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THE SPECIFIC ACTIVITY OF PROTEINS INVOLVED IN IRON METABOLISM DEPENDS ON COMPENSATION OF TYPE 2 DIABETES MELLITUS

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Objective. We aimed to analyze the alterations of activity of iron metabolism members i.e. ceruloplasmin (Cp) and transferrin (Tf), in relation to the percentage of glycated hemoglobin. The latter is one of biochemical criteria of chronic hyperglycemia compensation in case of type 2 diabetes mellitus.

Materials and methods. Concentration and activity of Cp and Tf, concentration of iron, copper and lipoprotein cholesterol were measured by biochemical methods in blood serum samples obtained from healthy donors and patients with type 2 diabetes mellitus divided in three groups according to glycated hemoglobin level.

Results. The significant decrease in serum copper, ferroxidase activity of Cp and iron-binding capacity of Tf, as well as an increase of Tf concentration, in groups with compensated and uncompensated type 2 diabetes mellitus was found.

Conclusion. Our data demonstrate a statistical link between the degree of type 2 diabetes mellitus compensation and alteration of iron metabolism members' activity. Thus, an increase of hyperglycemia is associated with a decrease of both Cp ferroxidase activity and the degree of Tf saturation with iron. These alterations may explain the efficiency of treatment with iron chelators of such type 2 diabetes mellitus complications as trophic ulcers. The said disease condition is directly connected with the changes in iron efflux.

Keywords: diabetes mellitus; glycated hemoglobin; transferrin; ceruloplasmin; iron; copper; ferroxidase activity; lipoproteins.

ЗАВИСИМОСТЬ УДЕЛЬНОЙ АКТИВНОСТИ УЧАСТНИКОВ ОБМЕНА ЖЕЛЕЗА ОТ СТЕПЕНИ КОМПЕНСАЦИИ САХАРНОГО ДИАБЕТА 2-го ТИПА

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Цель исследования — проанализировать изменения активности участников обмена железа, церулоплазмина и трансферрина у пациентов с сахарным диабетом 2-го типа в зависимости от процентного содержания гликированного гемоглобина, который является биохимическим критерием степени компенсации хронической гипергликемии.

Материалы и методы. С помощью биохимических методов измерены концентрация и активность церулоплазмина и трансферрина, концентрация железа, меди, холестерина липопротеинов в сыворотке крови, полученной от здоровых доноров и пациентов с сахарным диабетом 2-го типа, объединенных в три группы в зависимости от процентного содержания гликированного гемоглобина.

Результаты. Обнаружены достоверное снижение концентрации меди, ферроксидазной активности церулоплазмина и способности трансферрина насыщаться железом, а также увеличение концентрации трансферрина при компенсированном и некомпенсированном сахарном диабете 2-го типа.

Заключение. Установлена статистическая связь степени компенсации сахарного диабета 2-го типа с активностью участников обмена железа. Так, по мере усиления гипергликемии снижается как активность церу-

List of abbreviations

Cp — ceruloplasmin, HbA1c — glycated hemoglobin, HDL — high-density lipoproteins, LDL — low-density lipoproteins, T2D — type 2 diabetes mellitus, Tf — transferrin, TIBC — total iron-binding capacity (of blood serum).

лоплазмина, так и насыщение трансферрина ионами железа. Выявленные изменения объясняют причину эффективности лечения хелаторами железа таких осложнений сахарного диабета 2-го типа, как трофические язвы, которые связаны с изменением оттока железа.

Ключевые слова: сахарный диабет; гликированный гемоглобин; трансферрин; церулоплазмин; железо; медь; ферроксидазная активность; липопротеины.

Introduction

Type 2 diabetes mellitus (T2D) is associated with iron metabolism disorders, including those of hereditary etiology [1]. Indeed, T2D is found in about 80% of cases of hereditary hemochromatosis, which is associated with mutations in genes controlling iron metabolism: *HFE* (hemochromatosis protein), *HAMP* (hepcidin), *TFR2* (transferrin receptor), *SLC40A1* (ferroportin), and *HFE2* (hemojuvelin) [1]. Cp is the major ferroxidase in blood plasma [2]. Aceruloplasminemia being the result of a mutation in ceruloplasmin (*Cp*) gene is also associated with T2D. Recently double knockout of *Cp* and its homologue hephaestin (*Hp*) genes was demonstrated to cause T2D symptoms in mice [3].

Chronic hyperglycemia in T2D is connected with non-enzymatic biopolymers modification by glucose, as well as by products of glucose oxidation: glyoxal, methylglyoxal etc. The percentage of glycated hemoglobin (HbA1c) is the widely accepted biochemical criterion of chronic hyperglycemia and its compensation. HbA1c level between 5.7% and 6.4% marks prediabetes or hyperglycemia compensated by therapy, while in case of uncompensated T2D HbA1c level exceeds 6.5%. Continuously high level of glucose leads an increase of HbA1c percentage due to relatively low rate of red blood cells renewal (half-life period is about 60–90 days). However, other proteins, e.g. human serum albumin, are also targeted by non-enzymatic glycation [4]. Diminution of Cp activities due to aggregation, losing of copper ions and therefore the decreasing ferroxidase activity after *in vitro* modification by methylglyoxal or aminoacetone has been shown. The latter metabolites' levels are elevated in blood plasma in T2D patients [5, 6]. The level of the so-called non-ceruloplasmin copper is increased in blood serum of T2D patients [7]. Although Cp does not form a stable complex with transferrin (Tf) [8], its physiological function is acceleration of loading into Tf of ferric ions produced during Cp-catalyzed ferroxidase reaction [9]. Recently the glycation sites in both Cp and Tf were identified in T2D patients [10, 11]. However, no significant correlations between HbA1c and serum iron, as well as between HbA1c and TIBC were found in T2D [12]. Taking into account that alteration of Cp ferroxidase activity in case of hereditary pathology of copper metabolism is usually compensated by low-density lipoprotein (LDL)-associated ferroxidase activity [13], the study of the link between T2D and cholesterol level can also be informative.

This study aims at analyzing the alteration of activity of the proteins of iron-metabolism proteins, e.g. Cp and Tf. In parallel the lipoproteins and cholesterol, iron and copper levels were assayed in blood serum from healthy donors and T2D patients divided in three groups according to HbA1c level.

Materials and methods

This study included samples from 364 donors divided in three groups: Group 1 — 110 donors without T2D (HbA1c<5.8%); Group 2 — 195 patients with compensated stage of T2D (5.9%<HbA1c<6.9%); Group 3 — 59 patients with uncompensated T2D (HbA1c>6.9%). Acute phase of inflammation characterized by elevated level of C-reactive protein (>10 mg/L) was an exclusion criterion. Local institutional ethics committee approved the study (No. 2/19, 25.03.2019) and all participating subjects gave written informed consent to be included in the study for blood sampling. Blood serum samples were the courtesy of biochemical laboratories of Saint Petersburg State Clinical and Diagnostic Center No. 1.

Percentage of HbA1c was determined by the certified method [14] based on fast liquid chromatographic separation of hemoglobin obtained from red blood cells lysate using D-10 chromatograph and the reagent kit from Bio-Rad Laboratories (USA).

Concentrations of Cp and Tf in blood serum were determined with radial immunodiffusion according to Mancini [15]. Highly purified Cp and Tf were used to obtain monovalent rabbit antisera and to plot calibration curves reflecting the dependence of the area of immune precipitate on concentration of Cp or Tf [8, 16, 17].

Ferroxidase activity of Cp was determined with the help of automated method adapted for biochemical analyzer BS-200 (Mindray, China). The method is based on assaying the residual Fe(II) concentration after adding ferrozine as a chromogenic substrate and incubating the Fe(II)-containing reagent with a serum sample [18]. One unit corresponds to the amount of the enzyme (Cp) in serum that provides oxidation of 1 μM Fe(II) per 1 min in the medium containing 367 μM Mohr's salt (Fe(II) source) in 450 mM sodium acetate buffer (pH 5.8).

To determine serum copper concentration, 4-(3,5-dibromo-2-pyridylazo)-*N*-ethyl-*N*-sulfopropylaniline and trichloroacetic acid as chromogenic and deproteinization agents were used [19]. The ratio between copper and Cp concentrations was used to characterize copper saturation of Cp.

To determine serum iron concentration, 2-(5-nitro-2-pyridylazo)-5-(*N*-propyl-*N*-sulfopropylamino)phenol and thioglycolic acid as chromogenic and copper-, zinc-masking agents were used [20]. Total iron-binding capacity of serum (TIBC) was determined by adding 10 μL of 4.5 mM $FeCl_3$ to 0.25 mL of serum; iron excess was removed in 10 min with 20 mg $MgCO_3$. To measure concentration of iron [20] $MgCO_3$ was removed by centrifugation for 15 min at 3000 g and the supernatant was used.

Concentration of LDL and high-density lipoproteins (HDL) cholesterol was measured using the kits LDL-CHOLESTEROL-VITAL and HDL-CHOLESTEROL-VITAL (Vital Development Corporation, Russia). The methods are based on selective precipitation of LDL and chromogenic reaction of 4-aminoantipyrine with hydrogen peroxide produced in reaction of cholesterol with the mixture of cholesterol oxidase and cholesterol esterase.

To characterize the specific activity of Cp and Tf the ratios of ferroxidase activity to Cp concentration and TIBC to Tf concentration were used, correspondingly.

The results are represented as a mean ± standard error of the mean (M ± SEM), the hypothesis about law of distribution was analyzed by Kolmogorov's test, the equality of variabilities was analyzed as a result of Fisher's test. The equality of expectations was analyzed by one-way ANOVA and post hoc Fisher's LSD test. *P*-values <0.05 were considered statistically significant.

Results and discussion

The table summarizes biochemical variable differences obtained in this study for healthy donors (group 1), for patients with compensated T2D (group 2), and for patients with uncompensated T2D (group 3). The percentage of HbA1c used as a criterion for dividing the individuals into three groups is significantly different in all compared groups. Cp concentration measured using antibody is slightly higher in the 3rd group as compared with the first and the the second groups. On the contrary, the ferroxidase activity of Cp, as well as of serum copper, is decreased in the groups of patients with compensated and uncompensated T2D in comparison with healthy donors. As a result of these changes, a significant decrease of specific ferroxidase activity (FerOx/Cp) and saturation of Cp with copper were registered concomitantly with the increase of HbA1c percentage. In case of healthy donors the molar Cu/CP ratio very close to 6 was found, which practically corresponds to an expected ratio usually detected in highly purified preparations of Cp [16]. The data obtained demonstrate that with an increase of hyperglycemia the number of copper ions per one molecule of Cp significantly

Table

Biochemical variable differences in healthy donors (group 1), in patients with compensated T2D (group 2), and in patients with uncompensated T2D (group 3)

Biochemical variable	M ± SEM			*p*-values		
	group 1 (*n* = 110)	group 2 (*n* = 195)	group 3 (*n* = 59)	p_{1-2}	p_{1-3}	p_{2-3}
Glycated hemoglobin (HbA1c), %	4.57 ± 0.08	6.29 ± 0.02	8.33 ± 0.20	0.000	0.000	0.000
Ceruloplasmin (Cp), mg/L	459 ± 14	457 ± 9	518 ± 10	NS	0.003	0.001
Ferroxidase activity of Cp (FerOx), U/L	817 ± 23	712 ± 13	697 ± 14	0.000	0.001	NS
FerOx/Cp, U/mg	1.78 ± 0.04	1.56 ± 0.02	1.35 ± 0.02	0.000	0.000	0.000
Serum copper (Cu), μM	20.6 ± 0.7	17.8 ± 0.4	17.5 ± 0.4	0.000	0.003	NS
Cu/Cp, mole/mole	5.99 ± 0.01	5.15 ± 0.01	4.49 ± 0.02	0.000	0.000	0.000
Serum iron (Fe), μM	18.2 ± 0.6	18.9 ± 0.3	18.62 ± 0.8	NS	NS	NS
Total iron-binding capacity (TIBC), μM	49.0 ± 0.6	47.3 ± 0.5	47.0 ± 0.7	NS	NS	NS
Transferrin (Tf), mg/mL	2.51 ± 0.05	3.07 ± 0.04	3.86 ± 0.08	0.000	0.000	0.000
TIBC/Tf, mole/mole	1.62 ± 0.02	1.29 ± 0.01	1.02 ± 0.01	0.000	0.000	0.000
LDL cholesterol, mM	3.26 ± 0.12	3.38 ± 0.09	3.43 ± 0.17	NS	NS	NS
HDL cholesterol, mM	1.48 ± 0.04	1.39 ± 0.03	1.28 ± 0.05	NS	0.008	NS

N o t e. NS — *p*-value is higher than critical value ($p > 0.05$), 0.000 — non-zero digit is beyond 3th position after point.

decreases. In general, these changes correspond to the results of *in vitro* modification of Cp by metabolites typical of T2D. Indeed, incubation of Cp with methylglyoxal and aminoacetone is followed by the loss of its copper ions and a decrease of its ferroxidase activity [5, 6]. We hypothesized that changes in Cp ferroxidase activity might be compensated by increasing the LDL content. The latter also function as ferroxidase in association with copper ions [13]. However, no significant differences in LDL cholesterol were found among the results of the three groups. Only a slight decrease of HDL cholesterol in the group with uncompensated T2D was found in comparison with healthy donors and patients with compensated T2D.

No significant differences were found in serum iron and total iron-binding capacity of serum (TIBC) among all groups. However, concentration of serum Tf was higher in patients with compensated T2D as compared with healthy donors, and in patients with uncompensated T2D compared both with patients who had compensated T2D and healthy donors. In contrast, the ratio between TIBC and Tf, which characterizes the specific capacity of Tf to bind Fe(III), decreased in patients with compensated T2D in comparison with healthy donors. The same ratio was observed in patients with uncompensated T2D compared either with patients who had compensated T2D or with healthy donors. Indeed, one molecule of Tf can bind two ferric ions. In case of healthy donors, this ratio was 1.6 mole of iron per 1 mole of Tf, but in case of patients with compensated and uncompensated T2D this value was reduced to 1.26 and 1 mole of iron per 1 mole of Tf, respectively. It should be noticed, that such complications of T2D as trophic ulcers are connected with alterations of iron efflux from tissues and the failure of tissues' adaptation to hypoxic stress. Usin natural and non-natural iron chelators, e.g. lactoferrin, which is a homolog of Tf, can mitigate the severity of T2D and of metabolic syndrome [21].

Conclusion

In general, activity and concentrations of iron-metabolism proteins, involving in normal transport of iron in blood plasma, were in coincidence: during development of pathological features of hyperglycemia the concentrations of Cp and of Tf are rising, but specific activity of these proteins is suppressed. Under normal conditions the activity of proteins participating in iron metabolism, particularly in the transport of that element in plasma, is in conformity with their concentrations. However, the onset of hyperglycemia is followed by an increase of concentrations of both Cp and Tf and a concomitant drop of their specific activity. A careful suggestion can be made that elevated concentrations occur in response to the decreasing activity of Cp and Tf due to their modification by glucose and its metabolites. The changes observed can explain the efficiency of iron chelators in therapy of such complications of T2D as trophic ulcers that are connected with alterations of iron efflux from tissues.

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Conflict of interest. The authors declare no conflict of interest in financial or any other sphere.

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