Int J Diabetes & Metab (2010) 18: 124-131

The amidase activity of human tissue kallikrein is significantly higher in the urine of patients with either type 1 or gestational diabetes mellitus

Gilmar M Miranda,[1] Carolina A Magalhães,[1] Adriana Bosco,[2] Janice S Reis,[2] Antônio R Oliveira Jr,[3] Anelise I Nogueira,[3] Ricardo BS Leite,[4] Paulo AC Miranda,[4] Amintas FS Figueiredo[5]

Programa de Pós–graduação em Ciências Farmacêuticas, Faculdade de Farmácia, UFMG, Belo Horizonte, MG, Brazil[1]; Ambulatório de Diabetes e Enfermaria da Clínica de Endocrinologia da Santa Casa de Belo Horizonte, Belo Horizonte, MG, Brazil[2]; Ambulatório do Hospital Borges da Costa da UFMG, Belo Horizonte, MG, Brazil; [3]Ambulatório do Centro Metropolitano de Especialidades Médicas Dr. Dario de Faria [4]Tavares, da Santa Casa de Belo Horizonte, Belo Horizonte, MG, Brazil; [5]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFMG, Belo Horizonte, MG, Brazil

Abstract

The levels of tissue kallikrein are reduced in humans with hypertension, cardiovascular and renal diseases. There is little information on the participation of human tissue (renal/urinary) kallikrein (hK1) in type 1 diabetes mellitus (DM), and, no information concerning the role of hK1 was reported in gestational diabetes mellitus (GDM). The present study evaluated 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, HbA1c > 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 weeks of pregnancy, recently diagnosed, not under insulin therapy) were also selected. Thirty healthy pregnant and thirty healthy non−pregnant women (18−42 years) were selected as controls. A random midstream urine was used. hK1 amidase activity was estimated with D-Val-Leu-Arg-Nan substrate. Albumin was determined with Coomassie Brilliant Blue reagent. 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 and GDM patients, respectively, than in controls. These findings suggest that hyperglycemia rather insulin is involved in the mechanism of increased hK1 specific amidase activity in the urine of both type 1 DM and GDM patients. Albumin/creatinine ratio was increased in type 1 DM, GDM and healthy pregnant women.

Key words: *Human tissue kallikrein, tissue kallikrein, kallikrein, type 1 diabetes mellitus, gestational diabetes mellitus, diabetes mellitus.*

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders of carbohydrate metabolism in which glucose is underused, thereby producing hyperglycemia.[1] 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 to this category. Patients have insulinopenia, a deficiency of insulin, caused by the loss of pancreatic islet β–cells and are dependent on insulin to sustain life and prevent ketosis.[1] Type 2 DM is associated with insulin resistance. This group comprises approximately 90% of all cases of DM. Patients show a minimal amount of symptoms, at the onset. They are not prone to ketosis, and are not dependent on insulin to prevent ketonuria.[1] Gestational diabetes mellitus (GDM) is a carbohydrate intolerance of variable severity with onset or first recognition during pregnancy. The incidence of GDM probably is between 1 and 5%.[1] Normal pregnancy is associated with increased insulin resistance, especially in the late second and third trimesters.[1] Euglycemia is maintained by increased insulin secretion, with GDM developing in those women who fail to increase insulin sufficiently.[1] Other specific types of DM due to different causes, such as 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 as in the treatment of AIDS or after organ transplantation.[2]

Kallikreins (EC 3.4.21.8) are a subgroup of the serine protease family known to have several physiologic functions.[3] The kallikreins are divided into 2 main groups: plasma (EC 3.4.21.34) and tissue (EC 3.4.21.35) kallikreins.[4] The KLK1 gene, located on chromosome 19q13.4, expresses true tissue kallikrein (hK1), the principal known biochemical function of which is releasing the vasoactive and spasmogenic decapeptide kallidin or lysil-bradykinin (Lys–BK) from the plasma protein low molecular weight kininogen (LMWK).[3,5] The hK1 activity is significantly reduced in the urine of patients with hypertension and heart failure.[6,7]

Received on: 18/11/2009
Accepted on 3/7/2010

Correspondence to: Amintas Fabiano de Souza Figueiredo, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFMG, Av. Antônio Carlos, 6627, 30270–901 Belo Horizonte, MG, Brazil, E–mail: afsf@farmacia.ufmg.br

According to Margolius[8] urinary kallikrein was found to be significantly higher in poorly controlled (HbA1c > 11%), insulin-dependent diabetics than in either well controlled diabetics or normal subjects. Glycemic control was associated with a fall in kallikrein excretion. Still according to the author, one subsequent study in insulin-dependent, poorly controlled diabetics did not confirm this finding, while another study found significantly decreased urinary kallikrein level in type 2 (non-insulin dependent) diabetics with nephropathy, compared with control subjects, patients with diabetes without detectable nephropathy, or patients without diabetes and with nephropathy. These studies suggest that the role of the renal/urinary kallikrein (hK1) in DM is not clear yet. On the other hand, there is no information regarding the participation of hK1 in GDM.

To evaluate further the role of the urinary hK1 in type 1 DM and in GDM it was decided to measure the hK1 amidase activity in a midstream random sample collection of urine from 1): type 1 DM patients who have poorly controlled glycemia determined by glycated haemoglobin (HbA1c) > 7% or those receiving insulin or healthy individual as controls; 2): GDM patients who were not receiving insulin therapy, and of healthy pregnant women and healthy non-pregnant women as control individuals.

Materials and Methods

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 Comitee of Santa Casa de Belo Horizonte, Brazil. All patients and control subjects gave their written informed consent. Patients and control groups were enrolled between February 2005 and June 2009.

Type 1 DM patients

Patients of any gender and race were eligible for inclusion in the study as long as they were between 5 to 35 years old and had type 1 DM with the disease duration ranging from 1 year to 5 years. All patients were receiving insulin therapy and had high HbA1c levels. All type 1 DM patients and control subjects were evaluated by the physicians A. Bosco and J. S. Reis and diagnosed according to the criteria for DM diagnosis.[1]

GDM patients

Patients of any race were eligible for inclusion in the study as long as they were 18 to 42 years old, were between the 24th and the 37th weeks of pregnancy and had GDM recently diagnosed and were not receiving insulin therapy. Healthy pregnant women of any race were eligible for inclusion in the study as long as they were 18 to 42 years old, and were between 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 to 42 years old. None of the selected DM patients showed gestational hypertension or signs of nephropathy, retinopathy, or clinical evidence of diabetic neuropathy. 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. All GDM patients, pregnant women and healthy, non-pregnant women were evaluated by the physicians A. R. Oliveira Jr, A. I. Nogueira, R. B. S. Leite, and P. A. C. Miranda, and diagnosed according to the criteria for GDM diagnosis.[1] All patients and control subjects were submitted to a thorough clinical interview and to physical examination. All of their symptoms and signs that were indicative of type 1 DM and GDM or any other disease were analyzed, as well as their personal antecedents and the types of medications they were using.

Between February 2005 and December 2006, 212 type 1 DM patients were screened, only 43 having fulfilled the criteria for inclusion, without fulfilling the exclusion criteria, having been, thus, selected to constitute the type 1 DM patients subgroup. Forty three healthy individuals, paired according to gender and age (± 1), were used as normal controls, thereby constituting the type 1 DM control subgroup. On the other hand, between February 2008 and March 2009, 250 GDM patients and 110 healthy pregnant women were screened, only 30 GDM patients and 30 healthy pregnant women having fulfilled the criteria for inclusion without fulfilling the exclusion criteria were selected to constitute the GDM patients and the healthy pregnant subgroups. Thirty healthy non-pregnant women, paired according to age (± 1), were used as normal controls.

The subgroups were compared according to the following variables: HbA1c, urinary albumin, urinary creatinine, albumin/creatinine ratio (ACR), and hK1 specific amidase activity, respectively. The specific amidase activity was expressed as hK1 amidase activity/creatinine or μM/min/mg creatinine to correct for differences in urine flow rate.

Laboratory determinations

A random urine sample was used. In the laboratory, urine sample 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 blood and for all chemical compounds evaluated, except for glucose in type 1 DM patients.

Protein determination

Many methods have been developed to measure the total protein content of biological fluids. Dye-binding methods are based on the ability of proteins to bind dyes, such as Coomassie Brillant Blue (CBB). The dye-binding method of greatest contemporary interest uses CBB G-250 for assay of total protein in the cerebrospinal fluid or urine.[9] Only a small amount of protein is normally excreted in urine, most of it being albumin. The remaining excretion almost entirely consists of the Tamm-Horsfall glycoprotein (THGP), also known as uromucoid, which is probably secreted by the distal tubules.[9] In the present work, THGP was separated from albumin by adjusting the urine pH to 8.0.[10] The mixture was filtered on filter paper, and the clear filtrate was used for albumin determination. Urinary albumin was spectrophotometrically measured with CBB G-250 (Sigma Chemical Company, St. Louis, MO, USA) according to

Table 1: Comparison between some characteristics of type 1 diabetes mellitus patients, under insulin therapy, and control subjects

Parameter	Patients	Controls	*P* value
Number	43	43	—
Age (yr) [a]	15 (12-20)	14 (10-18)	—
Gender (male/female)	21/22	21/22	
HbA_{1c} (%) [a]	9.6 (7.9-11.2)	—	—
Urinary albumin [a, b]	30 (21-42)	37 (22-51)	0.244
Urinary creatinine [a,c]	0.69 (0.37-1.00)	1.50 (0.96-1.89)	0.000
ACR [a,d]	45 (33-64)	26 (23-29)	0.000
hK1 sp am act [a,e]	0.39 (0.25-0.60)	0.15 (0.13-0.17)	0.000

Abbreviations: ACR, albumin/creatinine ratio; hK1 sp am act, hK1 specific amidase activity; [a] median value; numbers in parentheses are the interquartile ranges 25% to 75%; [b] µg/mL; [c] mg/mL; [d] µg albumin/mg creatinine; [e]

Table 2: Comparison between some characteristics of gestational diabetes mellitus patients that are not under insulin therapy, and healthy pregnant women as control

Parameter	Gestational diabetes mellitus	Pregnant women	P value
Number	30	30	—
Age (yr) a	32 (24-38)	27 (20-29)	—
Urinary albumin a,b	129 (75-198)	59 (46-104)	0.002
Urinary creatinine a,c	0.74 (0.45-1.20)	0.67 (0.40-1.11)	0.063
ACR a,d	173 (113-237)	100 (72-150)	0.000
hK1 sp am act a,e	0.40 (0.24-0.66)	0.27 (0.15-0.50)	0.050

Abbreviations: ACR, albumin/creatinine ratio; hK1 sp am act, hK1 specific amidase activity; a median value; numbers in parentheses are the interquartile ranges 25% to 75%; b µg/mL; c mg/mL; d µg albumin/mg creatinine; e µM/(min . mg creatinine).

Table 3: Comparison between some characteristics of gestational diabetes mellitus patients, that are not under insulin therapy, and healthy non-pregnant women as control

Parameter	Gestational diabetes mellitus	Healthy non−pregnant women	*P* value
Number	30	30	—
Age (yr) [a]	32 (24-38)	27 (25-28)	—
Urinary albumin [a],[b]	129 (75-198)	33 (23-49)	0.000
Urinary creatinine [a,c]	0.74 (0.45-1.20)	1.02 (0.66-1.33)	0.063
ACR [d]	173 (113-237)	34 (27-43)	0.000
hK1 sp am act [d]	0.40 (0.24-0.66)	0.29 (0.20-0.38)	0.050

Abbreviations: ACR, albumin/creatinine ratio; hK1 sp am act, hK1 specific amidase activity; [a] median value; numbers in parentheses are the interquartile ranges 25% to 75%; [b] µg/mL; [c] mg/mL; [d] µg albumin/mg creatinine; [e] µM/(min.mg creatinine

Table 4: Comparison between some characteristics of healthy pregnant women and healthy non-pregnant women as control

Parameter	Healthy pregnant women	Healthy non− pregnant women	*P* value
Number	30	30	—
Age (yr) [a]	27 (20-29)	27 (25-28)	—
Urinary albumin [a,b]	59 (46-104)	33 (23-49)	0.000
Urinary creatinine [a,c]	0.67 (0.40-1.11)	1.02 (0.66-1.33)	0.063
ACR [a,d]	100 (72-150)	34 (27-43)	0.000
hK1 sp am act [a,e]	0.27 (0.15-0.50)	0.29 (0.20-0.38)	0.825

Abbreviations: ACR, albumin/creatinine ratio; hK1 sp am act, hK1 specific amidase activity; [a] median value; numbers in parentheses are the interquartile ranges 25% to 75%; [b] µg/mL; [c] mg/mL; [d] µg albumin/mg creatinine; [e] µM/(min . mg creatinine

Bradford[11] modified by Peterson.[12] Bovine serum albumin (Sigma) was used as standard. The result is expressed as mg/ml urine. The clear filtrate was also used for kallikrein amidase activity determination.

Creatinine determination

Creatinine was determined in urine samples separated before adjusting the urine pH to 8.0, using a kit of reagents based on the Jaffé's reaction (Bioclin/Quibasa Química

Básica Ltda, Belo Horizonte, MG, Brazil), and is expressed in mg/ml urine.

HbA1c determination
HbA1c was measured in hemolysates of fasting blood samples by ion-exchange chromatography (KATAL Biotecnológica Indústria e Comércio Ltda, Belo Horizonte, MG, Brazil), and is expressed as %.

Kallikrein amidase activity
Hydrolysis of the chromogenic substrate D-Val-Leu-Arg-Nan was assayed spectrophotometrically at 410 nm to allow the release of 4-nitroaniline (4-NAn) (ε_{410} = 8800 M^{-1}/cm) to be monitored as previously described.[13,14] The hK1 amidase activity was carried out as already described.[7]
Kallikrein specific amidase activity was calculated by dividing the reaction rate (v) [µM/min/ ml of urine filtrate by creatinine concentration (mg/ml urine)]. The result was expressed as µM/min/mg creatinine.

Statistical analysis
Descriptive statistics were reported as medians from the irregular distribution of the variables investigated. Differences between the groups were evaluated by the non-parametric Mann-Whitney test, because the population studied had a non-Gaussian distribution with non-homogeneous variances. A *P* value of ≤ 0.05 was considered to be statistically significant.

Results
Type 1 DM study
Comparisons between some characteristics of type 1 DM are shown in Table 1. The groups were similar in age and gender. Type 1 DM patients had high levels of HbA1c. There was no significant effect of type 1 DM on urinary albumin excretion. Urinary creatinine excretion was significantly lower in type 1 DM patients than in controls. ACR and hK1 specific amidase activity were significantly higher in type 1 DM patients than in controls.

GDM study
Comparisons between some characteristics of gestational DM patients and healthy pregnant women as controls are shown in Table 2. Urinary albumin, ACR, and hK1 amidase activity were significantly higher in GDM patients than in healthy pregnant women. There was no significant effect of GDM on urinary creatinine.

Comparisons between some characteristics of gestational DM patients and healthy non-pregnant women as controls are shown in Table 3. Urinary albumin, ACR, and hK1 amidase activity were significantly higher in GDM patients than in healthy non-pregnant women. There was no significant effect of GDM on urinary creatinine.

Comparisons between some characteristics of healthy pregnant women and healthy non-pregnant women as controls are shown in Table 4. Urinary albumin and ACR were significantly higher in healthy pregnant women than in healthy non-pregnant women. There was no significant effect of pregnancy on urinary creatinine and on hK1 amidase activity, respectively.

Our results demonstrate that the amidase activity of urinary hK1 is significantly higher in type 1 DM patients than in normal control individuals, and also in GDM patients than in healthy pregnant women and in healthy non-pregnant women, respectively, suggesting that renal hK1 is functioning abnormally in type 1 DM patients and also in the DMG patients.

Discussion
Type 1 DM study
HbA1c
The ADA recommends that the HbA1c goal for patients in general is an HbA1c goal of < 7%.[2] In this work, the insulin-dependent type 1 DM patients were in poor glycemic control with an HbA1c median of 9.6% (Table 1).

Microalbuminuria
Urinary albumin excretion
Urinary albumin excretion is a predictor of cardiovascular mortality in patients with diabetes mellitus and hypertension, also in an unselected population.[15] According to the ADA,[16] the gold standard for measuring urine albumin excretion is a 24-hour urine collection, because random specimens vary considerably in protein concentration. However, a more convenient alternative method for measuring urine albumin excretion is to determine, in random samples, the ratio of albumin to creatinine, the albumin/creatinine ratio (ACR),[17] in order to correct for differences in urine flow rate. In the present work, using a random urine specimen, no significant difference in the excretion of urinary albumin between patients and controls was observed.

Urinary creatinine excretion
The creatinine excretion rate in any one individual, in the absence of renal disease, is relatively constant and parallels endogenous production. The excretion of urinary creatinine was significantly lower in the type 1 DM patients than in the controls subjects. We have no explanation for this result.

Albumin/creatinine ratio (ACR)
According to Figueiredo and associates,[18] the ADA and the National Kidney Foundation (NKF) define microalbuminuria as an ACR between 30 and 300 µg/mL in both men and women.

In the present work ACR was significantly higher in type 1 DM than in controls subjects indicating the presence of microalbuminuria. From the 43 type 1 DM patients evaluated, 37 (86%) had microalbuminuria, and 6 (14%) had normal albuminuria.

According to Sacks and associates,[19] diabetes is the leading cause of end-stage renal disease in the US and Europe. Still according to the authors, early detection of microalbuminuria allows early intervention with a goal of delaying the onset of overt diabetic nephropathy.

Urinary hK1 specific amidase activity

Assay methods differing considerably in specificity and sensitivity are available to calculate urinary, pancreatic and salivary hK1.[14] In this study, the hK1 amidase activity was determined with the chromogenic substrate D-Val-Leu-Arg-Nan.[14] As the enzymatic activity in the urine samples was inhibited by Trasylol® (Bayer, Brasil), a strong kallikrein inhibitor also known as aprotinin, basic pancreatic trypsin inhibitor-BPTI, or Kunitz pancreatic trypsin inhibitor,[14] and not inhibited by soybean trypsin inhibitor-SBTI (Sigma), a strong serine proteinase and plasma kallikrein inhibitor,[14] it was concluded that only the amidase activity of hK1 was evaluated.

In this work, it was observed that the hK1 specific amidase activity was significantly higher in type 1 DM patients than in controls subjects. The duration of type 1 DM ranged from 1 year to 5 years, and all patients were receiving insulin therapy.

Our data support those of Mayfield and associates.[20] Those authors reported that in insulin-dependent diabetic patients in poor control (HbA1c > 11%), urinary kallikrein excretion was significantly higher than in patients in good or moderately good control (HbA1c < 11%) or a group of normal subjects. In addition, patients with an elevated kallikrein excretion rate had a fall in this rate during a period of strict glycemic control. The authors measured the excretion of kallikrein in 20 caucasian type 1, insulin-dependent diabetic patients and in 10 caucasian normal subjects as controls. The age of the diabetic patients was 24 ± 11 years, being compared to 32 ± 8.2 years for the normal subjects. Duration of diabetes ranged from 6 months to 20 years, and all patients were receiving insulin therapy. According to the authors, the reasons for increased kallikrein excretion in poorly controlled diabetic patients or the mechanisms responsible for its correction with glycemic control were uncertain.

The present results report that hK1 specific amidase activity is significantly higher in type 1 DM patients receiving insulin therapy, than in control subjects, support data by Jaffa and associates.[21] Those authors studied the effects of streptozotocin (STZ), diabetes and insulin on regulation of renal kallikrein in rats. Diabetes was induced by a single intravenous injection of STZ, 65 mg/kg body weight. After 24 hours diabetes was confirmed in STZ-treated rats by tail-vein plasma glucose level. One and two weeks after STZ injection, diabetic rats had reduced renal levels and urinary excretion of active kallikrein. Tissue and urinary prokallikrein levels were unchanged, but the rate of renal prokallikrein synthesis related to total protein synthesis was also reduced 30-45% 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. To further examine insulin's effects, nondiabetic rats were treated with escalating insulin doses in order to produce hyperinsulinemia. Renal active kallikrein increased in rats. Although renal prokallikrein was not increased significantly by hyperinsulinemia, its synthesis was. The authors concluded that their findings suggested that insulin modulates renal kallikrein production, activation and excretion.

The results now reported also supports data by Jaffa and associates,[22] who studied the effects of acute insulin and insulin-like growth factor I (IGF-I) treatment on renal kallikrein-kinin and rennin-angiotensin system components. The authors induced diabetes in rats by STZ injection. Three weeks after induction of diabetes, they 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 hours. In diabetic rats, kallikrein and renin mRNA levels were reduced by over 50%, being 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 and renin content or mRNA levels in diabetic rats. However, infusion of a fivefold higher IGF-I dosage resulted in a two-threefold increase in kallikrein and renin mRNA levels in 2 h. The authors concluded that: 1) diabetes suppressed kallikrein and renin gene expression, and these abnormalities were reversed by insulin or IGF-I; 2) the diabetic state produces resistance to IGF-I induction of kallikrein and renin gene expression. Still, according to the authors, these changes in regulated synthesis of kallikrein and renin in the kidney may underlie renal vascular changes that develop in diabetes.

Manto and associates[23] found that diabetic subjects with glomerular hyperfiltration have increased excretion of urinary kallikrein, if compared to diabetic patients with normal glomerular filtration rate or control subjects. Furthermore, a positive correlation was observed between urinary kallikrein excretion and glomerular filtration rate. According to the authors, their type 1 diabetic patients were in good metabolic control with similar HbA1c values (7.0 ± 1.3 %). According to the authors, eighty nine Caucasian type 1 diabetic patients without hypertension, renal disease or any diabetic complications were recruited: of these 78 were normofiltering while 11 showed an increased glomerular filtration rate (GFR); a group of 20 healthy subjects, matched for age and sex, with no family history of hypertension, served as controls.

We did not evaluate glomerular filtration rate (GFR) of our patients and control subjects. However, our data does not support those by Manto and associates.[23]

Our data does not support those of Pelikánová and associates,[24] who reported that, despite lack of alterations in renal hemodynamics, short-term insulin dependent diabetes mellitus (IDDM) is associated with decreased basal and furosemide-stimulated urinary kallikrein excretion, which is directly related to the blood glucose level. The authors examined 21 IDDM patients (23.1 ± 3.1 years) with disease duration from 1 month to 1 year, and HbA1c 10.6 ± 2.3%.

A control group consisted of 18 age-, weight-, and gender-matched healthy men. According to the authors, there were no significant differences in GFR between diabetic patients and controls, the values having not being affected by hyperglycemia. No significant differences between diabetic patients and controls were seen in renal plasma flow (RPF), filtration fraction (FF) or renal vascular resistance (RVR). Hyperglycemia decreased FF in either group. RVR was not affected by hyperglycemia. Urinary kallikrein excretion was equivalent in diabetic and control subjects during euglycemia. Hyperglycemia led to a significant decrease in kallikrein excretion in diabetic patients, while the excretion rate in controls did not change. Still according to the authors, their study demonstrated that, despite no alterations in renal hemodynamics, short-term IDDM is associated with decreased basal and furosemide-stimulated urinary kallikrein excretion, which is directly related to blood glucose level.

Concerning the effects of furosemide on hK1 excretion there are controversies: Croxatto and associates[25] reported that furosemide stimulates urinary kallikrein excretion. Guarda and associates[26] observed that, among their HF patients, those belonging to NYHA's functional class IV and who received the largest doses of furosemide showed the lowest urinary kallikrein values. On the other hand, Ohman and Karlberg[27] observed that furosemide increases the urine volume and the excretion of tissue kallikrein in normotensive individuals and in patients with primary hypertension. Bicknell and associates[28] reported that a rapid but short-lived rise in urinary kallikrein excretion was shown after furosemide administration in human beings, which could indicate a "washout" phenomenon rather than a direct involvement of kallikrein-kinin system in the action of furosemide. In 2006, we evaluated the hK1 amidase activity in the urine of 28 heart failure (HF) patients, from which 27 were making continuous daily use of furosemide (40-120 mg orally). In all patients, hK1 specific amidase activities were reduced.[7] We concluded that the diminished levels of hK1 specific amidase activities in HF patients treated with furosemide could be explained from their having HF rather than from the effect of furosemide.

In the present work the increased hK1 amidase activity observed in the type 1 DM patients may be due to either, the poor glycemic control or the insulin therapy.

To study further the mechanism of the increased hK1 specific amidase activity, 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.

GDM study

Microalbuminuria

Urinary albumin excretion

In the present study the urinary albumin excretion was significantly higher in GDM patients than in pregnant women, in GDM patients than in healthy non-pregnant women, and also in healthy pregnant women than in healthy non-pregnant women. These results agree with those in literature.

Urinary creatinine excretion

No significant differences in urinary creatinine excretion were observed between GDM patients and pregnant women, between GDM patients and healthy non-pregnant women, or between healthy pregnant women and healthy non-pregnant women. To our knowledge, this is the first study concerning the evaluation of urinary creatinine excretion between GDM patients, healthy pregnant women and healthy non-pregnant women, respectively.

Albumin/creatinine ratio (ACR)

To our knowledge, this is the first study concerning the evaluation of microalbuminuria in both GDM patients and in healthy pregnant women, between the 24^{th} and the 37^{th} weeks of pregnancy, using the ACR methodology.

In this study, significantly different values of ACR were observed between GDM patients and healthy pregnant women, between GDM patients and healthy non-pregnant women, and between healthy pregnant women and healthy non-pregnant women. 26 (87%) out of 30 GDM patients, between the 24^{th} and 37^{th} weeks of pregnancy had microalbuminuria (ACR between 30 and 300 µg/mg), and 4 (13%) had macroalbuminuria (ACR > 300 µg/mg). 30 (100%) out of 30 healthy pregnant women had microalbuminuria, while 20 (67%) out of 30 healthy non-pregnant women had microalbuminuria and 10 (33%) had normal albuminuria (ACR < 30 µg/mg).

According to Waugh and associates,[29] during uncomplicated pregnancy, the development of proteinuria is accepted as a poor prognostic sign of and associated with increasing maternal and perinatal mortality and morbidity. Physiological proteinuria increases with increasing gestation, having albumin as one of its largest constituent. Our data support those reported by Waugh and associates.[29]

We have no explanation for our data showing microalbuminuria in 67% of our healthy non-pregnant women.

hK1 specific amidase activity

To our knowledge, this is the first study concerning the evaluation of hK1 specific amidase activity either on the urine of GDM patients, healthy pregnant women and healthy non-pregnant women, respectively.

Our results show that hK1 specific amidase activity is higher in the urine of GDM patients, who are not under insulin therapy than in the urine of healthy pregnant women and in the urine of healthy non-pregnant women, respectively. This result suggests that hyperglycemia is responsible for the increase in the hK1 specific amidase activity of GDM patients.

As reviewed by Borgoño and associates,[30] experimental and bioinformatics data suggest that most, if not all, kallikreins are glycoproteins in vivo, and the glycosylation of many

proteins is important for their expression and function. The glycosylation of the "kallikrein loop" may serve to regulate kallikrein activity. An instance of which was reported by Oka and associates,[31] in which experiments on enzymatic activity confirmed that N-linked oligosacharides provided structural rigidity to the kallikrein loop, which determines the size of P_2 pocket in recombinant mouse K8 (neuropsin).

Our results possibly support the data from Borgoño and associates[30] and Oka and associates,[31] respectively.

The present report showed that hK1 specific amidase activity is higher both in the urine of type 1 DM patients, under insulin therapy and in poorly controlled glycemia, and in the urine of GDM patients, not under insulin therapy. This result is in agreement with those reported by Mayfield and associates.[20] However, those authors, according to their own words, could not explain the reasons for the increased kallikrein excretion in the poorly controlled insulin-dependent diabetes mellitus patients or the mechanism for its improvement after glycemic control. We believe that we are now able to state that hyperglycemia, instead of insulin, is the cause of the increased hK1 activity in poorly controlled IDDM.

In conclusion, the evidence presented here suggests that hyperglycemia is involved in the mechanism of the increase of hK1 specific amidase activity in the urine of both type 1 DM patients and GDM patients. This result suggests that further studies will be needed in order to explain the mechanism by which hyperglycemia enhances urinary hK1 specific amidase activity in both types of DM: type 1 DM and GDM, respectively.

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