Influence of β-Resorcylicidene Aminoguanidine on Selected Metabolic Parameters and Antioxidant Status of Rats with Diabetes Mellitus

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Summary
We studied the effects of administration of β-resorcylicidene aminoguanidine (RAG) to Wistar strain rats with experimental diabetes mellitus (DM) induced by streptozotocin. The effects studied included antioxidant levels in plasma and the liver, oxidative damage of lipids represented by the formation of substances reacting with thiobarbituric acid (TBARP) and selected biochemical indicators. The administration of RAG did not significantly affect antioxidant status of diabetic rats or hemoglobin glycation and plasma concentration of fructosamine. In diabetic rats, application of RAG decreased formation of TBARP in plasma but not in the liver. Moderate steatosis of liver and increased plasma levels of triacylglycerols in diabetic rats were significantly improved by application of RAG.

Key words
Diabetes mellitus • β-resorcylicidene aminoguanidine • Lipid peroxidation • Antioxidants

Introduction
Diabetes mellitus (DM) is a chronic metabolic condition characterized by disorder of glucose homeostasis. Numerous experimental and clinical observations have indicated that hyperglycemia may directly or indirectly contribute to an increased formation of free radicals (FR) and consequently to the onset of oxidative stress. Oxidation stress is a condition of imbalance due to excess formation of free radicals and decreased activity of antioxidant defense systems (Aruoma 1998, Ďuračková 1999). Increased formation of FR in Type 1 and Type 2 diabetes mellitus can be a risk factor of the disease. It occurs as a result of two processes: i) decreased activity of the body antioxidant systems (Muchová et al. 1999); ii) auto-oxidation of reducing saccharides and formation of adducts with proteins. Important processes in these reactions are glycation (Baynes and Thorpe 1999), glyoxidation and formation of advanced glycation end-products (AGE products, Oberley 1988, Čársky 1999). Increased oxidation stress and glycation of proteins are processes that can result in tissue damage and play an important...

Antioxidant levels in the blood and tissues are an important factor of sensitivity of individual tissues to oxidation stress (Ďuračková 1998, Baynes and Thorpe 1999). The exact role of oxidation stress in the initiation and progression of DM is not known in sufficient detail yet. Alterations in activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, induce changes in oxygen metabolism. So far, diverse data on activities of these enzymes have been reported in the literature. Similarly, there are diverse data on levels of low molecular weight antioxidants in diabetics, e.g. \( \beta \)-tocopherol, ascorbic acid, and glutathione. There are differences in the reactions of individual tissues to oxidative attack. Greater resistance to lipid peroxidation was observed in diabetic kidneys, heart and liver in comparison to the controls (Parinandi et al. 1990, Sukalski et al. 1993).

Along with a variety of antioxidants, numerous substances and drugs inhibiting oxidative stress and follow-up processes have been studied. Structurally and functionally, this is a diverse group of substances. The mechanism of their action is, as a rule, poorly understood. Generally, there are several types of inhibitors of diabetes development. These include, among others, substances (a) regulating Amadori product formation, (b) regulating formation of AGE products, (c) stimulating degradation of AGE products, and (d) influencing oxidation stress (use of antioxidants).

Aminoguanidine (AG) is an intensively studied inhibitor and is expected to act by a combination of two mechanisms – by its own antioxidant effect and by blocking of reactive o xo-groups and scavenging dicarboxyl intermediates formed in glycoxidation processes (Giardino et al. 1998). Thereby, AG inhibits development of follow-up processes producing AGE products. The disadvantages of AG are its toxic effect and, in certain conditions, its potential pro-oxidation effect (Skamarauskas et al. 1996). Therefore, the use of its Schiff base with resorcylic aldehyde – \( \beta \)-resorcylic aminoguanidine (RAG, Fig. 1), has been studied (Ćársky et al. 1978). In previous studies, RAG was found to regulate increased transmembrane potential, induced by alterations of surface charge of erythrocyte membranes in DM, and to decrease the fluidity of these membranes (Waculíková et al. 2000), as well as the fluidity of the myocardium sarcolema in vivo (Ziegelhöffer et al. 1997). It was found to have anti-mutagenic and bacteriostatic activity. Chelate-forming properties of RAG (Onuska et al. 1996) can take part in binding transition metal ions, catalyzing glycoxidation processes and formation of free radicals.

The mechanism of RAG action is not understood precisely. It is known to decrease the formation of AGE products that may take part in the development of post-diabetic complications. In vivo experiments confirmed anti-oxidation properties of RAG (Ďuračková et al., unpublished results).

The present study was aimed at determining the influence of RAG administration on the levels of antioxidants in plasma and in liver homogenate, on membrane lipid damage (formation of TBARP products), and on selected biochemical parameters in rats with artificially induced insulin-dependent DM.

**Methods**

**Chemicals**

Hexane, thiobarbituric acid (Merck, Germany), methanol (Fluka, Switzerland), luminol, \( 1,1,3,3 \)-tetraethoxypropane, streptozotocin, superoxide dismutase (Sigma, USA), trolox (Aldrich, Germany), ACL, ACW and SOD sets (F.A.T., Germany), heparin (Mercle, Germany), insulin MONO ID (Léčiva, Czech Republic). Resorcylic aminoguanidine was synthesized according to Čársky et al. (1978). All other chemicals used were from Lachema (Brno, Czech Republic) and were p.a. grade. Solutions were prepared in re-distilled water.

![β-resorcylic aldehyde](https://example.com/fig1.png)

**Fig. 1. β-resorcylic aldehyde**

**Experimental animals**

Male Wistar rats with weight of 280 – 350 g were used in the experiments. The animals were fed with standard Larsen food and had ad-libitum access to food and fresh water. The rats were divided into four groups:
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(1) Control group (C) – healthy animals that received fresh water by a stomach probe (10 ml.kg⁻¹) and physiological solution (0.5 ml.kg⁻¹) subcutaneously.
(2) RAG control group (R) – healthy animals that were given RAG in a physiological solution in a dose of 10 mg.kg⁻¹ by stomach probe, once daily for 8 weeks.
(3) Diabetic group (D) – animals with induced DM received fresh water by stomach probe (10 ml.kg⁻¹).
(4) Treated group (D+R) – animals with induced DM that received RAG in a physiological solution in a dose of 10 mg.kg⁻¹ by stomach probe, once daily for 8 weeks.

Diabetes mellitus was induced by administration of a single dose of streptozotocin (60 mg.kg⁻¹ in 0.5 mol.L⁻¹ citrate buffer, pH 4.5) into the tail vein. Insulin MONO ID in doses of 12 U.kg⁻¹ was administered daily to both diabetic groups (D and D+R) subcutaneously for 8 weeks.

National regulations for breeding and use of laboratory animals were observed in handling of the rats.

Methods

Plasma was taken from heparinized blood (25 U/ml). Liver homogenate (10 %) was prepared in physiological solution. The homogenate was centrifuged for 15 min at 18 000 x g (Janetzki, Germany). The supernatant was further centrifuged for 1 h at 100 000 x g (Himac CP 70, Hitachi, Japan). The resulting supernatant was used for further analysis.

The plasma and supernatant homogenate for determination of antioxidants and substances, reacting with thiobarbituric acid (TBARP) were put in a freezer (VXE 380, Jouan, Czech Republic) at –80 °C prior to further use.

Total proteins were determined in the supernatant according to Lowry et al. (1951).

TBARP were determined by a modification of the method of Uchiyama and Mihara (1978). 1,1,3,3-tetraethoxypropane was used as a standard. The results are expressed in µmoles of TBARP per liter of plasma or, in the homogenate, in µmoles of TBARP per gram of proteins.

Low molecular weight non-enzyme antioxidants, soluble in lipids (ACL), whose main component is β-tocopherol, were determined by the photochemiluminescence method of Popov and Lewin (1996). Antioxidant potential of ACL relates to trolox (T) and is expressed in µmoles of trolox per liter of plasma, or, in the homogenate, in µmoles of trolox per gram of proteins.

Water-soluble antioxidants (ACW) were determined by a photochemiluminescence method according to Popov and Lewin (1994). Their levels are expressed in µmoles of trolox per liter of plasma, or, in the homogenate, in µmoles of trolox per gram of proteins. Activity of Cu/Zn superoxide dismutase was determined by a photochemiluminescence method using luminol for formation and detection of superoxide (Popov et al. 1987). Bovine erythrocyte SOD was used as a standard.

Fig. 2. Levels of lipid soluble antioxidants (ACL) in plasma (A) and liver (B) of rats (n = 8 - 12). C - control group, R - RAG control group, D - diabetic group, D+R - animals with induced DM, which received RAG, T - trolox. Values are means ± S.E.M. Marginally significant differences from controls: * p = 0.06
Fig. 3. Levels of water soluble antioxidants (ACW) in plasma (A) and liver (B) of rats (n = 8 - 12). C - control group, R - RAG control group, D - diabetic group, D+R - animals with induced DM, which received RAG, T - trolox. Values are mean ± S.E.M. Significant differences from controls: * p < 0.01, ** p < 0.001.

Fig. 4. Formation of TBARP in plasma (A) and liver (B) of rats (n = 8 - 12). C - control group, R - RAG control group, D - diabetic group, D+R - animals with induced DM, which received RAG. Values are means ± S.E.M. Significant differences from controls: * p < 0.001. Difference D - D+R: o p < 0.001.

For photochemiluminescence determinations, we used the ACL, ACW and SOD sets and the PHOTOCHEM photochemiluminometer (F.A.T., Berlin, Germany).

Plasma levels of glucose, uric acid, cholesterol and triacylglycerols were determined by standard biochemical procedures, using the Hitachi 911 automatic analyzer (Roche, Switzerland). Cholesterol level in the liver tissue was determined according to Bálint (1962), and triacylglycerols according to Jover (1963). Glycated hemoglobin was determined according to Flückinger and Winterhalter (1976), and fructosamine according to Johnson et al. (1982).
Statistical significance of the observed effects was assessed using the Student t-test. Values are expressed as means ± S.E.M.

**Results**

The total antioxidant capacities of plasma and tissues depend on levels of known as well as of yet unknown antioxidants. At present, measurement of the total antioxidant potential is preferred to measurements of its individual constituents (Elangovan et al. 2000). This is considered a more appropriate approximation for studies of oxidation stress induced by hyperglycemia. In our work, we studied total low molecular weight antioxidants, soluble in water and in plasma lipids, and those in rat liver (Figs. 2 and 3).

In the plasma of diabetic rats, ACL level is marginally increased (111 %) compared to the controls. This can be attributed to induction of these antioxidants due to increased oxidation stress (Fig. 2). ACL levels in plasma and liver of diabetic rats are statistically significantly lower than in the controls (75.1 % and 73.6 %, respectively, Fig. 3). This indicates depletion of these substances in oxidation processes. The results indicate that individual antioxidants do not react to oxidation stress in the same manner, which can most likely be attributed to the nature of oxidants formed. Lipid-soluble and water-soluble antioxidants react differently to diabetes induced in rats.

RAG administered to diabetic rats did not significantly influence ACL and ACW levels in plasma or in the liver. The results obtained indicate that RAG in concentrations, in which it was administered to rats, did not significantly alter anti-oxidation balance, most likely because its antioxidant properties are not manifested under the given conditions.

We found that levels of SOD – a macromolecular antioxidant – are not significantly increased in diabetic rats (Table 1). RAG administered to diabetic rats induced a significant decrease of SOD levels in the liver – by 13 % in comparison to RAG control group (p < 0.05). No statistically significant differences in levels of SOD were found between diabetic rats with (D+R group) and without RAG (D group).

Formation of TBARP in the plasma of diabetic rats is significantly increased (121 %) with respect to controls (Fig. 4). Comparison of RAG control group (R) with the control group (C) shows that RAG statistically significantly decreased formation of TBARP in the RAG control group. Similarly, there is a statistically significant decrease of TBARP formation in the diabetic RAG group (D+R) compared to the diabetic group (D) (by 31 %). Detailed inhibitory mechanism of TBARP formation by RAG is not known yet. RAG did not statistically influence formation of RAG in the liver (Fig. 4).

**Table 1.** Selected biochemical parameters in the plasma and liver of rats (n = 8 - 18).

<table>
<thead>
<tr>
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<th>C</th>
<th>R</th>
<th>D</th>
<th>D+R</th>
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<tbody>
<tr>
<td><strong>Blood:</strong></td>
<td></td>
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<tr>
<td>Glucose (mmol/l)</td>
<td>7.71±0.16</td>
<td>7.50±0.08</td>
<td>34.50±2.92*</td>
<td>27.88±1.41**</td>
</tr>
<tr>
<td>Glycated Hb (%)</td>
<td>5.46±0.14</td>
<td>5.23±0.14</td>
<td>8.30±0.29**</td>
<td>7.71±0.21**</td>
</tr>
<tr>
<td>Fructosamine (mmol/l)</td>
<td>0.78±0.01</td>
<td>0.75±0.01</td>
<td>1.03±0.07**</td>
<td>1.14±0.05**</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>34.83±1.70</td>
<td>31.03±3.47</td>
<td>50.55±4.01**</td>
<td>41.71±3.24*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.79±0.07</td>
<td>1.83±0.15</td>
<td>2.17±0.12**</td>
<td>2.27±0.08**</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>1.58±0.08</td>
<td>1.51±0.12</td>
<td>4.56±0.57**</td>
<td>2.91±0.43**</td>
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<tr>
<td><strong>Liver:</strong></td>
<td></td>
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<tr>
<td>Cholesterol (µmol/g)</td>
<td>5.66±0.15</td>
<td>5.67±0.21</td>
<td>5.95±0.23</td>
<td>5.86±0.30</td>
</tr>
<tr>
<td>TAG (µmol/g)</td>
<td>11.86±0.71</td>
<td>14.46±1.42</td>
<td>26.44±5.12**</td>
<td>15.07±1.02**</td>
</tr>
<tr>
<td>SOD (mg/g)</td>
<td>19.28±4.99</td>
<td>21.60±2.09</td>
<td>20.29±2.57</td>
<td>18.83±3.15</td>
</tr>
</tbody>
</table>

C - control group, R - RAG control group, D - diabetic group, D+R - animals with induced DM, which received RAG, Hb - hemoglobin, TAG - triacylglycerols, SOD - superoxide dismutase. Values are means ± S.E.M. Significant differences from controls: * p < 0.05, ** p < 0.001. Difference D - D+R: * p < 0.05
Discussion

Several authors performed similar studies; however, their results were different. Elangovan et al. (2000) used cyclic voltametry and found a decrease in levels of water and lipid soluble low molecular weight antioxidants in plasma, but also in lungs, heart, and brain of diabetic rats. In the liver of rats with DM, these authors found an increased level of lipid soluble antioxidants. They determined damage of lipids in cell membranes based on conjugated diens. They found increased levels of conjugated diens in plasma and other organs with the exception of the liver. These authors conclude that diabetic liver is more resistant to oxidation stress, maybe due to increased levels of liver β-tocopherol. Sukalski et al. (1993) found that mitochondria from the liver of diabetic rats are more resistant to lipid peroxidation. Activity of SOD in liver of diabetic rats was decreased to 82% of that in control group, in contrast to our findings. The authors also found decreased activity of other antioxidant enzymes, glutathione reductase and glutathione peroxidase. Ascorbic acid, an important water-soluble scavenger of oxygen radicals, was significantly decreased in the liver, in accordance with our findings. However, the concentration of β-tocopherol was found to be 7 times higher in diabetic rats, in contrast to our study, where we found no significant differences in ACL levels.

The liver has an important function in maintaining glucose levels within physiological limits. In contrast to muscles and fatty tissue, insulin does not directly regulate the uptake of glucose by the liver. Insulin takes part in regulation of glucose metabolism in the liver by stimulating glycolysis, glycogen synthesis, and inhibition of gluconeogenesis.

Induction of artificial DM resulted in a significant increase of glucose levels in plasma (Table 1). At high glucose concentrations in blood, non-enzymatic glycation of proteins takes place along with glucose autooxidation (Jaing et al. 1990, Wolff et al. 1991, Čársky 1999, Muchová 1999). In diabetic rats, we also found a significant increase in glycation of hemoglobin and increased concentrations of glycated proteins (fructosamine) in plasma (Table 1).

In Type I diabetes mellitus, more or less severe lack of insulin induces qualitative and quantitative alterations of lipid and lipoprotein parameters. Fatty acids are increasingly taken up by the liver and, after esterification with glycerol phosphate, they are deposited as triacylglycerols. Thereby, diabetic liver steatosis is developed (Brixová 1981).

In the conditions of our experiment, we observed alterations in lipid metabolism of diabetic rats, as manifested by increased plasma levels of cholesterol and triacylglycerols (Table 1). There was a significant increase in levels of triacylglycerols in liver tissue, being a mark of moderate liver steatosis. There was also an increase in the level of uric acid in plasma of diabetic rats, compared to controls.

The administration of RAG had no significant effect on levels of glycated hemoglobin and fructosamine. It decreased concentration of triacylglycerols in plasma and in liver. Liver steatosis did not develop in diabetic rats, which had received RAG.

Uric acid is likely participant in the process of oxidation stress induced by diabetes. It is a matter of further studies to find whether anti-oxidation or pro-oxidation properties of uric acid operate in these processes.

In conclusion, we found that administration of RAG to diabetic rats induced a moderate decrease of glucose level in plasma and of triacylglycerols both in plasma and in liver tissue. As no significant effects of RAG administration on levels of antioxidants (ACL, ACW, SOD) were found, we conclude that the observed inhibition effect of RAG on the formation of TBARP was due to other mechanisms than direct anti-oxidation effect of RAG.

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References


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