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Sex and age-related changes in L-arginine metabolism in peripheral blood leukocytes in young caucasians with type 1 diabetes mellitus

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We found that hyperactivation of cytoplasmic (anti-inflammatory) and mitochondrial (pro-inflammatory) arginase isoforms in peripheral blood leukocytes (PBL) is more pronounced in women than in male patients with type 1 diabetes mellitus (T1DM) who received insulin for one year, especially in adolescents young adults 15 years old (12.0 - 25.0) compared with children/adolescents 9.3 years old (4.5-11.8). Long-term treatment with insulin up to 14 years (on average 5.3-5.9) reduces the activity of arginase, especially in puberty girls with a tendency to normalize mitochondrial arginase, while in prepubertal boys the activity of both arginase isoforms almost doubles and remains elevated in puberty boys and can be involved in inhibiting nitric oxide synthase (NOS) and decreasing the bioavailability of NO. This is confirmed by the concomitant continuous decrease in the levels of nitric oxide synthase (NOS) products, stable metabolites of NO (nitrite) and L-citrulline in the cytoplasm and mitochondria of PBL in prepubertal girls and boys, in the latter, regardless of age and insulin therapy, while in girls of puberty changes not found, apparently, due to the increased level of sex hormones that promote the expression and activity of NOS, which contribute to the inhibition of arginase. Further studies are needed to understand whether sex and age-related changes found in L-arginine metabolism in PBL can be useful in assessing the stage and progression of T1DM and the effectiveness of therapy.

Keywords: L-arginase, L-citrulline, Leukocyte, Nitrite, Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of insulin-producing pancreatic β-cells and systemic metabolic derangements[1,2]. A steady rise in the incidence of T1DM is happening globally, including Armenia[3,4]. Despite advances in insulin therapies, often glucose homeostasis cannot be restored, and subsequent common complications occur[5,6]. Interest in novel therapies for T1DM has grown in the past years and is supported by encouraging data on its metabolic control to overcome the autoimmune processes and disordered immunoregulation[1,7]. The induction and expansion of inflammatory cytokines and immune cells (T helper lymphocytes (Th), macrophages, dendritic cells, *etc*.) are accompanied by the production of reactive oxygen and nitrogen species, peroxynitrite and nitric oxide (NO), produced by constitutive and inducible NO. synthases (NOS) in the leukocyte and β-cell and contribute to the inhibition of insulin secretion by nitrosylation of proteins and the generation of neoantigens, leading to the destruction of pancreatic β-cells in patients with T1DM[8,9]. Inflammatory agents and oxidative/nitrosative stress stimulate the expression and activity of arginase isoforms[10]. It is noteworthy, that arginase is activated by peroxynitrite-mediated RhoA/Rho kinase signaling pathway, and their inhibition has a cardioprotective effect in T1DM[11,12].

Arginase and NOS are reciprocally regulated, sharing a common substrate, L-arginine, and are involved in the mechanisms of autoimmunity and immunosuppression[12,13]. The balance between the enzymes is competitively regulated by Th1 and Th2 lymphocytes, respectively, up-regulating inducible NOS (iNOS) and arginase and affecting the dichotomy of the macrophage M1/M2, *i.e.* M1 phenotype expressing iNOS for killing / fighting, against the M2 phenotype expressing A1 for healing/fixing[14,15]. Insulin can inhibit the development of diabetes by altering the balance in the islets from Th1 in destructive insulitis to Th2 in benign insulitis[16]. In turn, iNOS and arginase can also affect Th1/Th2 balance and macrophage differentiation and delay the development of

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T1DM[17,18]. Arginase can limit the level of L-arginine, which is an important metabolic modulator of macrophage and T cell functions[19]. L-arginine can also increase insulin and glucose levels, especially in plasma, both by NO-dependent and NO-independent mechanisms[20]. It is necessary for the growth of infants and children and promotes the expression and synthesis of insulin-like growth factor 1 (IGF-1)[21]. It is noteworthy that the L-arginine/NO pathway is affected in children and adolescents and is different from adults with T1DM[22]. The higher levels of glycated hemoglobin (HbA1c) detected in pre- and early puberty is associated with lower levels of IGF-1, which increases at higher doses of insulin, especially during puberty, and particularly in women with T1DM[23]. Differences in the immune systems of men and women play a crucial role in autoimmune diseases, including the severity and course of the disease, response to therapy, and overall survival[24]. However, sex and age-related differences in the metabolism of L-arginine in T1DM, and especially those associated with puberty, need to be elucidated. Our work is devoted to the study of issues related to sex and puberty associated differences in the metabolism of L-arginine in peripheral blood leukocytes (PBL) and plasma, as well as the effect of insulin treatment in young Caucasians with T1DM, which may be useful for assessing the stage and progression of T1DM in humans[25].

Materials and Methods

Chemicals

Dextran (Mr 100 000), diacetylmonoxime and bovine serum albumin were purchased from Carl Roth (GmbH, Karlsruhe, Germany). L-arginine·HCl, HEPES, (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride, 2(S)-amino-6-boronohexanoic acid, NADPH, FAD, FMN, ninhydrin and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Reanal (Hungary).

Participants

All patients with T1DM were recruited from Muratsan University Hospital, Department of Endocrinology (Yerevan, Armenia). The study population involved 270 Caucasians aged 4-22 years with newly diagnosed T1DM (duration up to one year from the time of the first manifestation) (male n=47, female n=44), and long-term T1DM (duration more than one year up to 14 years (male n=46, female n=40) and age- and sex-matched healthy volunteers (male n=46, female n=47). The informed consent was obtained from all participants studied in accordance with Good Clinical Practice (GCP) standards and the WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects[26]. The study was approved by the Ethics Committee of the Yerevan State Medical University after Mkhitar Heratsi. Patients with T1DM were diagnosed according to guidelines of American DiabetesAssociation[27]. At least two types of antibodies described in association with the development of T1DM - islet cell autoantibodies to insulin and glutamic acid decarboxylase – were positive at the time of diagnosis of T1DM. The exclusion criteria were macrovascular and microvascular (retinopathy, nephropathy) diseases, acute or chronic liver, kidney or cardiac diseases, malignancy, arterial hypertension, pregnancy. Diabetic patients were treated and monitored according to the standard medical protocol.

Procedures

Procedures were performed at the Department of Endocrinology of "Muratsan" University Hospital (Yerevan, Armenia). All patients received multiple daily insulin injections. The absence of microalbuminuria was determined by measurement of urinary albumin/ creatinine ratio, and of macro-vascular disease by the absence of a cardiovascular event or procedure, angina, ischemic ECG abnormalities. All the participants were examined early in the morning, fasted, having avoided caffeinated beverages, cigarettes and strenuous exercise since the previous evening.

Plasma HbA1c

Plasma HbA1c was measured at "Muratsan" Hospital clinical laboratory by a latex turbidimetric assay and marked as a percentage (%)[28].

Blood leukocyte and plasma isolation

Freshly obtained peripheral venous blood was anti-coagulated using 3.9% sodium citrate ($C_6H_9Na·5H_2O$), mixed with 6% dextran (Mr 100000, prepared with 0.9% NaCl) and incubated at 37°C for 60 min to remove red blood cells by gravity sedimentation. The layer containing plasma with leukocytes and platelets was decanted and centrifuged at 1000 rpm for 5 min, and precipitated leukocytes were washed twice before use, the supernatant was centrifuged at 6000 rpm for 20 min at 4°C to separate plasma from platelets[29].

Leukocyte cytoplasmic and mitochondrial fractions

Leukocyte cytoplasmic and mitochondrial fractions were obtained by differential centrifugation of the

leukocyte homogenates[30]. Leukocytes were resuspended in ice-cold 20 mM HEPES buffer pH 7.4, containing 0.25 M sucrose, (1:10, *w/v*) and homogenized using Potter homogenizer (1500 rpm for 3 min). Homogenates were centrifuged at 1200 rpm for 10 min at 4°C to remove nuclei and cell debris. Pellet was discarded and the supernatant further centrifuged at 11000 rpm for 20 min at 4°C to yield the cytoplasm in the supernatant and the crude mitochondrial preparation in the pellet, which was washed twice, resuspended and homogenized in the buffer used.

Arginase assay

The samples were added to the reaction mixture: 20 mM HEPES buffer (pH 7.4), 3.9 mM $MnCl_2 \cdot 4H_2O$, 15.4 mM L-arginine·HCl, incubated at 37°C for 60 min, followed by the addition of 10% TCA to stop the reaction[31]. Control experiments were conducted in the presence of 2(S)-amino-6-boronohexanoic acid hydrochloride the most potent, stable, and specific arginase inhibitor, which *in vivo* effectively blocks arginase activity and does not inhibit the NOS[32]. After centrifugation (15000 rpm, 3 min) protein-free supernatants were sampled and analyzed for L-ornithine content. The arginase activity is expressed as produced in an hour L-ornithine per mg of total protein.

Measurement of L-ornithine

Samples were mixed with 4.5% ninhydrin solution and heated (90°C, 20 min), then cooled to the room temperature and the absorbance was measured at 505 nm wavelength against blank control containing all the reagents minus the sample[31].

Measurement of nitrite

Samples deproteinized with 0.5 N NaOH and 10% $ZnSO_4 \cdot 7H_2O$, were centrifuged (15000 rpm, 3 min), and the protein-free supernatants were sampled and analyzed for nitrite using colorimetric technique based on diazotization reaction with Griess reagent and measured at 546 nm wavelength against blank control containing all the reagents minus the sample[33].

Measurement of L-citrulline

Samples deproteinized with 10% TCA, centrifuged (15000 rpm, 3 min), and the protein-free supernatants were mixed with reagent (mixture of equal amounts of 9.6% H_2SO_4 and freshly prepared solution (5 mM diacetylmonoxime, 0.9 mM thiosemicarbazide,and 0.025 mM $FeCl_3$, 1:1:1, *v/v*), heated in a boiling water bath for 10 min, cooled to the room temperature and the absorbance was measured at 490 nm wavelength against blank control containing all the reagents minus the sample[34].

Protein

Protein was determined using crystalline bovine serum albumin as standard[35].

Statistical analysis

The results were expressed as the mean (M) ± standard error of the mean (SEM). The differences among many groups were analyzed by analysis of variance (one-way ANOVA), followed by Holm-Sidak post hoc test. Comparisons between two groups were analyzed using Student's *t*-tests. *P*-value <0.05 was considered significantly different.

Results

Complex changes occur during T1DM in the differentiation and functioning of immune cells with the involvement of myeloid suppressor cells, macrophages, T-helper lymphocytes, *etc*.[36,37]. Therefore, we studied all subsets of peripheral blood leukocytes (PBL) to evaluate the overall picture of the immune response associated with the metabolism of L-arginine in the blood of patients with T1DM. We examined newly diagnosed (ND) non-treated patients with the first manifestation of T1DM (ND/NT), and those treated with insulin for up to one year (average 0.5-0.6 years) (ND/T), and treated for up to 14 years (average 5.3-5.9 years), *i.e.*, long-term treatment (LT/T). Patients were differentiated by sex and age: T1DM-FI - children/preadolescent females; T1DM-FII - adolescents/young females; T1DM-MI - children /preadolescent males; T1DM-MII - adolescents/young males, and healthy controls (HC) of corresponding sex and age. Clinical data and information regarding the T1DM patients, and HC are presented in the (Tables 1 & 2).

Arginase isoforms activity was measured in the cytoplasm (A1) and mitochondria (A2) of PBL in the mentioned above groups (Fig. 1). In the T1DM-FI group, at the first manifestation of T1DM (ND/NT) the activity of A1 remained the same and that of A2 only slightly increased, while after treatment in ND/T females the activity of A1 and A2 increased 2 and 2.4 times, respectively, resulting in the shift of balance towards A2, the activity of which became 1.5 times higher than that of A1 (P <0.001). These changes are important if we take into account different roles of arginase isoforms, as well as their opposite regulation of macrophage functions[38].

Table 1 — Clinical data of children/preadolscents with newly diagnosed T1DM (T1DM-ND) and long-term T1DM (T1DM-LT) and healthy controls (HC)

Variable	T1DM-ND	T1DM-LT	HC
Number of subjects	47	43	46
Male/female	24/23	23/20	24/22
The first manifestation/ up to a year Male//female	10/14//9/14	-	-
Age (years), median (range)	9.4 [5.3-11.5]	9.3 [4.5-11.8]	9.5 [4.0-11.5]
Age of debut (years), median (range)	8.0 [4.5-11.2]	6.1 [1.7-10.2]	-
Diabetes duration (years), median (range)	0.5 [0.01-1.0]	5.3 [1.6-9.1]	-
HbA1C (%), median (range)	8.0 [5.6-13.4]	7.3 [6.6-12.7]	4.7 [4.0-5.3]
BMI (kg/m2), median (range)	17.4 [14.9-22.1]	17.6 [15.1-21.0]	17.5 [15.1-23.0]
Cholesterol (mg/dL), median (range)	169 [141-192]	175 [145-197]	155 [137-175]
Triglycerides (mg/dL), median (range)	89 [71-105]	97 [72- 117]	79 [67-99]
Creatinine clearance (mL/min), median (range)	115 [95-128]	113 [89-133]	117 [99-133]

Table 2 — Clinical data of adolescents/young adults with newly diagnosed T1DM (T1DM-ND) and long-term T1DM (T1DM-LT) and healthy controls (HC)

Variable	T1DM-ND	T1DM-LT	HC
Number of children	44	43	47
Male/female	23/21	23/20	22/25
The first manifestation/up to a year Male//female	9/14//9/12	-	-
Age (years), median (range)	15.0 [12.0-22.1]	14.5 [12.2-20.7]	15.5 [12.2-21.5]
Age of debut (years), median (range)	13.9 [10.2-21.2]	10.4 [1.4-20.9]	-
Diabetes duration (years), median (range)	0.6 [0.1-1.0]	5.9 [1.8-9.9]	-
HbA1C (%), median (range)	8.9 [5,7-16.0]	8.5 [5.9-12.4]	4.8 [4.1-5.4]
BMI (kg/m^2), median (range)	19.0 [14.7-23.7]	19.7 [16.6-23.8]	20.6 [17.9-23.7]
Cholesterol (mg/dL), median (range)	168 [141-193]	177 [143-199]	159 [133-179]
Triglycerides (mg/dL), median (range)	85 [65-107]	92 [69-115]	81 [63-107]
Creatinine clearance (mL/min), median (range)	113 [94-127]	111 [91-132]	116 [98-129]

In long-term treated LT/T female patients, increased A1 activity remained, while A2 activity decreased, but was 1.4 times higher than in HC. Unlike children/preadolescent females, in the T1DM-FII group, arginase isoforms were activated in the cellular compartments of PBL at the first manifestation of T1DM. Moreover, the activity of A1 increased 1.95, 3.28, and 2 times in ND/NT, ND/T, and LT/T patients, respectively, compared with HC. The A2 activity also increased 1.3 and 2.8 times in ND/NT and ND/T patients, respectively, but normalized after prolonged treatment with insulin, resulting in the shift of balance towards A1, the activity of which was 1.5 times higher than that of A2 (P=0.003). Thus, long-term insulin treatment modulated the activity of A2 in children/preadolescent females and regulated the activity of both arginase isoforms in adolescents/young females.

In the T1DM-MI group, the activity of A1 and A2 increased by about 1.8 times in PBL at the first manifestation of diabetes and after treatment in ND/T male patients, remained basically at that level, while in long-term treated LT/T patients, their activity stimulated by about 2.5 times, compared with HC. In the T1DM-MII group, a less pronounced increase in the activity of arginase isoforms was observed, which increased about 1.5, 1.8, and 1.7 times in ND/NT, ND/T, and LT/T patients, respectively, compared with HC. Thus, in both age groups of male patients, insulin therapy did not reduce the time-dependent increase increase in the activity of arginase isoforms.

Sex- and age-dependent changes in the total activity of arginase in the blood plasma of patients with T1DM are presented in (Fig. 2). Interestingly, the activity of arginase remained unchanged in the plasma of female patients with T1DM in both age groups, both at the first manifestation of the disease, and after treatment with insulin, regardless of the duration, although it increased 1.4-fold in ND/T adolescents/young females, compared with HC. In the T1DM-MI group, plasma arginase activity increased 2.2, 1.7, and 2.1 times in ND/NT, ND/T, and LT/T children/preadolescent males, respectively, compared with HC, whereas, in the T1DM-MII group, it remained unchanged.

It was previously found that plasma arginase activation correlates with the degree of hyperglycemia and decreases markedly during 4 h of insulin infusion in patients with type 2 diabetes[39].In our study, only in adolescents/young females, insulin could efficiently modulate arginase activity in the cellular compartments of PBL.

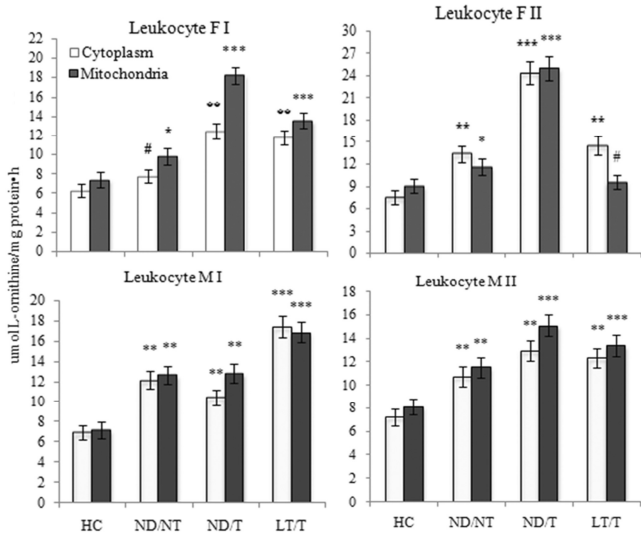

Fig. 1 — Effect of insulin therapy on arginase activity in the cell compartments of peripheral blood leukocytes of patients with T1DM. Hereinafter, the probability (P) is presented as # P >0.05, * P <0.05, ** P <0.01, *** P <0.001 in comparison with healthy controls.

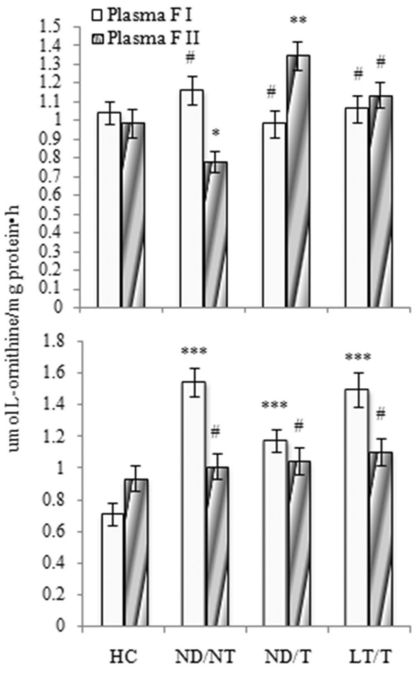

Fig. 2 — Effect of insulin therapy on arginase activity in peripheral blood plasma of patients with T1DM.

L-arginine is converted to L-ornithine and urea through arginase, and to NO and L-citrulline through NOS, and these metabolites are involved in the interaction of enzymes[40]. Nitric oxide is rapidly oxidized *in vivo* to nitrite and nitrate, which circulate in the blood and are commonly used to measure NO synthesis[41]. Sex and age-associated changes in the levels of nitrite and L-citrulline were examined in the blood of patients with T1DM to evaluate the activity of the nitrergic system. Nitrites measured in the cytoplasm and mitochondria of PBL and plasma in healthy children/preadolescent females were 1.8, 1.5, and 1.4 times higher, respectively, compared with adolescents/young females. This, apparently, is associated with a higher level of reactive oxygen species (ROS) in their blood, which are involved in the formation of peroxynitrite ($ONOO^-$) and reduce the bioavailability of NO[42]. Circulating levels of oxidative stress markers are higher in women than in men, who, in turn, have circulating concentrations of IL-8, IL-6, and TNF-α higher than women[43]. Our data showed that nitrite levels in the cytoplasm of PBL and plasma in healthy adolescents/young males are 1.4 and 2 times higher than in children/preadolescent males. Whether this is due to age-dependent activation of inducible NOS by TNF-α should be further investigated.

Sex- and age-dependent changes in nitrite levels in the cellular compartments of PBL in patients with T1DM are presented in (Fig. 3). In the T1DM-FI group, the levels of nitrite decreased 2.5, 2.0, and 1.9 times in the cytoplasm and 2.3, 1.9 and 1.9 times in

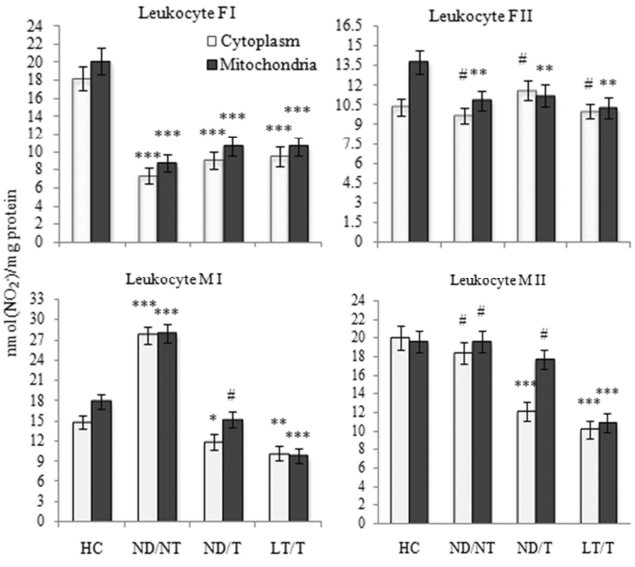

Fig. 3 — Effect of insulin therapy on nitrite level in the cell compartments of peripheral blood leukocytes of patients with T1DM.

mitochondria, in ND/NT, ND/T and LT/T female patients, respectively, compared with HC. In the T1DM-FII group, nitrite levels remained unchanged in the cytoplasm, but decreased in mitochondria 1.5, 1.2 and 1.3 times in ND/NT, ND/T, and LT/T females, respectively, compared with HC.

Thus, in children/preadolescent females, there is a persistent decrease in the levels of nitrite in the cellular compartments of PBL, which is somewhat attenuated by treatment with insulin. In adolescent/ young females with T1DM, changes in nitrite levels were detected only in the mitochondria and were less pronounced. It can be assumed that this is due to age-related differences in the share of macrophages M2 generating small amounts of NO and large quantities of L-ornithine from the same substrate L-arginine through arginase[44]. In addition, in adolescents/young females, elevated estrogen levels can prevent a drop in NO/nitrite levels affecting the expression and function of NADPH oxidases, as well as the activity of antioxidant enzymes, inhibiting the interaction of $O_2^{\bullet-}$ and NO and increasing the bioavailability of NO[45].

In the T1DM-MI group, at the first manifestation of T1DM, nitrite levels increased 1.9 and 1.5 times in the cytoplasm and mitochondria of PBL, as well as two times in plasma (vide infra). In ND/T male patients, nitrite level decreased 1.3 times in the cytoplasm and normalized in mitochondria, whereas in LT/T patients their levels decreased 1.5 and 1.8 times in the cytoplasm and mitochondria, respectively, compared with HC. In the T1DM-MII group, the nitrite levels remained unchanged at the first manifestation of T1DM, but decreased 1.7 times in the cytoplasm in ND/T patients and about two times in the cytoplasm and mitochondria of PBL in patients receiving long-term insulin treatment.

Figure 4 shows the sex and age-dependent changes in plasma nitrite levels in patients with T1DM. In the T1DM-FI group, at the first manifestation of T1DM, plasma nitrite level remained unchanged, but decreased 1.6 and 1.7 times in ND/T and LT/T patients, respectively, compared with HC, whereas, in the T1DM-FII group, it decreased 1.4 times at the first manifestation of T1DM, and normalized by insulin therapy, regardless of duration. We suggest that processes during puberty may contribute to insulin modulation of NO/nitrite production in PBL and plasma in females with T1DM.

In the T1DM-MI group, at the first manifestation of diabetes, a twofold increase in plasma nitrite level

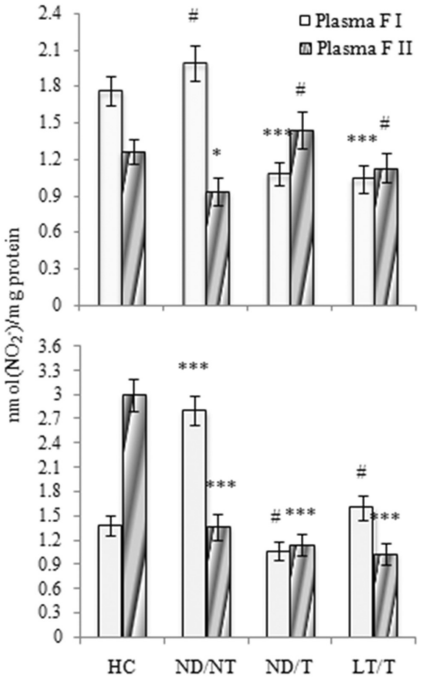

Fig. 4 — Effect of insulin therapy on nitrite level in peripheral blood plasma of patients with T1DM.

was detected, which normalized in ND/T and LT/T patients. In the T1DM-MII group, plasma nitrite level decreased 2.2, 2.6, and 2.9 times in ND/NT, ND/T and LT/T patients, respectively, compared with HC. Thus, in adolescents/young males insulin did not prevent a decrease in nitrite levels, regardless of the duration of treatment.

Our findings generally agree with the results, which show a fourfold drop in plasma nitrite levels in children and adolescents with long-term treated T1DM[22]. However, we found that insulin can modulate plasma nitrite levels in adolescents/young females with T1DM, unlike males, which indicates that the effectiveness of insulin therapy depends on sex and puberty.

L-citrulline, a byproduct of NOS, is recycled back to L-arginine by argininosuccinate synthase and argininosuccinate lyase, maintaining its level in many tissues and involving in citrulline-NO cycle[46]. Interestingly, in young Caucasians, both healthy and with T1DM, the level of plasma L-citrulline was about 5 times higher than in PBL, regardless of sex and puberty, and vice versa, the level of malondialdehyde, a stable product of lipid peroxidation and oxidative stress marker was significantly higher in PBL than in the plasma (unpublished data).

Figure 5 shows the sex and age-dependent changes in the level of L-citrulline in the cellular

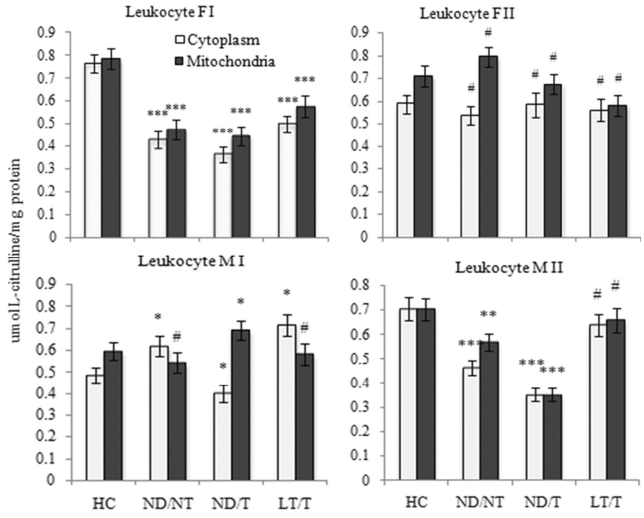

Fig. 5 — Effect of insulin therapy on L-citrulline level in the cell compartments of peripheral blood leukocytes of patients with T1DM.

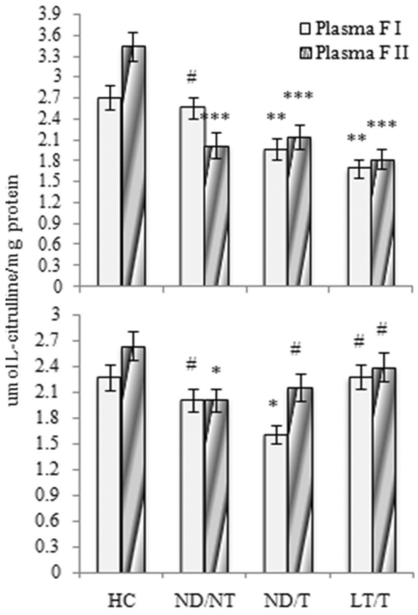

Fig. 6 — Effect of insulin therapy on L-citrulline level in peripheral blood plasma of patients with T1DM.

compartments of PBL in patients with T1DM. In the T1DM-FI group, the level of L-citrulline decreased 1.8, 2.3 and 1.5 times in the cytoplasm and 1.7, 1.8 and 1.4 times in mitochondria, in ND/NT, ND/T and LT/T female patients, respectively, compared with HC. This suggests that a decrease in the level of L-citrulline in leukocytes decreases with prolonged T1DM, however, whether this is a consequence of the influence of insulin requires further study. At the same time a simultaneous decrease in the levels of citrulline and nitrites observed in the compartments of PBL indirectly indicates a decrease in the NOS activity in children/preadolescent females with diabetes. In the T1DM-FII group, L-citrulline remained the same level in the cytoplasm and mitochondria of PBL before and after insulin treatment, regardless of its duration, and did not correlate with changes in the nitrite content.

Interestingly, in the T1DM-MI group, the level of L-citrulline in the cytoplasm increased by 1.3 and 1.5 times during the first manifestation of T1DM and after prolonged treatment with insulin, respectively, compared with HC, whereas in mitochondria it remained unchanged, with the exception of ND/T male patients, in which it also increased. In the T1DM-MII group, citrulline level decreased 1.5 and 1.2 times in the cytoplasm and mitochondria of PBL at the first manifestation of T1DM and two times in ND/T patients, respectively, compared with HC, but returned to normal after prolonged treatment with insulin.

Sex and age-dependent changes in plasma L-citrulline levels in patients with T1DM are presented in (Fig. 6). In children/preadolescent females with the first manifestation of T1DM, plasma L-citrulline level remained the same, but decreased 1.4 and 1.6 times in ND/T and LT/T patients. In the T1DM-FII group, plasma L-citrulline levels also decreased by 1.6 and 1.9 times in ND/T and LT/T patients, respectively, compared with HC. Thus, in both female age groups, plasma L-citrulline levels were not influenced by insulin, regardless of the duration of treatment.

Unlike females, in both age groups of male patients, plasma citrulline levels generally remained the same before and after insulin treatment, regardless of duration.

Discussion

Although, it is commonly known that pancreas is no longer viable, and incapable of producing insulin, during 1 or 2 years of diagnosis of T1DM, a significant proportion of patients with long-term disease have measurable levels of serum C-peptide and/or proinsulin indicating the presence of substantial β-cell mass that is dysfunctional but not destroyed[47,48]. Based on that, sex and age-dependent L-arginine metabolism was studied in different duration of T1DM.

Overactivation of arginase isoforms detected in all patients with T1DM, which is probably associated with a high level of extracellular D-glucose that promotes the activation of NADPH oxidase and increases ROS synthesis and L-arginine transport, through which it can stimulate the activity and

expression of arginase isoforms[12,49]. High arginase activity may also be associated with a high coefficient of oxidative stress (2.32) in Caucasian patients with T1DM[50]. It can also be assumed that arginase activity is stimulated in leukocytes as a response to the increase in the growth of *Candida albicans* in the gut, which we detected in patients with T1DM[51].Glucose concentration is known directly related to the growth rate of *C. albicans* in uncontrolled diabetic patients[52]. Moreover, *C. albicans* blocks NO production in human macrophages and induces arginase activity, blocking of which can restore NO production and increase macrophage kill potential[53].

Oxidative stress has a negative effect on insulin signaling and can be reduced by controlling hyperglycemia, which is shared by both type 1 and type 2 diabetes, and is a main factor of influencing oxidative stress either by the direct generation of ROS or by altering redox balance[54]. Short-term insulin pump therapy may significantly suppress oxidative stress affecting both ways[55]. When suppressing oxidative stress, insulin can inhibit the activation of arginase isoforms, but we were able to observe this effect only in adolescent/young females, presumably due to female sex hormones involvement in effect of insulin. Long-term insulin treatment can effectively modulate the activity of both A1 and A2, namely normalizes A2 activity and shifts the balance towards A1, which is necessary for the anti-inflammatory functioning of M2 macrophages, as well as for the fungicidal activity of human neutrophils[56]. In children/preadolescent females, long-term treatment with insulin does not affect A1 activity, but is accompanied by a decrease in A2 activity, which though remains two times higher than normal. Notably, endogenous estrogen, 17 β-estradiol (E2) can stimulate the M2 phenotype associated with A1 activity and inhibit M1 functions in both peripheral macrophages and those in the CNS[57]. E2 is also involved in protecting the function/survival of pancreatic β-cells and insulin secretion under conditions of oxidative stress[58]. It should be noted that in the cytoplasm, the A1-derived ornithine may provide proline or glutamate synthesis *via* ornithine aminotransferase, while in mitochondria A2-derived ornithine is involved in the synthesis of polyamines *via* ornithine decarboxylase[59].It is shown that A2 promote the inflammatory response of macrophages *via* NOS-independent stimulation of mitochondrial ROS, which contribute to insulin resistance and atherogenesis[60]. Recent data suggest that targeting the synthesis of polyamines partially protects against hyperglycemia by increasing Treg cells crucial for immune tolerance and reducing potentially pathogenic Th17 cells in the lymph nodes of the pancreas in T1DM[61]. At the same time, A1 can provide myeloid-derived suppressor cells (MDSCs) effect on the development of TH17 cell-associated autoimmunity (systemic lupus erythematosus, SLE), therefore targeting MDSCs or A1 may offer potential therapeutic strategies for treating SLE and other TH17 cell-mediated autoimmune diseases[62]. This echoes with the fact that a new function of the vascular endothelial growth factor (VEGF) receptor 1 signaling is to prevent the over-expression of arginase 1, associated with over-activation of macrophages, which causes autoimmune diseases[63]. Moreover, the healing of a diabetic wound is associated with increased neovascularization and tissue regeneration, which correlates with VEGF and the mitogen-activated protein kinase pathway, and endothelial nitric oxide synthase in streptozotocin-induced diabetes[64]. It was also found that the insertion/deletion gene polymorphism in the VEGF promoter region may play a role in the development of non-diabetic nephropathy[65]. However, nephropathy can eventually develop in both types of diabetes, taking into account the glycemic and genetic background[66].

Unlike females, children/preadolescent males who underwent prolonged insulin treatment, exhibit higher activity of arginase isoforms in PBL compared with patients who have received insulin for one year. Insulin also did not prevent an increase in arginase activity in adolescents/young male patients, regardless of the duration of treatment. This can aggravate the course of T1DM, especially since isoforms of arginase may contribute to the development of oxidative stress by local depletion of L-arginine, which leads to the forced simultaneous production of NO and superoxide through NOS and further peroxynitrite followed by peroxynitrite-mediated cell damage[67,68]. It should be also noted that signalling pathways involving endothelial and inducible NOS isoforms/NO, NADPH oxidase, and arginase could play key roles in chronic diabetes mellitus-associated cardiovascular abnormalities reviewed elsewhere[38,69]. Preventing these signalling alterations can also protect from the development of cardiovascular disease associated with diabetes.

It is commonly known that high arginase activity may reduce NO production, which leads to the

development of microvascular complications in diabetes[70]. Arginase can suppress the activity of NOS either by reducing the intracellular level of L-arginine or by producing urea, which inhibits the dimerization of NOS monomers[71]. Our data have shown that the activation of arginase isoforms in T1DM is accompanied by changes in the level of nitrite and citrulline, which are sex and age- dependent. So, in prepubertal females, levels of nitrite and citrulline are reduced in the cytoplasm and mitochondria of PBL at the first manifestation of T1DM, which possibly associated with suppression of the endogenous NO production. In adolescent/young female patients increased levels of estrogen and other sex hormones can upregulate NOS, particularly in leukocytes[72]. So, E2 can increase the ability of human neutrophils to produce NO and, therefore, contribute to the protection against cardiovascular disease[73]. Endothelial NOS (eNOS), in addition to the well-known estrogen-dependent stimulation, is constitutively expressed in humans in a sex-specific innate manner, namely, female endothelial cells express more eNOS mRNA and protein than men, both *in vitro*, and *ex vivo*[74].

Nitrites increased markedly in the cellular compartments of PBL and plasma in children/ preadolescent males with the first manifestation of T1DM and decreased during treatment with insulin. Interestingly, the nitrite level remained unchanged in the cellular compartments of PBL in adolescents/ young males with T1DM, and treatment with insulin reduced it below normal. It can be speculated that an increase in nitrite levels in PBL and plasma in prepubertal boys with T1DM may be associated with lower oxidative stress compared with adolescents/ young males. It is noteworthy that nanomolar concentrations of NO can promote cGMP-dependently glucose-induced $[Ca^{2+}]i$ oscillations and insulin secretion in β-cells and inhibit at submicromolar concentrations independently of cGMP[75]. Recent data suggest that NOS/NO may contribute to β-cell survival *via* selective suppression of DNA damage-response signaling and apoptosis, although NO cannot inhibit it in macrophages, hepatocytes, and fibroblasts[76]. Interestingly, NO readily reacts with cysteine residues in the key active site of both L-ornithine- and S-adenosyl methionine decarboxylases and inactivates the synthesis of polyamines, mediating anti-proliferative activity[77].

Limiting L-arginine can be improved with dietary supplements or by intravenous administration of arginine or its endogenous precursor citrulline, which also increases insulin sensitivity[78]. L-citrulline can enhance the formation of NO and suppress the activity of arginase, as its allosteric inhibitor[79]. It should be noted that changes in the level of citrulline are not directly dependent on the activity of NOS. The decrease in citrulline can be explained by its conversion to arginine, which in turn is metabolized by activated arginase, followed by depletion of arginine in plasma of diabetics. In addition, citrulline is produced by ornithine transcarbamylase, and even a slight deficiency of which is accompanied by a decrease in plasma levels of citrulline and arginine[80]. L-citrulline levels decreased in the cytoplasm and mitochondria of PBL and plasma in children/preadolescent females with T1DM, and slightly depended on the duration of insulin treatment. At the same time citrulline levels were less reduced in the cellular compartments in children/preadolescent males with T1DM than in adolescents/young ones, and long-term treatment with insulin completely restored it, regardless of age. It is important because L-citrulline can also restore the NO/ROS balance and play a protective role in T1DM as was shown in a mouse model of streptozotocin-induced diabetes[81]. In general, the effect of insulin therapy on sex and age- associated changes in the activity of arginase isoforms and levels of NOS metabolites in PBL and plasma of patients with T1DM may be partially related to the regulation of insulin functions *via* sex hormones directly through insulin-sensitive tissues and indirectly through oxidative stress[45].

Conclusion

Results of this study suggest that the activation of functionally different arginase isoforms and a corresponding decrease in the levels of nitric oxide synthase products, stable NO metabolites (nitrite) and citrulline in the cytoplasm and mitochondria in the peripheral blood leukocytes of young Caucasians with type 1 diabetes depend on sex and age, in particular puberty. Subcellular perturbations of L-arginine metabolic pathways in PBLare more pronounced in women than in men, and they are effectively modulated by insulin treatment in women, depending on the duration of insulin therapy, especially during puberty, presumably due to an increased level of estrogens that affect the metabolism of L arginine and insulin effects. In male patients, prolonged insulin therapy does not prevent hyperactivation of arginase isoforms and, accordingly, a decrease in the level of

nitrite, most pronounced in boys of puberty. Further research is needed to understand whether the sex and age-related changes observed in L-arginine metabolism in PBL can be useful for assessing the stage and progression of type 1 diabetes and the effectiveness of therapy, as well as to understand whether this should be taken into account in the differential targeting of arginase isoforms and nitrergic system for a beneficial effect on the course of type 1 diabetes.

Conflict of interest

All authors declare no conflict of interest.

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