Department of Medical Chemistry[1], Biochemistry and Clinical Biochemistry, Laboratory of Pharmacochemistry[2], III Clinic of Internal Medicine, Faculty of Medicine, Comenius University, Department of Biophysics and Chemical Physics[3], Faculty of Mathematics, Physics and Informatics, Comenius University and Department of Clinical Biochemistry and Hematology[4], State Hospital, Bratislava, Slovak Republic

Influence of 2,5-dihydroxybenzylidene aminoguanidine on lipid oxidative damage and on antioxidant levels in model diabetes mellitus

P. Korytár[1], M. Sivoňová[1], A. Maruniaková[1], O. Uličná[2], P. Kvasnička[3], A. Liptáková[1], P. Božek[4], J. Čársky[1], Z. Ďuračková[1]

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Prof. Zdeňka Ďuračková, PhD., Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Sasinkova 2, 813 72 Bratislava, Slovakia
zdenka.durackova@fmed.uniba.sk

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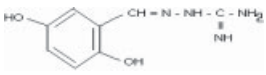
2,5-Dihydroxybenzylidene aminoguanidine (BAG) is a structural analogue of the antidiabetic compound aminoguanidine, and is an example of a substance protecting diabetic rats from lipoprotein oxidation arising in oxidative stress conditions characteristic of diabetes mellitus. We found that administration of BAG to diabetic rats decreases their susceptibility to lipoprotein oxidation, decreases formation of conjugated dienes and 4-hydroxy-2-nonenal, and increases antioxidant potential of plasma. On the other hand, our results show that BAG has a negative influence on lipoprotein oxidation in control rats. Increased formation of 4-hydroxy-2-nonenal and conjugated dienes and a decrease in plasma antioxidant potential was observed when BAG was administered to control rats. It is therefore necessary to search for other structural modifications of this substance that would combine higher antidiabetic activity with less toxicity in healthy individuals.

1. Introduction

Chronic hyperglycaemia in diabetics leads to alterations in the chemistry of glucose oxidation and thereby to an increased production of free radicals and reactive oxygen species, and to onset of oxidative stress. Free radicals derived from oxygen are generated mostly in auto-oxidation reactions of glucose and glycosylated proteins [1]. Elevated formation rates of oxidative damage end-products of lipids [2, 3], proteins [4, 5], and DNA [6, 7, 8] were found in the blood and tissues of diabetic patients, while antioxidant levels were found to be decreased [9, 10]. Oxidative stress plays an important role in ethiopathogenesis of diabetes and is a key player in onset and development of diabetic complications. These can be caused by a variety of mechanisms and develop at different levels. The mechanism that causes elevation of oxidative stress includes, along with non-enzyme glycation and auto-oxidative glycosylation, also metabolic stress, status of antioxidant protection systems and local tissue damage [1, 4, 11, 12].

Aminoguanidine (AG), a substance with considerable affinity to aldehydes, prevents alteration of apolipoprotein B arising from LDL oxidation by endothelial cells or copper ions. Aminoguanidine is supposed to act by antioxidant mechanism and also by blocking reactive oxo-groups or scavenging dicarbonyl intermediates arising in glycooxidation processes [13]. Thereby it inhibits follow-up processes resulting in formation of advanced glycation end-products (AGEs). Due to certain toxic effects and prooxidative effect in some circumstances, aminoguanidine is by itself problematic from the point of view of therapeutic application [14]. Therefore, a number of Schiff's base derivatives of AG were synthesised. One of them is the Schiff's base with resorcylidene aldehyde – β-resorcylidene aminoguanidine, RAG [15], that inhibits AGE production [16, 17], and displays antioxidant effects [18]. Some pro-oxidative effects were observed when the derivative was administered to control rats, so another derivative – 2,5-dihydroxybenzylidene aminoguanidine (BAG) – was synthesised.



2,5-Dihydroxybenzylidene aminoguanidine (BAG)

One of the manifestations of oxidative stress is oxidation of lipoproteins, which means oxidative damage of polyunsaturated fatty acids (PUFA). The more unsaturated a PUFA is, the more it is susceptible to oxidative damage. Oxidation of lipoproteins is a chain reaction initiated by free radicals. Biomembrane lipids and other lipoproteins are particularly susceptible to lipoperoxidation depending on the content of higher unsaturated carboxylic acids with two or more double bounds. A variety of cell types can be affected by oxidation and consequently take part in the development of atherosclerotic lesions (endothelial cells, unstriped muscle cells, monocytes, macrophagues, neutrophils, lymphocytes, fibroblasts). Cell damage and often cell death is caused by a variety of lipid peroxidation mechanisms initiated by free radicals and reactive oxygen

species (ROS) that are formed in the organism with diabetes mellitus and are insufficiently scavenged. Action of ROS on membrane lipoproteins and polyunsaturated fatty acids gives rise to various oxidation products, in particular, aldehydes, such as malondialedehyde (MDA) or 4-hydroxy-2-nonenal (4HNE), and other 4-hydroxyalkenals (HAKs) with various chain lengths. These aldehydic molecules are considered to be the ultimate mediators of toxic effects arising from oxidative damage of biological substrates. Through blood vessels, these products can reach other organs and tissues where they induce cell and tissue damage [19]. Besides that, ROS also react with proteins containing thiol groups and cause inactivation and fragmentation of such proteins. Alteration of nitrogen bases of nucleic acids and splitting of DNA strands is followed by DNA interlinking and tissue degeneration [11]. In contrast to short-lived free radicals, the relatively long-lived, lipid soluble aldehydes may be able to migrate from the place of origin through cell membranes to remote locations where they affect intracellular and extracellular targets. 4HNE and other aldehydes are designated as toxic messengers, capable to mediate oxidative damage of various molecules [20]. Intracellular level of 4HNE is a result of equilibrium between its formation and metabolic degradation. Fast and efficient metabolism of 4HNE in specific types of cells can lead to complete removal of the substance and thereby to protection of cells against damage. On the other hand, incomplete removal can result in considerable intracellular damage inflicted by 4HNE. 4HNE is subject to nucleophilic addition to the sulphur atom in cystein, to ε-amino group of lysine, and to the nitrogen of the imidazole ring [20–23].

High reactivity of 4HNE can make the molecule react with GSH, low molecular weight thiols and various macromolecules such as proteins or DNA. Binding of 4HNE to cellular proteins can give rise to various Schiff's base structures, cross bonds and Michael adducts by reaction with specific amino acid units. These reactions, especially those concerning –SH groups, combined with GSH depletion are very likely the basis of cytotoxic effects. Depletion of GSH changes redox state of cells, which may cause the onset and development of carcinogenesis [24–27]. 4-Hydroxy-2-nonenal can also react with amino groups of phospholipids, such as phosphatidylethanolamine, and thereby alter their properties. Hydroxyalkenals can also inhibit DNA reparative enzymes [28].

Antioxidant levels in blood and tissues significantly influence susceptibility of various tissues to oxidative stress. Antioxidants can inhibit or eliminate negative effects of oxidants. For characterisation of oxidative stress, it is not sufficient to know only oxidative damage products; levels of individual antioxidants should also be studied [19, 29].

This study was aimed at determination of the effect of the structural analogue of aminoguanidine, 2,5-dihydroxybenzylidene aminoguanidine (BAG), on production of selected oxidative damage products, as well as the antioxidant status in rats with diabetes mellitus induced by streptozotocin.

2. Investigations and results

Susceptibility of lipoproteins to oxidation was determined via determination of conjugated dienes. Lipid oxidation kinetics is shown in Fig. 1, which can be characterised by the following parameters: (**i**) slope of the curve, indicating lipid oxidation rate and rate of production of conjugated dienes; (**ii**) start time of lipoprotein oxidation (t_{ox}), indicating susceptibility of the sample to oxidation.

Fig. 2A shows the influence of BAG on oxidation of plasma lipoproteins in diabetic and control rats. Differences in time t_{ox} (seconds) are shown for individual groups (C, DIA, C + BAG, DIA + BAG). Time t_{ox} is a time to start of lipoprotein oxidation, i.e., a characteristic of their susceptibility to oxidative attack. Lower values of the time t_{ox} indicate early start of lipid oxidation and higher susceptibility of lipoproteins to oxidation.

In diabetic rats (DIA), time t_{ox} is significantly reduced compared to control rats (C) ($p = 0.022$). Administration of BAG to diabetic rats significantly delays the start of oxidation to values close to those found in control rats ($p = 0.031$) (Fig. 2A). However, administration of BAG decreases time t_{ox} in control rats, indicating increased susceptibility to oxidative attack with respect to control rats not treated by BAG (C) ($p = 0.049$, Fig. 2A).

4HNE was determined by HPLC. A typical chromatogram, showing results of 4HNE determination, is shown in Fig. 3.

Fig. 2B shows relative plasma levels of 4HNE in rats of individual groups (C, DIA, C + BAG, DIA + BAG). The 4HNE level was significantly higher in diabetic rats compared to control rats ($p = 0.046$). Administration of BAG markedly decreased ($p = 0.077$) 4HNE levels in diabetic rats (Fig. 2B). However, administration of BAG to control rats leads to an insignificant increase in 4HNE level compared to the C group. Fig. 2C shows values of antioxidant capacity of hydrophilic antioxidant components of plasma for individual groups (C, DIA, C + BAG, DIA + BAG) determined by ACW test.

We found a significant decrease ($p = 0.009$) of antioxidant capacity of hydrophilic antioxidant of plasma (ACW) in diabetic rats with respect to the control group. Similarly to results obtained from determinations of lipoprotein oxidation and 4HNE, administration of BAG to control rats induced negative reaction of the organism. Administration of BAG to control animals lead to a decrease of plasma ACW ($p = 3.14 \times 10^{-7}$). On the other hand, administration of BAG to diabetic rats increased plasma antioxidant capacity to levels close to those found in control rats ($p = 0.05$).

Fig. 2D shows values of antioxidant capacity of lipophilic antioxidant components of plasma (ACL) for individual groups (C, DIA, C + BAG, DIA + BAG). From Fig. 2D it is apparent that a significantly increased ACL capacity was found in plasma of diabetic rats compared to control ($p = 0.003$). This is most likely due to stimulation of pro-

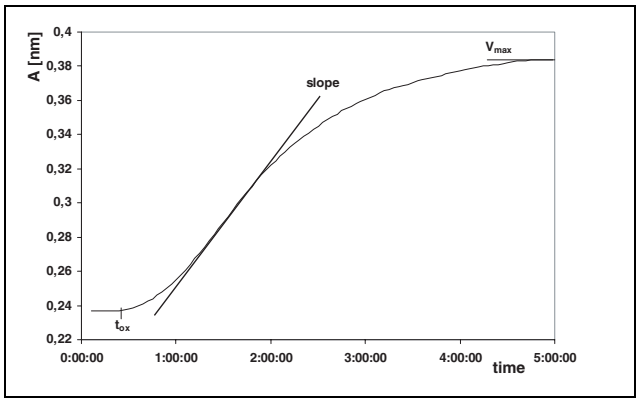


Fig. 1: Characteristic time course (h) of formation of conjugated dienes expressed by absorbance change at 245 nm in oxidation of lipoproteins induced by copper ions

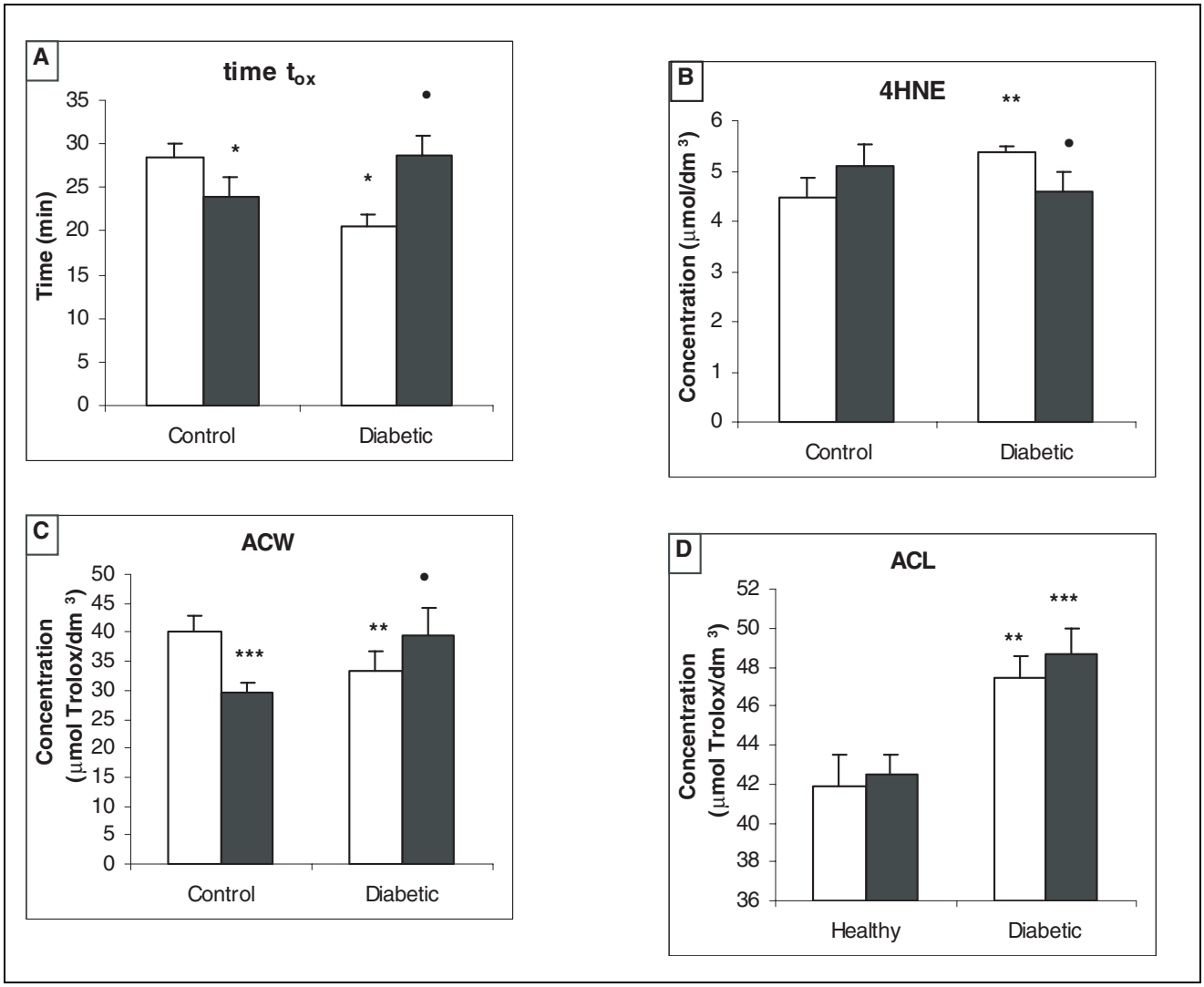


Fig. 2: Parameters characterised oxidative stress in diabetic and control rats without treatment with BAG (empty bars) and after treatment with BAG (filled bars). The details of experiments are given in chapters 4.1 and 4.3. A, time t_{ox} is characterising the start of lipoprotein oxidation (λ_{245}; duration of record 5 h). B, concentration of 4HNE in plasma of rats. C, antioxidant potential of water soluble antioxidants (ACW) of rat plasma. D, antioxidant potential of lipid soluble antioxidants (ACL) of rat plasma.
* = significant difference relative to control (C) ($0.01 < p < 0.05$);
** = significant difference relative to control (C) ($0.001 < p < 0.01$);
*** = significant difference relative to control (C) ($p < 0.001$);
• = significant difference relative to DIA group ($0.01 < p < 0.05$))

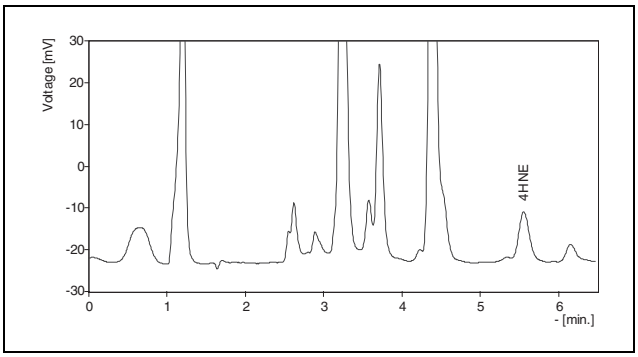


Fig. 3: Chromatography record from 4HNE determination. HPLC conditions: 250 × 4 mm Nucleosil 120–5 C18 column (Watrex); mobile phase of acetonitril: distilled water (70 : 30); flow rate of 1 ml/min; injection 20 µl; detection on a UV detector (DeltaChrome UVD 200) at 355 nm; 4-hydroxy-2-nonenal was employed as an internal standard. Standard area response was linear over the 4HNE concentration range 5–75 µmol/l

duction of antioxidants by a prolonged oxidative stress and due to their later regeneration. We also found that administration of BAG to diabetic rats significantly increases antioxidant capacity of ACL ($p = 9.7 \times 10^{-4}$).
The Table gives an overview of biochemical parameters of plasma for individual groups of rats. The values are reported as average of experimental values (n = 6 to 8) ± standard error of measurement.

Table: Average levels of glucose, cholesterol, TAG and urate in plasma of rats

Groups	Glucose (mmol/l)	Cholesterol (mmol/l)	TAG (mmol/l)	Urate (µmol/l)
Control	7.19 ± 0.36	1.07 ± 0.10	0.79 ± 0.10	48.65 ± 3.05
C + BAG	7.41 ± 0.35	1.24 ± 0.05	0.62 ± 0.10	45.3 ± 3.51
DIA	20.97 ± 1.04	1.56 ± 0.09	1.58 ± 0.28	57.04 ± 1.05
DIA + BAG	19.21 ± 4.11	1.57 ± 0.09	1.06 ± 0.19	46.96 ± 2.56

Values are reported as average ± standard error of measurement, n = 6 to 8

In diabetic rats, increased glucose levels of nearly 200% were found. Upon administration of BAG to diabetic rats, a slight and statistically insignificant decrease of plasma glucose was detected. In control rats, BAG had no influence on plasma glucose levels (Table). We found significant increased cholesterol levels in diabetic rats ($p = 0.086$). After BAG administration to diabetic rats cholesterol in plasma was not changed. In diabetic rats we found increased levels of triacylglycerols (TAG). Administration of BAG to diabetic rats marginally ($p = 0.07$) decreased TAG levels. Because hyperuricaemia is associated with diabetes, we also monitored levels of urate in the plasma of rats. In diabetic rats, we found a significantly increased urate level ($p = 0.013$). Administration of BAG to the controls had insignificant effect on urate levels. On the other hand, administration of BAG to diabetic rats was accompanied by a significant decrease ($p = 0.003$) of urate levels down to those of the controls (Table).

3. Discussion

One of the manifestations of oxidative stress associated with diabetes mellitus is an increased lipoprotein oxidation. LDL particles are a sensitive substrate for oxidative damage, which are functionally and structurally altered by oxidation. Both lipid and protein components of LDL undergo oxidative damage.

The resulting lipid oxidation in non-fractionated plasma depends also on other plasma components such as hydrophilic and lipophilic antioxidants and a relatively high concentration of albumin. Albumin acts against oxidation by binding transition metal ions, such as Cu^{2+} [30]. Cu^{2+} ions cause oxidation of lipids (in the presence of peroxides present in plasma) and generation of conjugated dienes. LDL oxidation also depends on the type of oxidants active during oxidative stress. If there is lack of physiological antioxidants, the impact of oxidative attack can be ameliorated by, for example, endogenously administered antioxidants.

Our results on lipid oxidative damage indicate that from the point of prevention against lipid oxidation and production of 4HNE, BAG is a potentially suitable substance that could prevent these negative effects in diabetes mellitus. This would help to avoid significant damage caused by lipid oxidation, specifically, cell membrane damage. It could also help to prevent oxidation of LDL lipid particles, which otherwise leads to deposition of lipids on the surface of blood vessels and formation of atherosclerotic plaques often encountered in patients suffering from this disease. However, the observed stimulation of 4HNE formation by BAG in control subjects predetermines further toxicology studies.

Determination of antioxidant capacity of plasma correlates with the results of determination of lipid oxidation and 4HNE. Decrease of antioxidant capacity of hydrophilic antioxidants of plasma indicates a decreased antioxidant protection of organism against ROS, accompanied by increased formation of oxidative stress biomarkers. This corresponds with our finding that in diabetic rats 4HNE levels are increased, and lipoprotein oxidation parameters are worse, i.e., there are shorter times to start of lipoprotein oxidation and an increased rate of production of conjugated dienes.

The role of urate in diabetic rats is unknown. Hyperuricaemia in diabetes mellitus seems to be associated with the insulin-resistant syndrome and with early onset or increased progression of nephropathy [35], with cardiovascullar mortality [36], and with risk factor for stroke [37]. Increased levels of urate in diabetic rats could be a result of induction of its production caused by increased oxidative stress. A similar induction of lipophilic antioxidants (ACL) by oxidative stress in diabetic animals was seen. The administration of BAG to control rats did not influence the level of urate. However, the administration of BAG to diabetic rats adjusted the level of urate to that of control rats. From this follows that the administration of BAG to diabetic rats may contribute to the inhibition of oxidative stress in diabetic organism.

So far, BAG has been at the stage of basic research. Studies of its effects should not be limited to a single parameter, but should comprise overall effects on an organism from the point of view of metabolism, compare advantages and disadvantages, determine its pharmaco-kinetic properties and toxicity. Although BAG administration causes inhibition of products of oxidative damage to lipids and TAG level in diabetic animals and control animals BAG apparently increases oxidability of lipids, other aminoguanidine derivatives should be searched which do not exert such negative influence of BAG on non-diabetic individuals. The knowledge acquired in studies with BAG could be found useful in the design of a new potential antidiabetical drug.

4. Experimental

4.1. Animals

We used Wistar rats, males with weight of 280–350 g. They were fed with the standard Larsen diet. Animals had the free access to the food and drinking water. We divided rats into four groups.

Control group (C) – control animals with administered water into their stomach (5 ml/kg) by the sounder. The NaCl solution ($c = 0.15$ mol/l) was applied subcutaneously (0.5 ml/kg).

Control group (C + BAG) – control animals with administered BAG in NaCl solution ($c = 0.15$ mol/l) into their stomach (5 mg/kg) once a day during eight weeks.

Experimental group (DIA) – animals with evoked DM. Drinking water (5 ml/kg) was administered into their stomach.

Experimental group (DIA + BAG) – animals with DM. BAG in NaCl solution ($c = 0.15$ mol/l) (5mg/kg) once a day during eight weeks was applied into their stomach by the sounder.

DM in both groups (DIA, DIA + BAG) was elicited by the single-dose administration of streptozotocine (60 mg/kg in 0.5 mol/l citrate buffer, pH 4.5) into tail's vein. Insulin MONO ID at the dosage 12 U/kg was injected subcutaneously to both diabetics groups every day during eight weeks. During the rat handling the national rules and instructions for the rearing the laboratory animals were followed.

4.2. Chemicals

4-Hydroxy-2-nonenal, luminol, steptozotocine (Sigma, Germany); HPLC-acetonitrile, hexane (Merck, Germany); HPLC-methanol (Fluka, Germany); heparin (Mercle, Germany), 2,4-dinitrophenylhydrazine (Lachema, Czech Republic), insulin MONO ID (Léčiva, Czech Republic), ACW, ACL sets (Analytikjena AG, Jena, Germany). All other chemicals were obtained from Lachema (Brno, Czech Republic) and were of analytical grade. Solutions were prepared in redistilled water. 2,5-dihydroxybenzylidene aminoguanidine (BAG) was synthesised by the condensation of water-alcoholic solution of aminoguanidine (AG) and 2,5-dihydroxybenzaldehyde.

4.3. Methods

Plasma was obtained from heparinised blood (25 U/ml). Liver homogenate (10%) was prepared in NaCl solution ($c = 0.15$ mol/l). Plasma samples were stored in deep freezer (VXE 380, Jouan) at $-80\,^{\circ}C$.

4.3.1. Oxidation of plasma lipoproteins

Oxidation of plasma lipoproteins was determined according to Schnitzer et al. [31]. Reaction mixture (2 ml) contained: 20 µl of plasma, $CuCl_2 \cdot 2\,H_2O$ (1×10^{-4} mol/l), 3.3×10^{-3} mol/l phosphate buffer (pH 7.4). Production of conjugated dienes was monitored through changes in absorbance at 245 nm during 300 min at 37 °C (Biochrom 4060, Pharmacia).

4.3.2. Determination of 4HNE

4HNE was determined according to Kinter [32]. 250 µl NaCl solution, c = 0.15 mol/l was added to 250 µl of blood plasma and 500 µl DNPH (5 mmol/l). After intense stirring on a vortex, the reaction mixture was left at rest in the dark for 1 h at room temperature. The reaction mixture was extracted three times against 1 ml of hexane. Collected extracts were evaporated under argon at 40 °C. 70% acetonitrile in distilled water was added to the evaporate. The sample processed in this manner was analysed using HPLC. We used a 250 × 4 mm Nucleosil 120-5 C18 column (Watrex), with mobile phase of acetonitrile : distilled water (70 : 30), flow rate of 1 ml/min, injection 20 µl, detection on a UV detector (DeltaChrome UVD 200) at 355 nm. 4-Hydroxy-2-nonenal was employed as an internal standard. Standard area response was linear within the 4HNE concentration range 5–75 µmol/l (y = 125.4x), where y = peak area and x = 4HNE concentration. The 4HNE concentration was quantified by comparing the peak area of sample with values from the standard curve. The recovery of three different concentrations of added 4HNE to the sample averaged 94%. The mean coefficient of variation for analysis of 9 replicate plasma samples was 4.8%.

4.3.3. Determination of water soluble antioxidants (ACW)

Water soluble plasma antioxidants were determined by ACW set (Analytikjena AG, Jena, Germany) using a luminol-dependent photochemiluminescence method according to Popov and Lewin [33]. Their method is based on a photo-induced, chemiluminescence-accompanied, and antioxidant-inhibitable autooxidation of luminol. The assay mixture (2.5 ml) consisted of 0.1 mol/l carbonate buffer, pH 10.6, 0.1 mmol/l Na_2EDTA, 30 µmol/l luminol and plasma or standard solution of trolox (20 µl). Activity of water soluble plasma antioxidants was compared with activity of trolox and expressed in µmol trolox per 1 l of plasma. Photochemiluminometer PHOTOCHEM (Analytical, Jena, Germany) was used for photochemiluminescence determinations.

4.3.4. Determination of lipophilic antioxidants (ACL)

Low molecular weight non-enzyme lipid soluble antioxidants were determined by ACL set (Analytikjena AG, Jena, Germany) with photochemiluminescence method according to Popov and Lewin [34]. The method is based on an antioxidant-sensitive inhibition of photo-induced chemiluminescence accompanied autooxidation of luminol. The lipid extraction from blood plasma was performed: 200 µl of plasma was mixed with 200 µl of H_2O and 400 µl of ethanol. After adding 800 µl of hexan, the sample was shaken for 1 min and centrifuged for 5 min at 1000 × g. 200 µl of hexan phase was dried under argon. The extract was dissolved in methanol and lipid soluble antioxidants were determined with ACL set. Antioxidant capacity of ACL was related to that of trolox and expressed in µmol trolox per liter of plasma. Photochemiluminometer PHOTOCHEM (Analytical, Jena, Germany) was used for photochemiluminescence determinations.
Concentrations of glucose, urate and triacylglycerols were determined by standard biochemical procedures using an automatic Hitachi 911 analyser (Roche, Switzerland). TRACE sets (Sydney, Australia) were used for the spectrophotometric determination of these compounds.
The results were statistically evaluated using t-test where applicable. Values are reported as average ± standard error of measurement.

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