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Full Length Research Paper

Relationship Between Plasma and Platelet Serotonin Levels in Adolescents with Type 1 Diabetes

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The aim of this study was to determine, in adolescents with type 1 diabetes, 5-hydroxytryptamine (5-HT, serotonin) levels in plasma and platelets, 5-HT transporter (SERT), platelet 5-HT$_{2A}$ receptor and transglutaminase 2. We performed a cross-sectional studying two groups: 1) adolescents with type 1 diabetes and 2) controls. Three milliliters of blood was obtained from all patients to determine 5-HT levels in plasma and platelets. Furthermore, platelets were obtained to evaluate SERT expression, 5-HT$_{2A}$ receptor and transglutaminase 2 using Western blot. Dot blot was used for 5-HT. Glucose, glycated hemoglobin, albumin, triglycerides, cholesterol, hemoglobin, mean platelet volume and platelet count were also measured. Results were analyzed using Student *t* and Mann-Whitney U tests; $P < 0.05$ was accepted as statistically significant. Adolescents with type 1 diabetes had an elevation of plasma glucose and glycated hemoglobin ($P < 0.001$) as well as an increase the 5-HT levels in plasma and platelets along with a decreased expression of SERT, 5-HT$_{2A}$ and increased transglutaminase 2 in platelets compared to controls ($P < 0.05$). Increased the 5-HT levels in plasma and platelets, together with the decreased expression of SERT and 5-HT$_{2A}$ receptors and increase in transglutaminase 2 platelets, may have an important role in the prothrombotic state frequently presented by patients with diabetes.

Keywords: Serotonin, type 1 diabetes mellitus, platelets, SERT, 5-HT$_{2A}$ receptor, transglutaminase 2

INTRODUCTION

5-Hydroxytryptamine (5-HT, serotonin) in the body regulates multiple biological functions. In the brain, its roles as a trophic factor, neuromodulator and neurotransmitter have been widely studied (Lauder, 1993; Jacobs and Azmitia,1992; Mercado and Hernandez,1992). At the peripheral level, 5-HT is also synthesized from L-tryptophan (L-Trp) through two biochemical reactions: the first is catalyzed by L-tryptophan-5-monooxygenase (Tph) (Walther and Bader, 2003) to produce 5-hydroxytryptophan (5-HTP). A second

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reaction is catalyzed by a decarboxylase to synthesize 5-HT(Jacobs and Azmitia, 1992; Boadle-Biber, 1993; Hernandez and Illnerova, 1970). 5-HT at the peripheral level acts as a hormone, promotes smooth muscle contraction (Majno and Palade, 1961), modifies the permeability of blood vessels (Tamir, 1982), regulates the processes of secretion and osmosis (Mössner and Lesch,1998), acts in the inflammatory processes (YuB et al., 2008; Alderman et al., 1996), and actively intervenes in the mechanisms of platelet aggregation and blood coagulation (Lake et al., 2000; McNicol and Israels, 1999). With regard to its participation in the process of platelet aggregation, currently little significance has been attributed to it because 5-HT has been considered a mild platelet agonist compared to adenosine diphosphate (ADP). ADP and 5-HT are highly concentrated in the dense granules of platelets together with adenosine triphosphate (ATP) and Ca^{2+} (McNicol and Israels, 1999). 5-HT stimulates platelet aggregation and requires the concomitant activation of at least one Gi and one Gq protein coupled to the 5-HT_{2A} receptor (Maayani et al., 2001; Killam and Cohen,1991; Hoyer et al., 2002) which through its stimulation the phosphatidyl inositol pathway is activated, resulting in an increase of cytoplasmic Ca^{2+} (Offermanns et al., 1997; Schins et al., 2003). Moreover, a high expression of serotonin transporter (SERT) has been demonstrated in platelets (Lesch et al., 1993; Mercado and Kilic, 2010). It has also been observed that its inhibitors such as sertraline decrease the aggregation and secretion of α-granules in the platelets (Serebruany et al., 2001; Atar et al., 2007). Therefore, stimulation of platelets by 5-HT activates the phosphatidyl inositol pathway, producing an increase in intracellular Ca^{2+}. This signaling path is mediated by the Gαq protein coupled to the 5-HT_{2A} receptor (Offermanns et al., 1997). The increase of Ca^{2+} in platelets also activates the transaminases that utilize 5-HT as a substrate for small GTP ases such as α-RhoA and α-granules associated with Rab4, there by blocking the activity of GTP (Walther et al., 2003; Dale et al., 2002; Szasz and Dale, 2002; Shirakawa et al., 2000). When RhoA and Rab4 bound to GTP interact with effector molecules, rearrangement of the cytoskeleton and exocytosis of α-granules to the bloodstream takes place (e.g., von Willebrand Factor) or are exposed to the plasma membrane (e.g., glycoprotein IIb/IIIa and P-selectin), thus supporting irreversible platelet aggregation (Mercado and Kilic, 2010; Walther et al., 2003; Dale et al., 2002; Szasz and Dale, 2002; Shirakawa et al., 2000; Williams, 2012).

In summary, these data emphasize that the concentration of 5-HT levels in plasma depends on the expression of SERT and 5-HT_{2A} receptor located in the platelet membrane. However, at present, neither the contributions of these biological phenomena nor the order of appearance in the diabetic clinical state are known. Give in the clinical relevance of the diabetic state, all mechanisms summarized here are basic and important as the role of 5-HT in the clotting mechanisms. Therefore, the objective of this study was to determine, in adolescents with type 1 diabetes, the concentration of 5-HT levels in plasma and platelets as well as the expression of SERT and 5-HT_{2A} and their potential changes in the pathophysiological mechanisms regulating the concentration of5-HT levels in plasma

MATERIALS AND METHODS

The study was approved by the research and ethics committees of the National Commission for Scientific Research in Health of the Instituto Mexicano del Seguro Social (IMSS). All parents of patients provided written informed consent after a detailed explanation regarding the procedures to which their children would be subjected. A cross-sectional study was planned in 80 adolescents selected from the Endocrinology Service of the Pediatric Hospital, Centro Medico Nacional Siglo XXI, IMSS. Two groups were formed. The first group included 40 adolescents of both genders (22 males and 18 females) with type 1 diabetes and with a clinical course of 4.93 ± 2.9 (SD) years at the time of the study. Study participants were aged 13.50 ± 0.48 (SD) years and had a body mass index 20.2 ± 1.8 (SD) according to the national criteria of the Diabetes Group (American Diabetes Association, 2012). The second group was comprised of 40 normal adolescents (21 males and 19 females, aged 13.31 ± 0.43 (SD) years and with body mass index 20.8 ± 3.5 (SD). This group served as the control group. There was no observed concurrent pathology in either group. Exclusion criteria were the recent ingestion (<15 days) of medications with serotonergic activity (fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram, fenfluramine, buspirone, lithium, sumatriptan, or others) or anticholinergics, antihistamines, and H1 blockers as well as diabetic patients with a history of severe hypoglycemia (loss of consciousness or convulsions).

All adolescents were fed a normal diet of 55 kcal/kg/day (15% protein, 55% carbohydrates, 30% fats). Patients were under treatment with a combination of fast-acting and intermediate insulin at a rate of 1.0 to 1.5 U/kg/day. Three milliliters of blood was collected by venipuncture in borosilicate tubes containing 450µl anticoagulant citrate dextrose (ACD) solution (3.6 mg sodium citrate, citric acid 9.9 mg, 11 mg dextrose) between 07:00 and 08:00h and 12h after the last meal. Blood samples were immediately centrifuged at 500 x g for 3 min in a refrigerated centrifuge (AvantiJ-31, Beckman Instruments, Fullerton, CA) to obtain platelet-rich plasma (PRP). It was then centrifuged for 15 min at 6000 rpm to obtain platelet-poor plasma (PPP) and platelet concentrates (PC), where the 5-HT levels in plasma and platelets were determined by high-performance liquid chromatography (HPLC) with

fluorimetric detection as reported by Peat and Gibb, 1983. In the platelet membranes, the SERT expression, 5-HT_{2A} receptor and transglutaminase 2 by Western blot and Dot blot for 5-HT in platelets were evaluated. In addition, in the two groups of adolescents, blood glucose was determined using the glucose-oxidase method (Bentley, 1963), glycosylated hemoglobin by cation exchange chromatography (Cole et al., 1978), albumin according to the method of Doumas et al.,1971, triglycerides and cholesterol were quantified using a Flex reagents cartridge kit (Dade Behring Inc., Newark, DE, USA), which uses an enzymatic colorimetric method. Hemoglobin and platelet count were also evaluated by a colorimetric method and by flow cytometry.

Biochemical assays

5-HT levels in plasma and platelets

Aliquots of PPP were deproteinized with $HClO_4$ 0.1 M (10 mosm)+ 4 mM sodium metabisulfite and centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge (AvantiJ-31, Beckman Instruments). The supernatant was filtered with 0.2 µM nylon membranes and 5-HT levels in plasma were assessed. PC was re-suspended with $HClO_4$0. 1M + 4 mM sodium metabisulfite, homogenized and centrifuged at 10,000rpm for 10 min. The supernatant was filtered with 0.2 µM nylon membranes and 5-HT was determined. 5-HT levels in plasma and platelets were measured by HPLC according to the method of Peat and Gibb, 1983. A C_{18} reverse-phase symmetry column (5-∝m particle size, 3.9 x 150 mm) was used. A binary system was used with a monobasic 2 mM potassium phosphate solution, pH 3.40, plus heptanesulfonic acid,1 g/L of solution and a mixture of methanol/water at a ratio of 3:2 v/v and at a rate of 1 mL/min. 5-HT was assessed using a fluorometric detector (model 474, Waters) with a 290 nm excitation and 330 nm emission. The response was obtained with an analogous system (Empower2 Chromatography Data Software, Waters Corporation, Milford, MA). 5-HT concentration was considered as the maximum height of the signal according to a standard curve of known amounts and expressed in µmol/L.

Immunotransference of SERT, 5-HT_{2A} and transglutaminase 2 by Western blot

The PC was homogenized using a Glas-Col (Terre-Haute, USA) homogenizer (one minute at 1400 rpm)in Tris-HCl 50 mM solution, pH 7.4, plus protease inhibitors (Protease Inhibitor Cocktail, Sigma Chemical, St Louis MO, USA), to obtain samples enriched with platelet membranes. The samples were then centrifuged at 10,000 g for 15 min at 4ºC. The protein concentration was quantified by the Bradford method; 30 ∝g of protein was then placed in each of the channels of a 1-mm-thick 12% SDS-polyacrylamide gel. Electrophoretic run was carried out at 100 V for 2h. Electrotransference of the corresponding proteins was performed on nitrocellulose membranes, and the run was carried out at 10 V, 1.30 mA for 45 min. Nitrocellulose membranes with the transferred proteins were placed in a blocking solution (Millipore Chemiluminescent Blocker, Bedford, MA, USA) to 50% for 30 min. The membranes were incubated with primary monoclonal antibody specific for SERT (Chemicon, Temecula, CA), 5-HT_{2A} (Merck-Millipore) and transglutaminase 2 (Gene Tex International Corp.) at a 1:500 dilution in the same blocking solution. On the following day, membranes were incubated with anti-mouse secondary antibody (Chemicon) at a dilution of 1:5000 in the same blocking solution. Membranes were immediately incubated with Elite ABC system (Vector Laboratories, Burlingame, CA) (Naish, 1989) for 1 h. After the membranes were washed with PBS, they were revealed with diaminobenzidine (DAB). Internal control used was glyceraldehyde-3-phosphate dehydrogenase (GADPH). The bands obtained were analyzed and quantified by densitometry.

Dot blot for 5-HT in platelets

PC was homogenized with a Tris-HCl 50 mM, pH 7.4 solution. Four µL of the homogenate was placed in the center of the nitrocelluose membrane. The membrane was then placed in a Bio-Dot Bio-Rad apparatus, and the membranes were allowed to dry for 1 h. When finished, a block with TBS-BSA at 5% for 30 min was made. It was then incubated with the primary polyclonal antibody specific for 5-HT (Chemicon, Billerica, MA, USA) at a dilution of 1:1000 in TBS-BSA at 5% plus Tween 0.1% for 1 h. Three washes were then carried out with TBS-Tween 0.1%. Following this, the secondary antibody was added with a dilution ratio of 1:10,000, IDRye 800CW (alcohol) in TBS-Tween at 0.1% for 45 min. Membrane was immediately washed three times with TBS-Tween and developed with the Odyssey CLx alcohol system. Densitometry analysis was carried out using the ChemiImager 4400 software.

Statistical Analysis

To compare the results of the clinical and biochemical data from patients and controls, averages and standard deviations were first obtained. Differences among adolescents were carried out using Student *t*-test for normally distributed variables. For comparing the relative optical densities of the SERT and 5-HT_{2A} bands obtained by Western blot in platelets membranes and of the 5-HT Dot blot, averages and SD were first obtained. Groups were then compared using the Mann-Whitney U test; $p < 0.05$ was accepted as statistically significant.

Table 1. Clinical data of adolescents with type 1 diabetes and controls

	Controls		Type 1 diabetics	
	***n* = 40**	**CV**	***n* = 40**	**CV**
Age (years)	13.31 ± 0.43	0.03	13.50 ± 0.48	0.03
Gender				
Male	21		22	
Female	19		18	
Body weight (kg)	46.55 ± 2.47	0.05	48.33 ± 2.78	0.05
Height (m)	1.50 ± 0.09	0.06	150. 2 ± 0.14	0.09
BMI	20.8 ± 3.5	0.16	20.2 ± 1.8	0.09
Time of evolution(years)	-	-	4.93 ± 2.90	0.58

Values are expressed as average ± standard deviation.
Differences between groups were determined using Student t test.
CV, coefficient of variation; BMI, body mass index.

Table 2. Biochemical data of plasma of adolescents with type 1diabetes and controls

	Controls		Type 1 diabetics	
	***n* = 40**	**CV**	***n* = 40**	**CV**
Glucose (mg/dL)	90.1 ± 6.3	0.06	180.7 ± 15.5*	0.08
Hb_{A1c}	5.6 ± 0.04	0.07	8.90 ± 2.1*	0.23
Albumin (g/dL)	4.5± 0.2	0.04	4.9 ± 0.3	0.06
Cholesterol mg/dL	147.9 ± 10.5	0.07	148.1 ± 7.9	0.05
Triglycerides (mg/dL)	136.0 ± 5.6	0.04	85.5 ± 11.4*	0.13
Hemoglobin (g/dL)	12.35 ± 0.51	0.41	14.37 ± 0.21*	0.01
MPV (fl)	9.75± 0.19	0.02	9.74 ± 0.48	0.05
Platelet count (x10^3/ml)	274.0± 19.50	0.07	247.5 ± 12.01	0.05

Values are expressed as average ± standard deviation. All determinations were carried out in duplicate.
Differences between groups were determined using Student t test.
*p< 0.001.
CV, coefficient of variation; MPV, mean platelet volume.

RESULTS

Glucose and glycosylated hemoglobin in the plasma were significantly elevated in adolescents with type 1 DM ($p < 0.001$) (Table 1). The plasma albumin concentration was similar in both diabetic and control groups. Note that plasma triglycerides of diabetic adolescents were significantly less compared to the controls ($p < 0.001$) (Table 1). With regard to data reported for blood count, only a significant increase in the hemoglobin of diabetic patients was noted ($p < 0.001$) (Table 2).

Figure 1A shows the concentrations of 5-HT. As can be seen, there was a significant increase of 5-HT levels in plasma and platelet in adolescents with type 1 diabetes compared to normal controls ($p < 0.001$). To confirm the increase of 5-HT in platelets, a Dot blot-specific polyclonal 5-HT antibody was tested. As shown in Figure 1B[a], greater immunoreactivity was demonstrated in diabetic adolescents. Figure 1B[b] shows the arbitrary units of optical density. It is important to note that adolescents with diabetes demonstrated a significant increase of 5-HT in platelets when compared with controls ($p < 0.05$).

SERT expression in platelets was determined using Western blot. A 75-kDa band that corresponded to the SERT and another 37-kDa band for GADPH were observed both in control adolescents as well as diabetic subjects (see Figure 2A). Figure 2B shows the arbitrary optical densities of SERT. Interestingly, adolescents with diabetes showed a significant decrease of protein expression when compared with the controls ($p < 0.001$). Figure 2C shows the expression of the 5-HT_{2A} receptor in platelets of control and diabetic patients by Western blot. There was only one 54-kDa band in both groups of adolescents. Expression of GADPH was also observed. Note that in the same manner as demonstrated for SERT expression, adolescents with diabetes had a significant

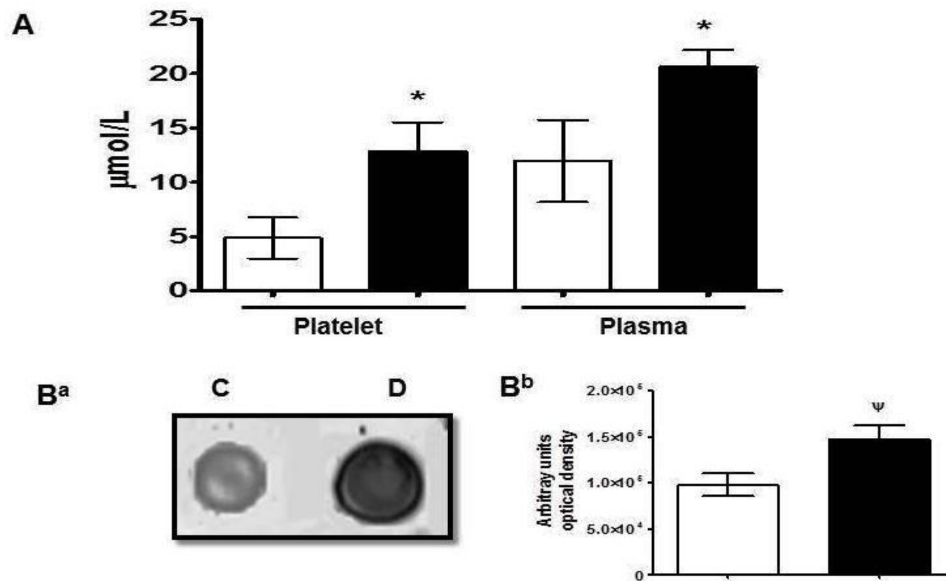

Figure 1. (A) Concentration of 5-HT plasma in adolescents. □, C, controls and ■, D, type 1 diabetics. Each bar corresponds to the average values ± SD of 40 controls and 40 patients with diabetes. The difference between groups was done using Student *t*-test. *p < 0.001. (B[a]) Dot blot, was made in separated samples of platelets (it was not a pool of platelets) in both C and D groups. (B[b]) Quantification of 5-HT. Each bar corresponds to the average values ± SD of 20 adolescents in each group. Between-group differences were obtained using Mann-Whitney U test. ψ< 0.05.

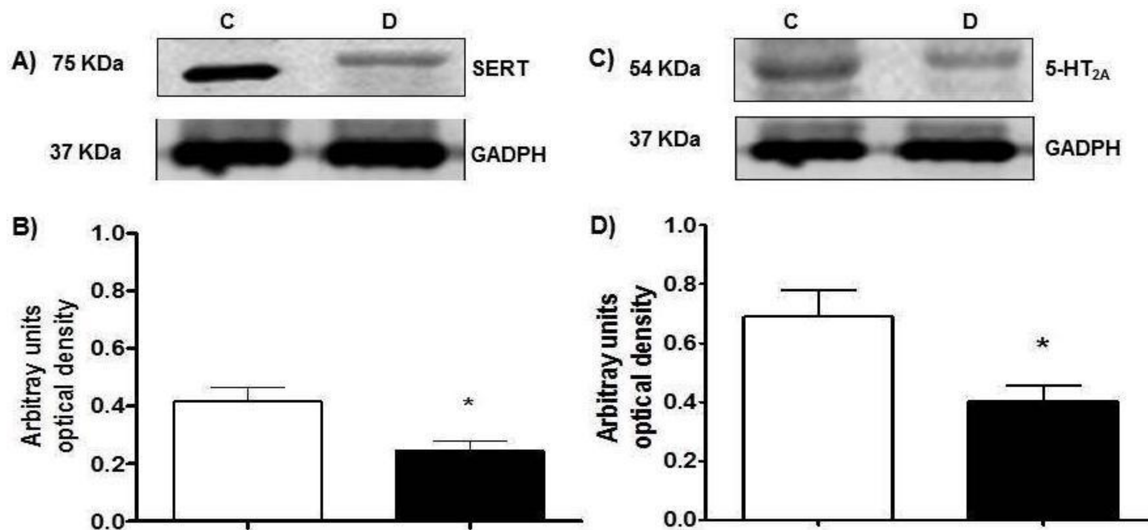

Figure 2. Western blot analysis of the expression SERT and 5-HT_{2A} receptor in control adolescents (□, C) and type 1 diabetics (■, D). (A) Immunoreactivity detected in the platelets of the 20 adolescents in each group. A 75-kDa band for SERT and GADPH of 37 kDa is observed. (B) Quantification of SERT in each of the groups. (C) Immunoreactivity detected in the platelets. Only one 54-kDa band for 5-HT_{2A} and GADPH of 37 kDa is observed. (D) Quantification of 5-HT_{2A} in each of the groups. Each bar corresponds to the average values ± SD. Between-group differences were obtained using Mann-Whitney U test. *p < 0.001.

decrease in the expression of the 5-HT_{2A} receptor when compared to controls (Figure 2D) ($p < 0.001$).

Transglutaminase 2 in platelets is shown in Figure 3. Diabetic patients and controls expressed a 95-kDa band (Figure 3A). Interestingly, it was noted that diabetic patients had a significant increase of the enzyme expression compared to controls (Figure 3B) ($p < 0.05$).

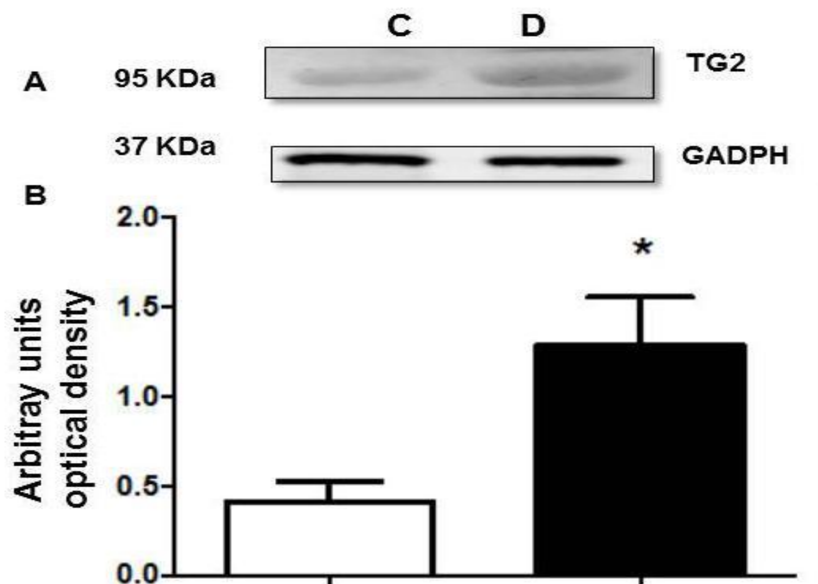

Figure 3. Western blot analysis of the transglutaminase 2 expression in control adolescents (□, C) and type 1 diabetics (■, D). (A) Immunoreactivity detected in the platelets of 20 controls (line 1) and 20 diabetics (line 2) with specific antibodies for each protein. Only one band of 95 kDa for transglutaminase 2 and GADPH of 37 kDa is observed as internal control. (B) Quantification of transglutaminase 2 in each of the groups. Each bar corresponds to average values ± standard deviation. Between-group differences were obtained using Mann-Whitney U test. *p < 0.05.

DISCUSSION

The purpose of this work was to determine in adolescents with type 1 diabetes the concentration of 5-HT levels in plasma and platelets as well as the expression of SERT and the 5-HT$_{2A}$ receptor in platelet membranes as possible mechanisms that regulate the concentration of 5-HT levels in plasma. It is known that SERT is a member of the family of cellular membrane transporters dependent on Na^+ and Cl^- ions and intracellular K^+. SERT works by uniting a Na^+ ion, a 5-HT molecule and a Cl^- ion. This union produces a conformational change in its chemical structure, which allows the translocation of 5-HT to the interior of the cytoplasm of the platelets (Lesch et al., 1993; Mercado and Kilic, 2010; Walther et al., 2003; Murphy and Lesch, 2008). Once the 5-HT is liberated in the cytoplasm, SERT unites to a K^+ ion, finalizing in this manner 5-HT reuptake. Therefore, the efficient function of SERT in the platelet membranes represents the principal mechanism that regulates the concentration of 5-HT levels in plasma (Mercado and Kilic, 2010; Walther et al., 2003).

In the present work we also observed that adolescents with type 1 diabetes had a significant increase of 5-HT levels in plasma and platelets. As far as we know, this is the first study to report plasma 5-HT concentrations, both in normal control adolescents as well as in type 1 diabetic patients. It was previously noted and accepted that SERT is the principal mechanism that regulates the concentration of 5-HT levels in plasma (Lesch et al., 1993; Mercado and Kilic, 2010; Walther et al., 2003). For this reason, we believe it necessary to evaluate the expression of SERT in the platelet's membranes in both groups of adolescents. A significant decrease of SERT was demonstrated in patients with type 1 diabetes compared to controls. This finding suggests that 5-HT uptake by SERT in platelets is decreased, which may explain the observed increase of 5-HT levels in plasma. Decrease of SERT expression in platelets of adolescents with diabetes accompanied by an elevation of 5-HT levels in plasma suggests that the mechanism of regulation of its expression is directly related with the increase of the quantity of 5-HT levels in plasma (Lesch et al., 1993; Mercado and Kilic, 2010; Walther et al., 2003; Murphy and Lesch, 2008). It has recently been noted that the relationship that exists between 5-HT levels in plasma and SERT expression in platelet membranes is biphasic (Mercado and Kilic, 2010). Specifically, SERT expression in membranes and reuptake of 5-HT initially increase when platelets are exposed to high levels of 5-HT in plasma. However, when the increase of 5-HT levels in plasma persists, a decrease of SERT expression is produced in the platelets with the purpose of limiting the reuptake of 5-HT through a feedback mechanism (Mercado and Kilic, 2010; Walther et al., 2003).

Furthermore, in the present study an increase of the 5-HT concentration in platelets of diabetic patients was also observed (see Figure 1). The high concentration of 5-HT

in platelets may be secondary to an increase in the capacity of the SERT reuptake (Mercado and Kilic, 2010; Martin et al., 1995), independent of the decrease in protein expression in membranes. This may also be due to a decrease of the activity of monoamine oxidase A, as has been observed in patients with type 1 diabetes (Mosnaim et al., 1979) and in patients with significant depression (Bakkaloglu, 2008). The increase of 5-HT in platelets may also cause a greater exocytosis of α-granules in platelets as a consequence of the serotonylation and concomitant constitutive activation of small GT Pases such as Rho and Rab4 (Mercado and Kilic, 2010; Walther et al., 2003). Rab 4 is associated with early endosomes, regulates membrane recycling and stimulates secretion of α-granules in platelets (Mercado and Kilic, 2010; Walther et al., 2003). Platelets also contain transglutaminase 2 dependent on Ca^{2+} that catalyzes a reaction of transamidation between 5-HT and small GTPases including Rab4 (Mercado and Kilic, 2010; Walther et al., 2003). In this study it was also observed that adolescents with type 1 diabetes showed a significant increase in the expression of transglutaminase 2 suggesting that, together with the increase of 5-HT in the cytoplasm of platelets, serotonylation and activation of Rab4 may also take place. Therefore, the association of SERT and Rab4 in the cytoplasm could avoid the traffic of SERT to the plasma membrane and concomitantly decrease its expression on the surface of the platelets, which would explain the decrease of 5-HT reuptake in accordance with the increase of 5-HT levels in plasma of patients.

Another interesting finding shown in both groups was the expression of the 5-HT_{2A} receptor in platelet membranes. The 5-HT_{2A} receptor is coupled to G proteins, and the union of 5-HT to the receptor activates C phospholipase producing diacylglycerol and inositol 1,4,5-triphosphate (Killam and Cohen, 1991; Hoyer et al., 2002;Schins et al., 2003;Mercado and Kilic, 2010; Walther et al., 2003). Both molecules induce the liberation of Ca^{2+} from intracellular storage, which produces platelet activation (Mercado and Kilic, 2010; Walther et al., 2003). It was demonstrated in this study that adolescents with diabetes had a significant decrease of the expression of the 5-HT_{2A} receptor, suggesting that the increase of 5-HT levels in plasma produces a desensitization of the receptor through a feedback mechanism where the C-kinase protein intervenes (Kagaya et al., 1990).

In conclusion, our study findings demonstrate that during the diabetic state there is an increase of 5-HT levels in plasma that probably limits its platelet reuptake because it produces a significant decrease in SERT expression in the membrane by a mechanism of down regulation. In a similar manner, a modification of the 5-HT_{2A} receptor was also demonstrated. However, a significant elevation of 5-HT concentration in platelets was paradoxically observed, suggesting that 5-HT plays a primordial role in the regulation of the serotonylation of Rab4 that, together with the increase of transglutaminase 2 expression, participates in the liberation of α-granules and in the decrease of SERT expression on the surface of the platelet. Therefore, elevation of 5-HT levels in plasma and platelets may participate in platelet aggregation during the prothrombotic event frequently present in patients with type 1 and type 2 diabetes.

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