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Increased tissue kallikrein amidase activity in urine of patients with type 1 diabetes under insulin therapy, and in those with gestational diabetes mellitus not under insulin therapy ☆

Gilmar Machado Miranda [a], Carolina Antunes Magalhães [b], Adriana Aparecida Bosco [c], Janice Sepulveda Reis [c], Antônio Ribeiro-Oliveira Jr. [d], Anelise Impelizieri Nogueira [d], Ricardo Barsaglini da Silva Leite [e], Paulo Augusto Carvalho Miranda [e], Amintas Fabiano de Souza Figueiredo [f,*]

[a] Departamento de Bioquímica Clínica, Faculdade Pitágoras, Ipatinga, MG, Brazil
[b] Laboratório da Prefeitura de Belo Horizonte, Venda Nova, Belo Horizonte, MG, Brazil
[c] Ambulatório de Diabetes e Enfermaria da Clínica de Endocrinologia da Santa Casa de Belo Horizonte, Belo Horizonte, MG, Brazil
[d] Ambulatório do Hospital Borges da Costa da UFMG, Belo Horizonte, MG, Brazil
[e] Ambulatório do Centro Metropolitano de Especialidades Médicas, Dr. Dario de Faria Tavares, da Santa Casa de Belo Horizonte, Belo Horizonte, MG, Brazil
[f] Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFMG, Av. Antônio Carlos, 6627, 30270-901 Belo Horizonte, MG, Brazil

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ABSTRACT

Human tissue kallikrein (hK1) is reduced in hypertension, cardiovascular and renal diseases. There is little information on the participation of hK1 in type 1 diabetes mellitus (DM), type 2 DM, and gestational diabetes mellitus (GDM), respectively. The aim of this study was to evaluate the roles of insulin and hyperglycemia on urinary hK1 activity in type 1 DM and in GDM. Forty-three type 1 DM patients (5–35 years, disease duration ⩽5 years, receiving insulin, HbA_{1c} > 7.6%) were selected. Forty-three healthy individuals, paired according to gender and age, were used as controls. Thirty GDM patients (18–42 years, between the 24th and 37th week of pregnancy, recently diagnosed, not under insulin therapy) were also selected. Thirty healthy pregnant (18–42 years, between the 24th and 37th week of pregnancy) and 30 healthy non-pregnant women (18–42 years) were selected as controls. Random midstream urine was used. hK1 amidase activity was estimated with D-Val-Leu-Arg-Nan substrate. Creatinine was determined by Jaffe's method. hK1 specific amidase activity was expressed as μM/(min mg creatinine) to correct for differences in urine flow rate. hK1 specific amidase activity was significantly higher in the urine of type 1 DM than in controls, and in the urine of GDM patients than in healthy pregnant women and healthy non-pregnant women, respectively. The data suggest that hyperglycemia, rather than insulin, is involved in the mechanism of increased hK1 specific amidase activity in both type 1 DM and GDM patients, respectively.

1. Introduction

Kallikreins (EC 3.4.21.8) are a subgroup of the serine protease family known to have several physiologic functions [1]. They are divided into two main groups: plasma (EC 3.4.21.34) and tissue (EC 3.4.21.35) kallikreins [2]. The KLK1 gene, located on chromosome 19q13.4, expresses true tissue kallikrein (hK1) in kidney, pancreas, salivary glands and other tissues [2–5]. The principal known biochemical function of which is releasing from the plasma protein low-molecular-weight kininogen (LMWK) the vasoactive and spasmogenic decapeptide kallidin or lysil-bradykinin (Lys-BK), which is involved in the control of blood pressure, electrolyte balance, inflammation, and other diverse physiological processes [2–4]. The hK1 activity is significantly reduced in the urine of patients with hypertension and heart failure [6,7].

Diabetes mellitus (DM) is a group of metabolic disorders of the carbohydrate metabolism in which glucose is underused, thereby producing hyperglycemia [8]. The classification of DM comprises four clinical classes: type 1 DM results from β-cell destruction, usually leading to absolute insulin deficiency. Approximately 5% of all cases of DM belong in this category. Patients have insulinopenia,

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* Corresponding author. Fax: +55 31 3491 1686.
E-mail address: amintasfigueiredo@gmail.com (A.F.S. Figueiredo).

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insulin deficiency from the loss of pancreatic islet β-cells and therefore rely on insulin injections to sustain their lives and prevent ketosis [8]. Type 2 DM results from an increasing insulin secretion failure owing to insulin resistance. This group comprises approximately 90% of all cases of DM. Patients show a minimal set of symptoms, not being prone to ketosis or depending on insulin to prevent ketonuria [1]. Gestational diabetes mellitus (GDM) is a carbohydrate intolerance of variable severity with onset or first recognition during an ongoing pregnancy. The incidence of GDM is probably between 1% and 5% [9]. Normal pregnancy is associated with increased insulin resistance, especially in the late second and third trimesters [2]. Euglycemia is maintained by increased insulin secretion, with GDM developing in those women who fail to increase insulin levels sufficiently [9]. Other specific types of DM are due to different causes, e.g., genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas, such as cystic fibrosis, and drug- or chemical-induced such in AIDS treatment of or after organ transplantation [9].

Urinary kallikrein was found to be significantly higher in poorly controlled ($HbA_{1c} > 11\%$) insulin-dependent diabetics than in either well-controlled diabetics or normal subjects. Glycemic control was associated with a decrease in kallikrein excretion [10]. A subsequent study of insulin-dependent, poorly controlled diabetics of short duration did not, however, confirm that finding [11]. Those studies suggest that the role of the renal/urinary hK1 in type 1 DM is not clear yet. Another study reported that kallikrein excretion in type 2 DM patients with nephropathy was significantly lower than in control subjects and in type 2 DM patients without nephropathy [12]. Urinary hK1 was significantly increased in pregnant women at 18 and 34 weeks of gestation, whether compared with the non-pregnant state, but it was significantly reduced towards non-pregnant levels at term. According to the authors, kallikrein excretion rate in pregnancy is a controversial issue [13].

The purpose of the present study was to evaluate the roles of insulin and hyperglycemia on the activity of urinary hK1 in both type 1 DM and in GDM, respectively.

2. Materials and methods

2.1. Design and study population

The study was approved by the Ethical Research Committee of the Federal University of Minas Gerais and by the Ethical Research Committee of Santa Casa de Belo Horizonte, and all patients and control subjects gave their written informed consent. Patients and control groups were enrolled between February 2005 and June 2009.

2.2. Patients

Patients of any gender and race were eligible for inclusion in the study as long as they were between 5 and 35 years old (±1 year) and had type 1 DM, with the disease duration ranging from 1 to 5 years. The definition of type 1 DM was based on American Diabetes Association (ADA) criteria [9]. Healthy subjects of any race were eligible for inclusion in the study, as long as they were between 5 and 35 years old (±1 year).

Women of any race were eligible for inclusion in the study as long as they were 18–42 years old (±1 year), within the 24th and the 37th weeks of pregnancy and had GDM recently diagnosed, besides not being receiving insulin therapy. The definition of GDM was based on ADA criteria [9]. Healthy pregnant women of any race were eligible for inclusion in the study, as long as they were 18–42 years old (±1 year), and within the 24th and 37th weeks of pregnancy. Healthy non-pregnant women of any race were eligible for inclusion in the study, as long as they were 18–42 years old (±1 year). All patients and control subjects were submitted to a thorough clinical interview and to physical examination. All of their symptoms and signs that could be indicative of type 1 DM, GDM, or any other disease were analyzed, as well as their personal background and the type of medications they were making use of.

Type 1 DM and GDM patients were submitted to a normal sodium diet (140 mmol/L).

The criteria for patient exclusion encompassed the non-agreement in participating in the study, nephropathy, retinopathy, neuropathy, hypertension, hepatic alterations or any other disease within 30 days before their enrollment.

2.3. Laboratorial determinations

A random urine sample, the 1- to 2-h timed collection in clinic-morning, midstream specimen, was used [1]. In the laboratory, urine was visually and chemically examined with dipstick test (Urofita 10U bioBrás Diagnósticos, Biobrás S.A., Belo Horizonte, MG, Brazil). All urine samples were negative for all chemical compounds evaluated (bilirubin, blood, glucose, ketone bodies, nitrites, protein, urobilinogen) except for glucose in type 1 DM patients.

2.3.1. Tissue kallikrein amidase activity

Assay methods differing considerably in specificity and sensitivity are available to calculate urinary, pancreatic and salivary hK1 activity [14]. In this paper, the hK1 amidase activity was determined with the chromogenic substrate D-Val-Leu-Arg-Nan [14].

Hydrolysis of the chromogenic substrate D-Val-Leu-Arg-Nan (Chromogenix AB, Italy), was assayed spectrophotometrically at 410 nm in order to allow the release of 4-nitroaniline (4-NAn) ($\varepsilon_{410} = 8800\ M^{-1}\ cm^{-1}$) to be monitored as previously described [14,15]. The hK1 amidase activity assay was carried out as already described [9]. Reaction rate (v) was expressed in μM/(min mL urine).

2.3.2. Creatinine determination

Creatinine was determined spectrophotometrically with a kit of reagents based on the Jaffé's reaction (Bioclin/Quibasa Química Básica Ltda, Belo Horizonte, MG, Brazil), and expressed as mg/mL urine. The assays were carried out in quadruplicate.

Kallikrein specific amidase activity was calculated by dividing the reaction rate (v) [μM/(min mL urine)] by creatinine concentration (mg/mL urine). The result was expressed as μM/(min mg creatinine) to correct for differences in urine flow rate.

2.4. Statistical analysis

The data are expressed as medians from the irregular distribution of the variables investigated. Differences between the groups were evaluated by the non-parametric Mann–Whitney test, for the population studied had a non-Gaussian distribution with non-homogeneous variances. A P-value of $\leqslant 0.050$ was considered to be statistically significant.

3. Results and discussion

3.1. Human tissue kallikrein and type 1 diabetes mellitus

Forty-three type 1 DM patients (21 males and 22 females), median age 15 years (interquartile range12–20 years) fulfilled the criteria for inclusion without fulfilling the exclusion criteria, having been, thus, selected to constitute the type 1 DM patients subgroup. HbA_{1c} median value was 9.6 (interquartile range 7.9–11.2%). The ADA recommends that the HbA_{1c} goal for patients in general is

an HbA_{1c} goal of <7% [2]. Thus, in this work, the insulin-dependent type 1 DM patients were in poor glycemic control. Duration of type 1 DM ranged from 1 year to 5 years, and all patients were receiving insulin therapy. Forty-three healthy individuals, (21 males and 22 females), median age 14 years (interquartile range 10–18 years) were used as normal controls, thereby constituting the type 1 DM controls subgroup.

The urinary hK1 specific amidase activity was significantly higher in type 1 DM patients [0.39 μM/(min mg creatinine), interquartile range 0.25–0.60 μM/(min mg creatinine)] than in controls [0.15 μM/(min mg creatinine), interquartile range 0.13–0.17 μM/(min mg creatinine)] ($P = 0.000$) (Fig. 1).

In 1984, Mayfield et al. evaluated in 20 Caucasian type 1 DM patients (24 ± 11 years) and 10 Caucasian normal subjects (32 ± 8.2 years) the urinary excretion of hK1 in order to determine whether an abnormality in kallikrein was present in patients. Twelve of the type 1 DM patients, poorly controlled, showed HbA_{1c} levels higher than 11% (range 11.8–15.8%) (mean 14.2 ± 0.5%), and 8, good to moderately good controlled, showed HbA_{1c} levels below 11% (range 7.3–11.0%) (mean 9.4 ± 0.5%). DM duration ranged from 6 months to 20 years, and all patients were receiving insulin therapy. None of the patients had hypertension (diastolic blood pressure > 90 mmHg). Urinary kallikrein was measured by both an esterase activity assay, with *N*-Tosyl-L-arginine-O-methyl ester (Tos-Arg-OMe) as substrate, and a RIA. According to the authors, urinary kallikrein esterase activity was significantly higher ($P < 0.05$) in 12 poorly controlled type 1 DM patients (9.4 ± 1.0 EU [esterase unity]/24 h) than in 8 type 1 DM patients in good or moderately good control ($HbA_{1c} < 11\%$) (6.1 ± 1.4 EU/24 h) or in 10 normal subjects (6.7 ± 0.5 EU/24 h). Corresponding kallikrein excretion values measured by RIA were 171 ± 14 μg/24 h, 118 ± 26 μg/24 h and 123 ± 11 μg/24 h, respectively. In addition, patients with an elevated kallikrein excretion rate showed a decrease in this rate during a period of strict glycemic control. According to the authors, the reasons for increased kallikrein excretion in poorly controlled DM patients or the mechanisms responsible for its correction with glycemic control were uncertain [10]. Our results with the type 1 DM patients group support the results of Mayfield et al. [10].

In 1985, Esmatjes et al. evaluated the excretion of urinary kallikrein in 21 patients with short duration type 1 DM and 15 normal subjects. According to the authors, no significant differences were found in the urinary kallikrein excretion in the two groups [11]. Our results with the type 1 DM patients group are in disagreement with the results of Esmatjes et al. [11].

In 1987, Jaffa et al. evaluated the effects of streptozotocin (STZ) diabetes and insulin on regulation of renal kallikrein in rats. Active kallikrein was measured in urine and kidney tissue by a RIA. According to the authors, 1 and 2 weeks after STZ injection, diabetic rats ($n = 13$) showed significantly reduced renal levels of urinary excretion of active kallikrein (25.6 ± 2.2 ng/mg protein *vs.* 54.7 ± 2.7 ng/mg protein in 12 control rats, $P < 0.001$)). Tissue and urinary prokallikrein levels were unchanged, but the rate of renal prokallikrein synthesis related to total protein synthesis was reduced 30–40% in diabetic rats. Treatment of diabetic rats with insulin prevented or reversed the fall in tissue level and excretion rate of active kallikrein and normalized prokallikrein synthesis rate. Non-diabetic rats were also treated with escalating insulin doses to produce hyperinsulinemia. In these rats ($n = 14$), renal active kallikrein was increased (74.9 ± 4.1 ng/mg protein *vs.* 59.9 ± 3.7 ng/mg protein) ($P < 0.02$). The authors suggested that insulin modulates renal kallikrein production, activation, and excretion [16].

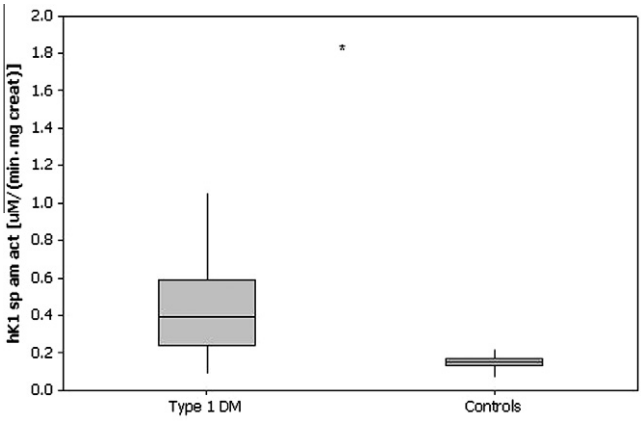

Fig. 1. Comparison of hK1 specific amidase activity (hK1 sp am act) expressed as μM/(min mg creatinine) between type 1 DM patients and healthy subjects as controls, The symbol (*) indicates a statistically significant difference ($P < 0.050$).

In 1992, Jaffa et al. reported that they previously showed that renal prokallikrein synthesis and plasma rennin activity were reduced in STZ-diabetic rats. In order to investigate the molecular mechanism underlying these changes they investigated the effects of diabetes and insulin treatment on renal kallikrein and renal renin mRNA levels and the activity of these enzymes. Rats made diabetic by STZ were either treated with 1.5–1.75 UPZI insulin daily ($n = 7$) to maintain moderate hyperglycemia (D + I rats) (plasma glucose 200–300 mg/dL) or left untreated ($n = 7$) as to produce severe hyperglycemia (plasma glucose > 400 mg/dL, D). Control (C) rats ($n = 8$) were also studied. Active and prokallikrein levels were measured by a RIA that incorporates a monoclonal antibody specific for active kallikrein. After three weeks, a reduction in total kallikrein in D rats was principally accounted for by reduced active kallikrein (active: 23.4 ± 1.5 *vs.* 36.3 ± 3.9 ng/mg protein, D *vs.* C, respectively, $P < 0.001$). Renal kallikrein mRNA was reduced in 50% in D rats. A proportional reduction in immunoreactive kallikrein was also observed (37.8 ± 2.5 *vs.* 55.8 ± 6.8 ng/mg protein, D *vs.* C; $P < 0.001$). Kallikrein mRNA and immunoreactive kallikrein levels in D + I rats were not different from in C rats. Renin mRNA level was also markedly reduced in kidney of D rats, compared to kidney of C rats. This was associated with reduced plasma renin concentration (4.5 ± 0.2 *vs.* 10.5 ± 1.6 ng Ang I/mL h, D *vs.* C, $P < 0.01$). Renal rennin concentration was increased (1.49 ± 0.27 μg Ang I/mg protein h) compared to C rats (0.84 ± 1.3 μg Ang I/mg protein h, $P < 0.05$). The authors concluded that the diabetic state reduces both kallikrein and renin mRNA levels in the kidney, and that insulin treatment reverses these abnormalities [17].

In 1997, Jaffa et al. evaluated the effects of acute administration of insulin and insulin-like growth factor I (IGF-I) on components of the kallikrein-kinin system (KKS) and renin-angiotensin system (RAS) in diabetic and control rats by using complementary molecular biochemical, and immunocytochemical techniques. Three weeks after induction of diabetes, the authors measured renal kallikrein and renin mRNA levels, renal kallikrein and renal renin activity, and plasma renin activity in control and diabetic rats and diabetic rats treated with insulin or IGF-I for 2 or 5 h. Active and prokallikrein levels were measured by a RIA specific for active kallikrein. Plasma and renal renin activity were determined from the amount of generated angiotensin I (Ang I) measured by a RIA for Ang I. In diabetic rats, kallikrein and renin mRNA levels were reduced >50% compared with control rats. Renal tissue kallikrein levels and plasma renin activity were decreased, whereas renal renin content was unchanged. Insulin increased kallikrein and renin mRNA levels after 2 h. IGF-I, at a dosage that stimulated kallikrein mRNA levels in control rats, had no effect on renal kallikrein mRNA levels in diabetic rats. Infusion of a fivefold higher IGF-I dosage resulted in a two- to threefold increase in kallikrein mRNA levels in 2 h. According to the authors, diabetes suppresses kallikrein and renin gene expression, and these abnormalities are reversed by

insulin or IGF-I, and the diabetic state produces resistance to IGF-I induction of kallikrein and renin gene expression [18].

In 1998, Pelikánová et al. evaluated the urinary kallikrein excretion in 21 type 1 DM patients with disease duration from 1 month to 1 year. None showed signs of nephropathy or retinopathy. The DM patients were in poor glycemic control (HbA_{1c} = 10.6 ± 2.3%), all of whom were receiving human insulin therapy (38 ± 9 units [U]/day). A control group consisted of 18 age-, weight-, and gender-matched healthy men. Urinary kallikrein activity was determined by radioesterolytic method using ^{3}H-*p*-tosyl-L-arginine methyl ester and Tos-Arg-OMe as substrates. According to the authors, hyperglycemia led to a significant decrease in kallikrein excretion in diabetic patients (5.77 ± 3.22 mUE/min), while the excretion rate in controls did not change (10.38 ± 3.73 mUE/min) ($P < 0.001$). The authors concluded that, despite no alterations in renal hemodynamics, short-term type 1 DM is associated with decreased basal and furosemide-stimulated urinary kallikrein excretion, which is directly related to the blood glucose level [20]. Our results with the type 1 DM patients group are in disagreement with the results of Pelikánová et al. [19].

In the present work the increased hK1 specific amidase activity observed in type 1 DM patients may be due either, to hyperglycemia (HbA_{1c} median = 9.6%) or insulin therapy.

In order to investigate further the mechanism of the increased hK1 specific amidase activity, which was observed in the urine of type 1 DM patients, we decided to determine the hK1 specific amidase activity in the urine of GDM patients who were not under insulin therapy, and in the urine of both healthy pregnant women and healthy non-pregnant women as controls.

3.2. Human tissue kallikrein and gestational diabetes mellitus

Thirty GDM patients, with median age 32 years (interquartile range 24–38 years) and not under insulin therapy, 30 healthy pregnant women, with median age 27 years (interquartile range 20–29 years), and 30 healthy non-pregnant women, with median age 27 years (interquartile range 25–28 years), fulfilled the criteria for inclusion without fulfilling the exclusion criteria, having been, thus, selected to constitute the GDM and the control subgroups, respectively. None of the GDM patients showed gestational hypertension or signs of nephropathy, retinopathy, or clinical evidence of diabetic neuropathy. None of the healthy pregnant women as well as none of the non-pregnant women had any diseases nor were taking any medications.

The median urinary hK1 specific amidase activity was significantly higher in GDM patients [0.40 μM/(min mg creatinine), interquartile range 0.24–0.66 μM/(min mg creatinine)] than in healthy pregnant women [0.27 μM/(min mg creatinine), interquartile range 0.15–0.50 μM/(min mg creatinine)] ($P = 0.050$) (Fig. 2) and in GDM patients [0.40 μM/(min mg creatinine), interquartile range 0.24–0.66 μM/(min mg creatinine)] and healthy non-pregnant women [0.29 μM/(min mg creatinine), interquartile range 0.20–0.38 μM/(min mg creatinine)] ($P = 0.050$) (Fig. 2). The hK1 specific amidase activity was not significantly different between healthy pregnant women [0.27 μM/(min mg creatinine), interquartile range 0.15–0.50 μM/(min mg creatinine)] and healthy non-pregnant women [0.29 μM/(min mg creatinine), interquartile range 0.20–0.38 μM/(min mg creatinine)] ($P = 0.825$) (Fig. 2).

These results suggest that hyperglycemia is responsible for the increase in the hK1 specific amidase activity of GDM patients. Our conclusion finds support in an article by Homko et al. [20] published in 2001, in which the authors evaluated pre-hepatic insulin secretion rates (ISRs) in 7 GDM patients and in 8 age- and weight-matched non-diabetic pregnant women during late gestation (third trimester) and again in the postpartum. They found that women with GDM were more insulin resistant than non-diabetic controls and also had a significantly lower ISRs and glucose uptake rates in response to hyperglycemia. Postpartum, insulin resistance and ISRs showed improvement in both groups, whereas ISRs were no longer significantly different in GDM patients and in controls. Insulin resistance, however, remained higher in GDM patients and their glucose uptake remained lower. The authors concluded that the GDM patients had a major -cell defect that made it impossible for them to compensate for their increased level of insulin resistance which occurred during late pregnancy [20].

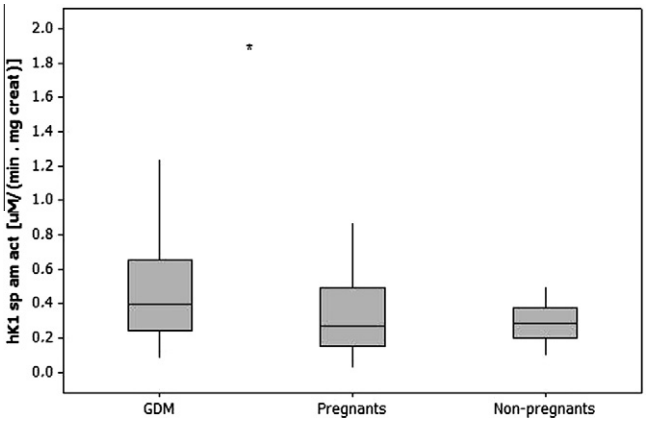

Fig. 2. Comparison of hK1 specific amidase activity (hK1 sp am act) expressed as μM/(min mg creatinine) between GDM patients, healthy pregnant women and healthy non-pregnant women as controls, The symbol (∗) indicates a statistically significant difference ($P < 0.050$).

In 2004, Borgoño et al. reported that experimental and bioinformatics data suggest that most, if not all, kallikreins are glycoproteins in vivo. According to them, glycosylation of many proteins is important for their proper expression and functions. Still according to the authors, the classical kallikreins (hK1 for instance) has a unique surface loop named "kallikrein loop". Glycosylation of the kallikrein loop, among others, may function in regulating kallikrein activity [21].

In 2009, Campbell et al. reported, and that was the first time that was done, the effects of type 2 DM on the kallikrein-kinin system (KKS). They found increased levels of tissue kallikrein as evidenced by increased circulating levels of tissue kallikrein, increased tissue kallikrein immunoreactivity in atrial myocytes and increased tissue kallikrein mRNA levels in atrial tissue. According to the authors, the mechanism of increased tissue kallikrein gene expression in type 2 DM is uncertain. Therefore, they did not discard the possibility that the increased tissue kallikrein gene expression in type 2 DM was caused by hyperinsulinemia [22].

We believe hK1 glycosylation from hyperglycemia, can increase its enzymatic activity as observed in both type 1 DM and GDM. However, we should not dismiss the possibility of hK1 activity modulation by insulin. Further studies are needed in clearing out this assumption.

In conclusion, our study suggests that hK1 may be involved in DM physiopathology.

4. Conflict of interest

There is no conflict of interest.

Acknowledgments

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