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Influence of Magnesium and Metformin on Advanced Glycation End Products Receptors Expression in Ovary

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Abstract

Advanced glycation end products(AGEs) and cellular receptors for advanced glycation end products(RAGEs)are involved in development of some diabetes mellitus complications(nephrophaty, neurophaty, micro and macro vascular complications). The aim of the study is to show the influence of magnesium and metformin on the expression of RAGEs at the ovarian level in diabetes mellitus. This influence was tested using four groups of non-pregnant female adult rats. The first group was control group and did not receive any substance. The second group received streptozotocin 60mg/kg ip. in unic dose. Groups III and IV received streptozotocin just like lot II but the third

group received also $MgCl_2$ 1mmol/ kg b. wt. /day ip. daily 8 weeks and the fourth group received metformin 100 mg/kg b. wt. /day (by endogastric probe)daily ,8weeks.The plasma concentration of magnesium, glucose and total antioxidant capacity(TAS)was determined at the beginning of the study, at 48 h and after 8 weeks. RAGEs expression was determined after harvesting the ovaries from all animals by immunohistological technique. The obtained results show an increase of TAS in group III and IV versus

second group(30.62+/-2.19 and 31,88+/-1,41 vs. 20,52 +/-2,41 Trolox units p<0.01) Ovarian expression of RAGEs was significantly increased by streptozotocin and reduced by magnesium and metformin. The conclusion of the study is that magnesium and metformin significantly decreased the RAGEs expression and can therefore may reduce the tissue action of AGEs.

Keywords

Magnesium, Metformin, RAGE, Diabetes, Ovary.

Introduction

Diabetes mellitus is one of the most and severe diseases in human clinic. This disease affects all tissues and organs in the human body.

There are many pathogenic mechanisms involved in the development of diabetes complications[1, 2].Among these, the excess production of free radicals is probably the main mechanism, but other factors as advanced glycation products (AGEs)and the overproduction of some cytokines are also involved[3] .

The receptor for AGEs is RAGEs . This is a member of the immunoglobulins superfamily[4]. It is a cell surface receptor and is a signal-transducing receptor for AGEs .

In normal conditions, RAGEs is expressed at a low and very low level in the cells of human and and animal body (endothelial cells, neurons, leukocytes, pancreatic cells, glomerular cells, and others)[5].

An important fact is the stimulation by AGEs (after RAGEs activation) of reactive oxygen species (ROS) generation. AGEs plays an important role in increasing oxidative stress in diabetic patients. There is evidence that AGE and the RAGE receptor are involved in the diabetes complications and especially in the renal and vascular complications of this disease[6, 7].

Metformin is one of the most widely used oral antidiabetics in the treatment of diabetes mellitus.This oral antidiabetic drug reduces some of the tissue damages caused by the disease [8].

Magnesium is an important biavalent cation from human body largely located at the intracellular level.There are over 300 magnesium dependent enzymes .

The plasma and cellular magnesium concentration is reduced in the body of diabetic patients[9-11].

Although RAGE is a multiligand receptor involved in various pathological processes in the human body, there is very little data on how metal cations influence the functions and expression of this receptor.

Purpose of this study was to highlight the effect magnesium and metformin on RAGE expression in ovary of nonpregnant female rats during experimental induced diabetes mellitus.

Material and methods

The study involved four groups, each one comprising ten non-pregnant adult female Wistar rats, weighing 170-250 g.Animals were obtained from Laboratory Animal Center of Cantacuzino Institute of Research, Bucharest, Romania and were housed in groups of five in Plexiglas cages (65 x40 x 30 cm) with the floor covered with sawdust. Animals were maintained in a controlled environment (12 h light/dark cycle with lights on at 07.00,temperature of 20 ± 1°C) before and throughout the experimental period with free access to water and standard granulated food. The first group was a control group and did not receive any substance. Experimental diabetes mellitus was induced in three other groups.

To induce diabetes mellitus, following overnight fasting, the other three groups of animals received streptozotocin (STZ) (Sigma Aldrich, St. Louis, USA), 60 mg/kg b.wt., dissolved in a citrate buffer i.p. (in a single dose), as described elsewhere [12]. The second group received only STZ.

The third group received STZ just like the second group but received also $MgCl_2$(Sigma-Aldrich St. Louis, USA) 1 mmol/kg/day i.p., daily for eight weeks. The fourth group received STZ just like the second group but received also metformin(Bristol-Myers Squibb UK) 100 mg/kg b.wt. per os (endogastric probe), daily, for eight weeks. The first dose of magnesium and the first dose of metformin were given on the same day as STZ in separate administrations.

The weight of the animals was determined daily and the dose of MgCl2 and metformin was adjusted according to changes in body weight.

Rat mortality after STZ administration was: control group - 0, STZ group - 2, STZ+$MgCl_2$ group - 1, STZ+metformin group - 1 rat. The following parameters were determined: blood glucose concentration (using Randox Daytona analyzer, Randox LTD Laboratories, UK and diagnostic kits Randox)), total-plasma magnesium concentration (atomic absorption spectrophotometry, (using AAS1N Carl Zeiss Jena spectrophotometer, Germany) and the serum oxidative status (total antioxidant capacity)(TAS).TAS and plasma glucose levels were determined before the start of the experiment, at 48 hours and eight weeks after STZ dministration. Plasma magnesium level was determined prior to onset of the experiment and after 8 weeks. Blood sampling was performed by venipuncture of the rats tails. The blood was collected in tubes. To obtain serum, the blood was allowed to clot for 30 minutes at room temperature before centrifugation for five minutes at 4.C. To obtain plasma, blood tubes treated with heparin were immediately centrifuged for five minutes at 2.000 g, at 4C.

Total antioxidant status was measured in serum as a Trolox Equivalent Antioxidant Capacity (TEAC) , Total antioxidant status (TAS) was assayed with a slightly modified chemiluminometric method using the luminol–horseradish peroxidase system (Berthold Lumat 9507 chemiluminometer, Berthold, Bad Wildbad, Germany).In this method, constant light emission results from luminol degradation in the presence of a catalyst(horseradish peroxidase) with an enhancer (p-iodo-phenol), and is recorded kinetically. When a biological fluid is introduced into this system, the level of light emission decreases for a period; this is proportional to the total antioxidant capacity.

The principle of the antioxidant assay(TAS determination) is formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2¢-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, ABTS·+ , a soluble chromogen that is green in color and can be determined spectrophotometrically at 405 nm. After 150 µl of ABTS Substrate Working Solution to each well - incubation time is 5 minutes at room temperature.The plate should be read within an hour. Antioxidant Assay Kit - CS0790 Sigma-Aldrich has been used to quantify TAS.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble, alpha-tocopherol analogue, was used as the standard. Calibration was performed with Trolox (hydro-soluble vitaminE) (Sigma Aldrich), and the final results relate to Trolox equivalents. The pro-oxidant system, which generates light, was brought to five million relative units of light

(RLU), and serum samples were used at a dilution of 1/10 [13].

This method determines the antioxidative effect and results are expressed in Trolox units.

After eight weeks, all animals were anesthetized and killed by decapitation after blood sampling. The both ovaries of each animal were removed and examined under optical microscopy. The ovaries were fixed in 10% formalin for 48 hours and then cut into 5-10 mm transversal slices. Slices were embedded in paraffin blocks. The antiRAGE antibody (Polyclonal, ab65965, Abcam)(7TM Antibodies UK) was used for the immunohistochemical study For the immunohistochemical study, tissue samples were keeped for 24 hours in the fixation solution, included in paraffin and sectioned in sections with a thickness of 4 microns by Microm HN 325 microtom. The sections were shown on K8020 glue blades and were processed in four distinct sessions for uniform working conditions. After deparaffinization and hydration, the sections were treated by HIER technique with sodium citrate at 97 °C for 20 minutes, blocking endogenous peroxidase (with Peroxidase Block solution, Novocastra UK). The sections were initially incubated cold with the primary antibody and subsequently with the secondary antibody for 30 minutes at room temperature. Reactions were developed using 3.3'- diaminobenzidine tetrahydrochloride (DAB Chromogen, Novocastra UK) for 5 minutes. The system Zeiss Observer Z1 Tissue Gnostics 9Tissue Faks was used to scan and retrieve images Determination of changes in RAGE expression was performed by computer analysis of color density in optical microscopy. The expression RAGE was quantified according to the intensity of the color density. The following score was used: 0-absence of color, 1-weak color density, 2-medium color density, 3-intense color density. Five sections of each ovary were examined. The observation was done by two independent observers.

The obtained data were statistically interpreted with the ANOVA test.The data were presented as aritmetic mean+/- standard deviation(SD)and analysed using SPSS program for Windows 10,version 17.0 ANOVA. P values <0.05 were considered statistically significant.

The research was performed after the aproval by Ethical Committee for Research of Gr. T. Popa University of Medicine and Pharmacy Iasi. All animal procedures were performed acording to the European Union low(86/609/EEC)which regulates the use of animals for experimental and other scientific purposes in the EU.

Results and Discussion

The used dose of STZ produced a sever diabetes mellitus and strongly increased plasma glucose level in all animals (Figure 1).

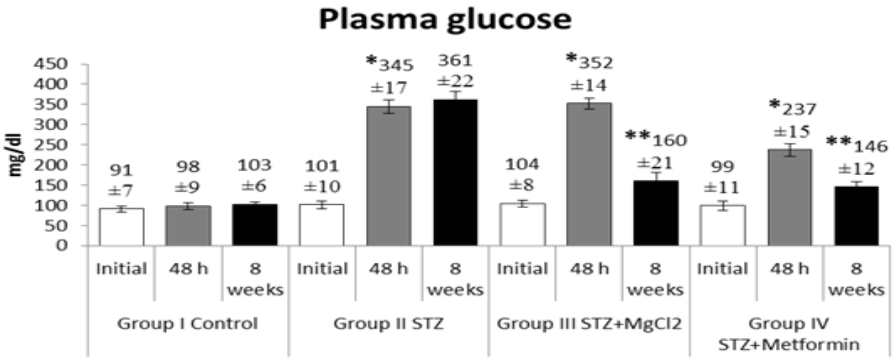

Figure 1. Level of plasma glucose in all animal groups included in the study.

* p< 0.01 versus initial

* * p<0.05 versus initial

Daily $MgCl_2$ administration induced a significant increase of plasma total magnesium concentration in the third group (Figure 2).

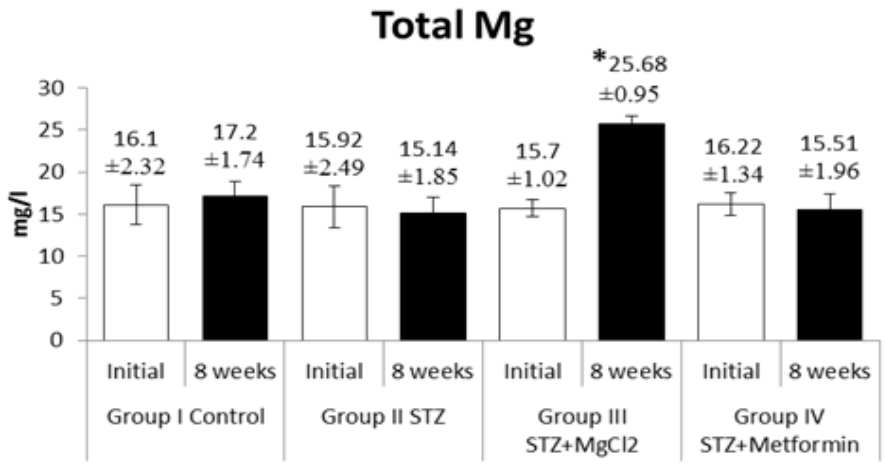

Figure 2. Plasma magnesium concentration in all animal groups included in the study.

* p<0.01 versus initial

In the case of diabetes in human patients, the low plasma level of magnesium has been shown by numerous studies. In the case of experimental diabetes, the existing data are variable. In some studies the plasma level of magnesium is low but in other studies it has been found unchanged[14]. Our data do not show significant changes in the plasma concentration of magnesium after the induction of diabetes with STZ.

Magnesium and metformin reduced the intensity of RAGE al the endometrial and ovarian level (compared to control group) . Intensity of RAGE expression by computer analysis of color density in optical microscopy is reduced after the administration of magnesium and metformin(Table 1)

Group	Group I Control	Group II STZ	Group III STZ+MgCl2	Group IV STZ+metformin
Color density (Score M+/-SD	0.56+/-0.21**	2.84+/-0.37	1.76+/-0.28 **	2.05+/-0.16*

Table 1. Intensity of RAGE expression by analysis of color density is reduced after the administration of magnesium and metformin.

*p<0.05 versus STZ group **p<0.01 versus STZ group

Antioxydant capacity (TAS) decreases after STZ administration. In the group received STZ+magnesium and in the group received STZ+metformin the decrease of TAS was significantly lower compared to the group receiving only STZ.(Table 2)

Group	Initial	After 48h	p	After 8weeks	P
Control	39,85+/-2	40,18+/1,46	<0.01	40,08 +/-2,11	<0.01
STZ	39.75+/-1.93	20,97+/2,02		20,52 +/-2,41	
STZ+Mg Cl2	39.4+/-1.03	25.8+/ 1.13	<0.05	30.62+/-2.19	<0.01
STZ+Metformin	40,52+/ 1,69	27,45+/1,24	<0.05	31,88+/-1,41	<0.01

Table 2. TAS values in all animal groups included in study. (Results are in Tolox Units)

(p versus STZ group). TAS values are increased in groups that received STZ + Mg or STZ + metformin compared to the group that received only STZ.

Hyperglycemia increases AGEs formation and also induces RAGEs hyperexpression[15]. Interactions between AGEs and their cellular receptors RAGE play an important role in the pathogenesis of diabetes complications (neurophaty, micro and macro vascular complications). AGE and RAGE are present in ovary [16]. In some inflammatory diseases, the level of RAGE is also higher [17]. The most important intracellular signaling pathways activated after RAGE stimulation are MAPK / ERK, and NF-κB. As a consequence of NF-kB activation, there is an increase in ROS formation, but also an increased synthesis of proinflammatory interleukins (IL-6 and IL-1) and TNF[18].

Magnesium is required for insulin metabolism [19].A higher magnesium intake reduces risk of disturbances of glucose and insulin metabolism and development of diabetes mellitus. Dietary magnesium intake improves insulin resistance[20] and plays a role in reduction of diabetic lesions in various organs [21].The present data are in agreement with those showing that reducing RAGEs expression is an important way to decrease the tissue diabetic damages[22].The RAGE have become a target for the development of future antidibetic therapy[23].

The obtained results are consistent with the data showing that ROS synthesis, inflammatory response to AGEs and expression of RAGE in other cells are also reduced by this oral antidiabetic drug[24].

Metformin and other drugs which reduced the RAGEs expression has had a protective action against renal damages in dibetes mellitus. Our obtained data show that metformin significantly reduced also RAGE expression in ovary.

In our research, achievable magnesium concentrations in humans in clinical practice significantly reduced the ovary RAGEs expression in nonpregnant female rats with experimental diabetes mellitus. The reduction of RAGE expression is a way for magnesium protective effect against diabetes damages in ovary. Other ways for magnesium protective effect against the lesions induced by diabetes are : reducing the formation of ROS, decreased NF-kB activation after RAGE stimulation [25]and reducing TNF alpha formation.

Conclusions

Magnesium and metformin significantly reduce ovarian RAGEs expression in experimental diabetes. We hypothesize that the association of magnesium with metformin may be beneficial in reducing the diabetes effects on ovaries.

Limitation

An important limitation of this study is that it was not possible to determine the plasma concentration of AGE.

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