Melatonin inhibits insulin secretion and decreases PKA levels without interfering with glucose metabolism in rat pancreatic islets

Abstract: The effect of melatonin (0.1 μM) on freshly isolated islets from adult rats was investigated. Melatonin caused a marked decrease of insulin secretion by islets in response to glucose. The mechanism involved was then examined. Melatonin did not interfere with glucose metabolism as indicated by the measurement of glucose oxidation. However, the content of the protein kinase A (PKA) catalytic α-subunit was significantly decreased in islets exposed to melatonin for 1 hr in the presence of 8.3 mM glucose, whereas that of the protein kinase C (PKC) α-subunit remained unchanged. Melatonin also inhibited forskolin-induced insulin secretion, a well known activator of adenylyl cyclase (AC) activity. This may explain the low content of insulin found in islets incubated in the presence of melatonin for 3 hr. In fact, 3',5'-cyclic adenosine monophosphate (cAMP), a product of AC activity, stimulates insulin synthesis. These findings led us to postulate that a down-regulation of the PKA signaling pathway may be the mechanism involved in the melatonin inhibition of the process of glucose-induced insulin secretion.

Introduction

Blood glucose concentration is the most important regulatory stimulus for B-cell insulin secretion. The process of glucose-induced insulin secretion is fully dependent on the metabolism of the sugar in the B-cell. The products of the glucose metabolism, especially adenosine triphosphate (ATP), close the ATP potassium channel (K_{ATP}), increasing the intracellular potassium concentration that leads to a membrane depolarization [1]. This phenomenon induces the opening of the voltage sensitive calcium channels (VSCC) provoking a rapid increase in calcium influx and in the intracellular calcium concentration [Ca], that triggers granule exocytosis [2, 3]. On the other hand, the metabolism of glucose in the B-cell also activates several enzymes, particularly phospholipase C (PLC) and adenylyl cyclase (AC), which induce an increase in protein kinase C (PKC) and protein kinase A (PKA) activities, respectively [4, 5]. These kinases have an important role in the mechanism of glucose-induced insulin secretion, including opening of the VSCC [6].

The insulin secretion, however, is complex and several other stimuli interfere in the process of glucose-induced insulin release, including autonomic nervous system effects, circulating metabolites and hormones. Recently, in a series of perfusion experiments with isolated islets, Peschke et al. [7, 8] showed that melatonin inhibits glucose- and forskolin-induced insulin secretion. The data indicated that melatonin probably acts on cultivated neonate pancreatic islets by inhibiting AC activity through the pertussis-toxin sensitive G-protein system via Mel₁β receptors [8]. The elegant study of Peschke’s group [7] was performed in islets isolated from the pancreas of neonate rats and after an overnight period in culture. The authors did not investigate if melatonin affects glucose metabolism of the pancreatic islets. Also, effect of melatonin on insulin secretion by islets freshly obtained from adult rats remains to be investigated. These points were addressed in the present study.

For this purpose, pancreatic islets were isolated from adult rats and the effect of melatonin (0.1 μM) was immediately tested for its effect on glucose- and forskolin-induced insulin secretion, glucose oxidation, and the content of PKA and PKC α-subunits.

Materials and methods

Animals

Male albino rats weighing 150–200 g (45–60 days old) were obtained from the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. The animals were kept in groups of five at 23°C in a room with a light/dark cycle of 12/12 hr (lights on at 07:00 hr). Ethics approval was granted for this study by the Institute of Biomedical Sciences, Animal Experimental Committee, University of São Paulo, São Paulo, Brazil.

Chemicals

Collagenase type V, bovine albumin-fraction V, Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT) and melatonin were purchased from
Sigma Chemical Co. (St Louis, MO, USA). [U-14C]-Glucose and 125I-insulin were obtained from Dupont NEN products (Boston, MA, USA). Rat insulin standards and antirat insulin antibody were a gift from Dr Leclercq-Mayer, Université Libre de Bruxelles, Belgium. The reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA, USA). Protein A sepharose 6MB was from Pharmacia (Upsala, Sweden). Polyclonal anti-PKA and anti-PKC antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Insulin secretion during incubation of islets**

Rat pancreatic islets were isolated as described by Lacy and Kostianovsky [9]. Incubations of live islets were carried out at 37°C for 60 min in 0.5 mL of Krebs-Henseleit (KH) buffer (Na+ 139 mM, K+ 5 mM, Ca2+ 1 mM, Mg2+ 1 mM, Cl– 124 mM, HCO3– 24 mM) and 0.2% albumin in the presence of glucose (5.6; 8.3 or 16.7 mM). The buffer was equilibrated in a mixture of O2 (95%) and CO2 (5%). When indicated, melatonin was added to the medium at 0.1 μM. After incubation, the islets were washed three times with KH buffer, and resuspended in 1 mL KH buffer for sonication. Samples of the experiments were frozen and stored at −20°C for insulin determination.

**Perfusion experiments**

The method used was slightly modified from our previous publications [10]. Islets (n = 100) were preincubated for 60 min at 37°C in the presence of 16.7 mM glucose and 0.1 μM melatonin. After this period, the islets were washed five times with KH albumin buffer and perfused in a chamber containing a special filter (cellulose acetate-SCWP100, 8 μm pore size). The perfusion was performed with KH albumin buffer at a constant rate of 1 mL/min (Fig. 2). The perfusion medium was collected at 1-min intervals from the 31st until to the 70th minute using a fraction collector (ULTRORAC II, LKB, BROMA 2070). The composition of the perfusion medium was changed as follows: 5.6 mM glucose only from the 31st to the 40th min, 16.7 mM glucose without (control) or with 0.1 μM melatonin (Mel) from the 41st to 60th minutes and again 5.6 mM glucose only form the 61st to the 70th min. Aliquots of all experiments were frozen and stored at −20°C for insulin assay.

**Measurement of [U-14C]-glucose decarboxylation**

Groups of 30 islets were incubated in glass tubes containing 100 μL of KH albumin buffer solution, 2 μCi/mL [U-14C]-glucose and 5.6, 8.3 or 16.7 mM glucose in the presence and absence of 0.1 μM melatonin, equilibrated against a mixture of O2 (95%) and CO2 (5%). The tubes were transferred into counting vials that were carefully closed with a rubber stopper and incubated for 120 min in a shaker bath at 37°C. After this period, 0.1 mL of HCl (0.5 N) was added, by syringe, to the tubes containing the islets to stop the reaction, without loss of the gas produced during the experiment. The 14CO2 produced was exposed for 2 hr to NaOH solution (1 N) injected outside the tube containing the islets, in the same manner as for HCl. Biodegradable scintillation liquid (Amersham, Pharmacia, Upsala, Sweden) was added and radioactivity of the 14CO2 adsorbed by NaOH was counted.

**Insulin content of the islets**

Batches of 30 islets were incubated for 3 hr at 37°C in the KH buffer containing 8.3 mM glucose in the presence or absence of melatonin 0.1 μM. After incubation, the islets were washed three times with KH buffer, and resuspended in 1 mL KH buffer for sonication. Samples of the experiments were frozen and stored at −20°C for insulin determination.

**Determination of the content of PKA and PKC catalytic α-subunit by Western blotting**

Batches of 100 islets were incubated for 1 hr at 37°C in the KH buffer containing 8.3 mM glucose in the presence or absence of melatonin 0.1 μM. After incubation, 800 μL of ice-cold buffer A (100 mM Tris, 50 mM Hepes, pH 7.4, 100 mM sodium pyrophosphate, 10 mM sodium vanadate, 100 mM sodium fluoride, 1% triton-X-100, 2 mM PMSF and 0.1 mg/mL aprotinin) were transferred to Eppendorf tubes containing the islets, sonicated for cell disruption and centrifuged at 20,000 × g at 4°C for 15 min. The protein of the supernatant was measured [11] and used for immunoprecipitation with 10 μL of anti PKA catalytic α-subunit or 10 μL of anti-PKC catalytic α-subunit antibodies and protein A Sepharose 6MB or used as total extracts followed by 8% SDS-PAGE and immunoblot analysis [12].

**Statistical analysis**

Results of perfusion experiments, insulin secretion and CO2 produced are presented as means ± S.E.M. The statistical treatment was carried out using ANOVA. The mean values of incremental integrated areas in perfusion experiments was calculated by subtraction of each point after the 40th minute, until to the end of the experiment, from the mean of the basal values (from the 35th to 40th min). For analysis of the immunoblotting data, Student’s unpaired t-test was used. The level of significance was set for P < 0.05.

**Results**

Progressive increments of glucose concentrations (5.6, 8.3 and 16.7 mM) proportionally raised the amount of insulin released during 60 min incubation by control islets as expected (Fig. 1). Melatonin caused a marked inhibition of insulin secretion in the presence of all glucose concentrations. The indole also inhibited insulin secretion by pancreatic islets in the perfusion experiments (Fig. 2). Under these conditions, insulin release by 16.7 mM glucose stimulus was abolished by melatonin (Fig. 2).

The total content of insulin was determined in pancreatic islets incubated for 3 hr in 8.3 mM glucose in the presence
or absence of melatonin (0.1 μM). The indole caused a significant reduction of insulin content (from 9634 ± 258 to 6665 ± 771 μU per islet; mean ± S.E.M. of 10 samples).

The rates of [U-14C] glucose decarboxylation were also measured in islets incubated for 120 min in the presence of increasing glucose concentrations (5.6, 8.3 and 16.7 mM). Melatonin did not cause significant change of [U-14C] glucose decarboxylation (Fig. 3). This result indicates that glucose metabolism in pancreatic islets is not affected by melatonin and led us to investigate alterations in other important pathways involved in the process of glucose-induced insulin secretion. The effect of melatonin on forskolin-induced insulin secretion was then examined (Fig. 4). Forskolin per se caused a significant increase of insulin secretion. This effect of forskolin was abolished by melatonin, especially at 5 and 7 μM concentrations.

Discussion

The carbohydrate metabolism is influenced by melatonin both in humans [13–16] and in rodents [17–19]. Pinealectomy induces insulin resistance characterized by decreased glucose tolerance and a reduced glycogenesis in liver and muscle of rats [20–22]. Pinealectomy is followed by a reduction in blood sugar levels, and after alloxan treatment, the blood glucose rise is higher in pinealectomized than in intact rats [23]. Conversely, the infusion of pineal extracts [17] leads to hypoglycemia and increases glucose tolerance and hepatic and muscle glycogenesis after glucose loading. The changes in carbohydrate metabolism are, at least
in the presence of 16.7 mM glucose and with 0.1 
observed under static conditions, islets were preincubated
verify in a dynamic and more detailed way the inhibition
with higher glucose concentrations in the medium. To
of melatonin on insulin secretion became more pronounced
exposed to 16.7 mM glucose only. These findings confirm
the 60th min perfusion) in comparison with the islets
induced by pulses of glucose (50 mM) or KCl (100 mM).
Authors showed that melatonin decreases insulin secretion
by pulses of glucose (50 mM) or KCl (100 mM). Their experiments, however, were carried out in islets
isolated from neonate rats after being cultivated overnight.
Their experiments, however, were carried out in islets
on overnight-cultivated neonate pancreatic islets by inhib-
lating the AC/cAMP-system. This possibility was investi-
gated in our experimental model. We incubated isolated
islets in the presence of 5.6 mM glucose, in the absence or
presence of melatonin and melatonin plus forskolin
(Fig. 4). Confirming Peschke’s observations, melatonin
inhibited forskolin-induced insulin secretion. To better
clarify this point we then carried out experiments to
determine the content of PKA z-subunit in islets incubated
in the presence of melatonin. The content of PKA was
significantly decreased in islets exposed for 60 min to
melatonin in the presence of 5.6 mM glucose with no
alteration in the content of PKC z-subunit (Fig. 5). A
decreased content of cAMP may explain the lower content
of insulin observed in pancreatic islets after 3 hr incubation.
In fact, cAMP has been shown to stimulate insulin mRNA
transcription rate (30, 3129).

The results presented herein led us to conclude that
0.1 ε melatonin acutely inhibits insulin secretion by fresh
islets isolated from adult rats as reported by Peschke’s
group for overnight cultivation of neonate islets. This effect
is not associated with alteration in B-cell glucose metabo-
lism but with a decrease of PKA content. It remains to be
examined, however, if other pathways in the process of
insulin release are also affected by melatonin.

Acknowledgments
This research has been supported by FAPESP, CAPES,
CNPq and PRONEX. The authors are grateful to the constant technical assistance of Marlene S. da Rocha.

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