretion in isolated islets from aged and CTL female rats
For static insulin secretion, groups of four islets were incubated for 1 h with 2.8, 8.3 or 16.7 mM glucose. Data are shown as means + SEM obtained from 25–54 groups of islets. $^*P < 0.05$ *versus* CTL for the same stimulus.

8.3 or 16.7 mM glucose ($P < 0.05$; Fig. 4*C* and *D*). In order to exclude differences between groups for glucose metabolism and to analyse the Ca^{2+} influx profile at β-cell depolarization, islets were perifused with 30 mM K^+ (Fig. 5). In these conditions, aged islets also showed a lower amplitude and AUC, when compared with CTL islets ($P < 0.05$; Fig. 5*C* and *D*).

Carbachol-, PMA-, forskolin- and IBMX-induced insulin secretion

We also investigated the contribution of PKC and PKA pathways to β-cell secretion in islets isolated from aged rats. Figure 6*A* shows insulin release in response to 8.3 mM glucose with or without CCh (100 μM), a cholinergic agonist, or PMA (100 nM), a PKC activator. These stimuli increased insulin secretion in both groups, but to a lesser extent in islets from aged rats compared with CTL animals ($P < 0.05$ and $P < 0.005$, respectively). However, the amount of Ca^{2+} mobilized from internal stores was similar for islets from aged and CTL rats when perifused with 100 μM CCh in a Ca^{2+}-free medium (0.15 ± 0.02 and 0.18 ± 0.02 F_{340}/F_{380} min^{-1}, respectively).

Figure 6*B* shows insulin secretion in response to 8.3 mM glucose with or without 10 μM forskolin, an AC activator, or 1 mM IBMX, a phosphodiesterase inhibitor. Both stimuli increased insulin secretion in older and CTL groups, whereas in response to forskolin, islets from aged rats released less insulin compared with islets from CTL animals ($P = 0.01$).

Protein expression of PKCα, PKAα, Synt 1 and SNAP-25

Figure 7*A* and *B* shows PKCα and PKAα protein expression, respectively. No difference was observed between groups. We also hypothesized that lower insulin

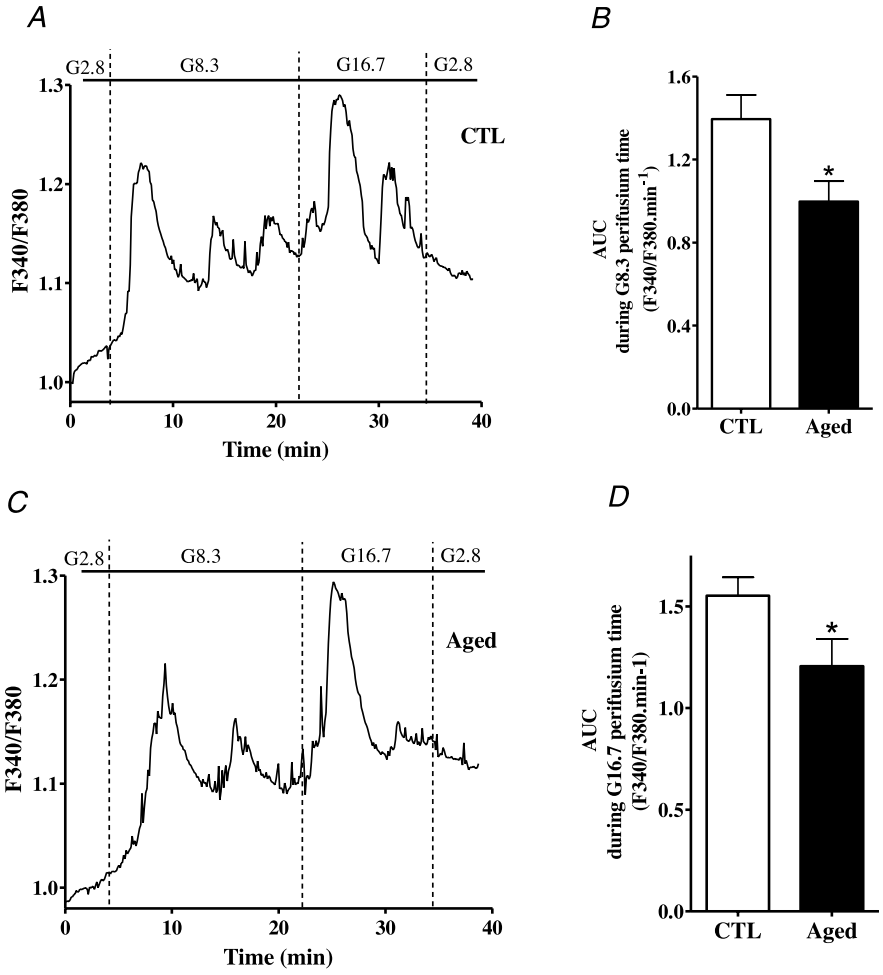

Figure 4. Representative curves of glucose-induced intracellular Ca^{2+} oscillations in islets isolated from aged (*A*) and CTL rats (*B*), and the AUC of the $[Ca^{2+}]_i$ in response to 8.3 (*C*) and 16.7 mM glucose (*D*)
The experiments were performed in a perifusion system in a medium containing 8.3 (G8.3) or 16.7 mM glucose (G16.7). Values are the flurorescence ratios, F_{340}/F_{380}, registered for each group. Data are shown as means + SEM obtained from eight to 10 independent experiments. $^*P < 0.05$ *versus* CTL.

secretion in islets from aged rats could be due to alterations in the exocytosis machinery, as previously reported (Nagamatsu *et al.* 1999; Vikman *et al.* 2006). Figure 7*C* and *D* shows that Synt 1 and SNAP-25 protein expressions were also similar between islets from aged and CTL animals.

Discussion

In the present study, we show that aged rats have a normal glucose tolerance, whereas in the fasting and fed states the plasma glucose levels were lower in aged rats compared with CTL rats. In addition, fed insulinaemia was reduced in these animals. Several reports have shown a higher age-related incidence of type 2 diabetes (Coon *et al.* 1992; Cowie *et al.* 2006, 2009; Gómez-Pérez *et al.* 2011). The appearance of glucose intolerance with the age is difficult to explain. It has been hypothesized that insulin resistance increases with age due to increased adiposity, decreased lean muscle mass, changes in dietary habits and reduced physical activity (Coon *et al.* 1992; Chang & Halter, 2003; Gunasekaran & Gannon, 2011). In our study, aged rats showed a lower IRβ protein expression in the liver, whereas a contradictory profile of this protein content in the gastrocnemius muscle was observed, with increased IRβ and $Akt_{1/2/3}$ expressions in older rats (Fig. 2). Although conflicting, our results are similar to observations on the gastrocnemius muscle of 18-month-old female rats reported by Gómez-Pérez *et al.* (2011). These authors showed that there is more age-associated oxidative damage

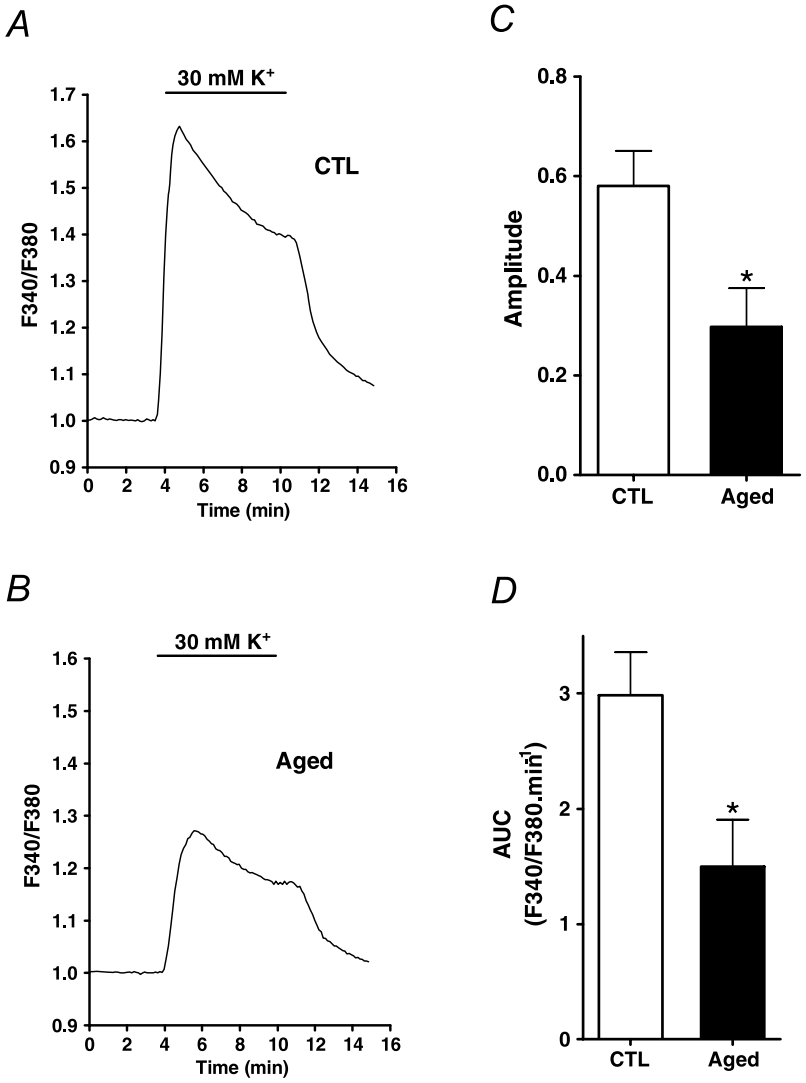

Figure 5. Calcium influx induced by 30 mM K^+ in isolated islets from aged (*A*) and CTL rats (*B*), and amplitude (*C*) and AUC (*D*) of the $[Ca^{2+}]_i$ influx induced by the depolarizing agent
The experiments were performed in a perifusium system in the presence of 2.8 mM glucose with or without 30 mM K^+ (indicated by the bars). Values are the fluorescence ratios, F_{340}/F_{380}, registered for each group. Data are shown as means + SEM obtained from five independent perifusion experiments. $^*P < 0.05$ *versus* CTL.

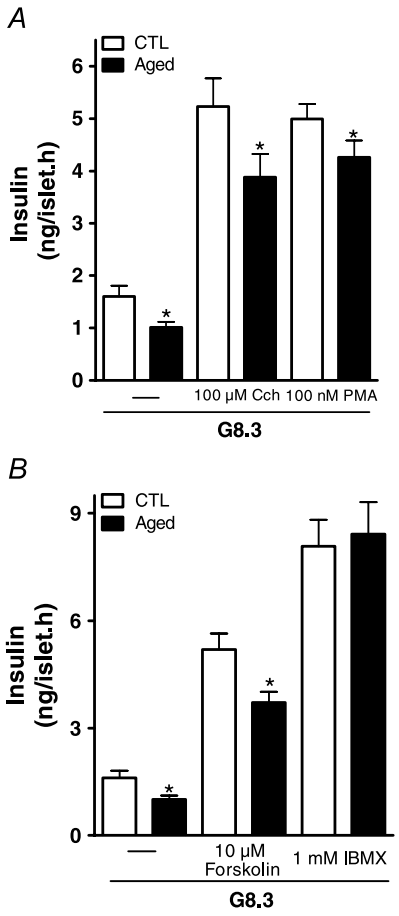

Figure 6. Insulin secretion induced by 100 μM carbachol (CCh) or 100 nM phorbol 12-myristate 13-acetate (PMA; *A*) and in response to 10 μM forskolin or 1 mM 3-isobutyl-1-methyl-xanthine (IBMX; *B*) in islets from aged and CTL rats
Groups of four islets were incubated for 1 h at 8.3 mM glucose (G8.3), with or without CCh, PMA, forskolin or IBMX. Each bar represents mean + SEM ($n = 15$–24). $^*P < 0.05$ *versus* respective CTL.

and insulin resistance in the gastrocnemius muscle than in the soleus, as well as increased IRβ and $Akt_{1/2/3}$ protein expression.

Our study also demonstrated an age-related decreased β-cell function. Isolated islets from aged rats presented a decreased insulin secretory capacity in the presence of increasing glucose concentrations (Fig. 3), an effect that was due to smaller glucose-induced intracellular Ca^{2+} oscillations (Fig. 4). Decreased insulin secretion in aged rats has been previously reported (Ammon *et al.* 1984, 1987; Muzumdar *et al.* 2004). These effects may be associated with a possible ATP-sensitive K^+ (K_{ATP}) channel alteration, because Ammon *et al.* (1987) showed that, in islets isolated from 24-month-old rats, a higher K^+ efflux was observed in 16.7 mM glucose, indicating that the normal inhibition of the K_{ATP} channels is lost with age. Furthermore, our data are in agreement with those of Ammon *et al.* (1987), who showed a lower $^{45}Ca^{2+}$ uptake following glucose stimulation in islets from 24-month-old rats. Also, aged rats demonstrated a significant decrease in glucose oxidation via the pentose phosphate shunt (Ammon *et al.* 1984). Thus, decreased Ca^{2+} handling and glucose signalling contribute to the decreased coupling between stimulus and secretion in the β-cells of the aged group.

Notably, our study is the first to show Ca^{2+} dynamics following a glucose stimulus in islets isolated from 24-month-old rats. In addition, we observed that the Ca^{2+} influx was impaired by the depolarization stimulus (30 mM K^+; Fig. 4), an effect that suggested a lower activation β-cell L-type Ca^{2+} channels. Pancreatic β-cells express the α1.2 and α1.3 isoforms of the L-type Ca^{2+} channels, which regulate Ca^{2+} influx during the first phase of insulin secretion (Schulla *et al.* 2003; Yang & Berggren 2006). In addition, L-type Ca^{2+} channels not only promote the cation influx, but directly interact with exocytotic proteins (Yang & Berggren 2006; Eliasson *et al.* 2008). Impairment in their action has been reported to decrease β-cell function in different models of type 2 diabetes (Kato *et al.* 1996; Roe *et al.* 1996; Iwashima *et al.* 2001).

In addition, in the β-cell, nutrient stimulation leads to membrane depolarization and Ca^{2+} influx, which activates the PLC–PKC and AC–PKA pathways (Tengholm & Gylfe, 2009). Activity of PLCδ1 has been found to increase in the presence of stimulatory glucose concentrations, resulting in a cycle of synthesis and degradation of the β-cell plasma membrane phosphatidylinositol 4,5-bisphosphate (Thore *et al.* 2005, 2007). Also, $[Ca^{2+}]_i$ may stimulate formation of cAMP in β-cells, because AC type VIII, present in β-cells, is a Ca^{2+}-sensitive isoform and, in the presence of glucose, may account for the increase in cAMP levels (Delmeire *et al.* 2003). In fact, a direct coupling between Ca^{2+} and cAMP has been reported by Dyachok *et al.* (2008). They showed that in β-cells, glucose induces oscillations in cAMP levels and that each oscillation is preceded and enhanced by the increase in $[Ca^{2+}]_i$. In our study, aged islets secreted less insulin in response to glucose (in an effect associated with a lower cytoplasmatic Ca^{2+} influx induced by glucose); therefore, the PLC–PKC and AC–PKA pathways may have been disrupted in the older group, decreasing glucose-induced secretion.

In this regard, we observed that isolated islets from aged rats also secreted less insulin in response to CCh, PMA and forskolin (Fig. 6). However, this effect was not accompanied by any alteration in islet PKC, PKA or exocytotic protein content (Fig. 7). We suggest that the impaired glucose-induced Ca^{2+} influx decreased PLC/PKC and AC/PKA activation, thereby lowering the β-cell secretory response in the aged group.

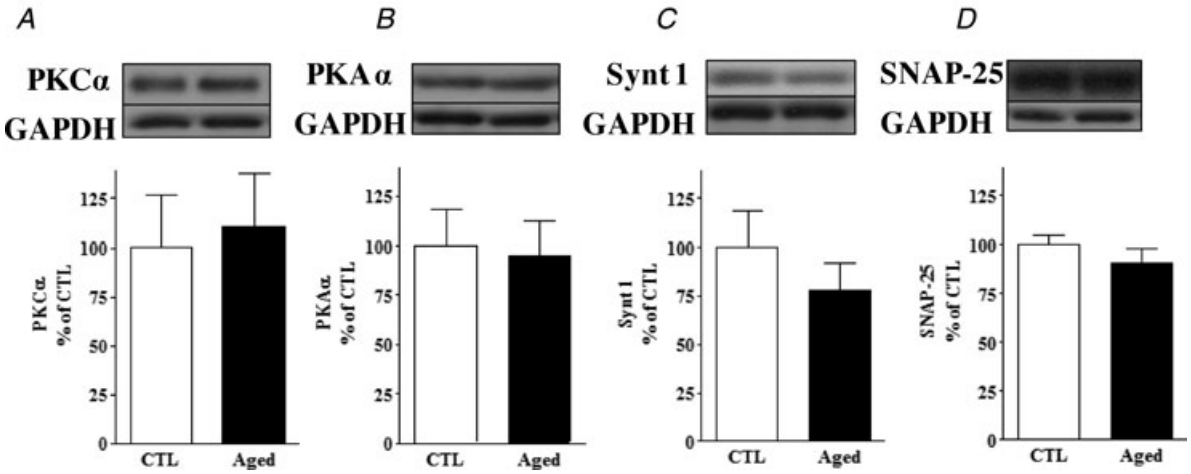

Figure 7. Protein expression of protein kinase C (PKCα), protein kinase A (PKAα), syntaxin 1 (Synt 1) and synaptosomal-associated protein 25 kDa (SNAP-25) in islets from aged and CTL rats
Protein extracts were processed for Western blot detection of PKCα (*A*), PKAα (*B*), Synt 1 (*C*), SNAP-25 (*D*) and GAPDH (internal control). The bars represent the means + SEM of the values, determined by optical densitometry ($n = 4$ or 5 rats).

Some evidence suggests that β-cell sensitivity to incretin hormones may be decreased with ageing. Treatment with exendin-4, a glucagon-like peptide-1 analogue, increases β-cell mass in young (6-week-old) mice but not in 8-month-old mice, indicating that the aged β-cell does not respond very well to glucagon-like peptide-1 (Tschen *et al.* 2009). This evidence, together with our observations, suggests that the lower activation of PKC and PKA may be a negative factor for maintenance of β-cell mass and proliferation during ageing. Furthermore, it has been reported that 8-month-old rats present a lower number of insulin-positive cells that express pancreatic duodenal homeobox-1 (Maedler *et al.* 2006), and islets from 22-month-old mice demonstrate a 50% reduction in pancreatic duodenal homeobox-1 mRNA content when compared with islets from 2-month-old mice (Ihm *et al.* 2007).

In conclusion, aged rats showed a normal glucose tolerance due to a compensatory increase in target proteins of the insulin transduction pathway in the muscle. Additional experiments are necessary to determine how long this compensatory action persists. Pancreatic islets from older rats showed a decline in the secretory function, with lower glucose-induced insulin release and Ca^{2+} influx. The reduction in Ca^{2+} handling may disrupt the coupling between stimulus and secretion in aged rats, probably via lower activation of the PLC/PKC and AC/PKA pathways, because the insulin secretion in aged islets was compromised in conditions in which these kinases are activated. This effect, together with a lower islet Ca^{2+} handling, in the aged group may compromise the activation of the PLC/PKC and AC/PKA pathways, which exert an amplifying action upon insulin granule exocytosis.

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