Original Article

ANTI-AGE ANTIBODIES AND MARKERS OF OXIDATIVE STRESS IN SPONTANEOUSLY HYPERTENSIVE RATS AT DIFFERENT AGES

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Summary

The aim of this study was to investigate anti-AGE antibodies (AGE Abs) and two markers of oxidative stress, copper (Cu) and iron (Fe) in sera of spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) at 2, 4 and 8 months. AGE Abs were assessed with direct enzyme-linked immunosorbent assay (ELISA). Significant differences were observed between AGE Abs in 4 and 2 month old SHR ($p < 0.001$) and between 8 and 4 month old SHR ($p<0.001$). There was a positive correlation between the levels of AGE Abs in SHR and WKY and age ($r = 0.636$; $p = 0.005$), ($r = 0.420$; $p = 0.015$). The same relation was observed for the serum content of iron and copper in both SHR and WKY. The increase in levels of AGE Abs is directly connected with the increase of advanced glycated end-products with age and their relation with oxidative stress. The evidence for activated processes of oxidative stress is confirmed from the elevation of the serum concentrations of the iron and copper. Iron and copper take part in maintaining the oxidative balance in the organism, and the elevation of their serum concentrations is significant in pathogenicity of hypertension and aging.

Key words: advanced glycation end products, anti-AGE antibodies, copper, iron, spontaneously hypertensive rats

Introduction

Advanced glycation end-products (AGEs) form as a result of non-enzymatic reactions, in which glucose forms adducts with proteins, lipids and nucleic acids. AGEs accumulate naturally as a result of chronological ageing but this process is greatly accelerated under conditions of hyperglycemia and oxidative stress [**1**]. AGEs act directly, as well as via specific receptors, to alter the function of many intra- and extracellular proteins, including antioxidant and metabolic enzymes, calcium channels, lipoproteins, transcription and structural proteins. The accumulation of endstage products of the Maillard reaction alters the structural properties of tissue proteins and reduces their susceptibility to catabolism [2]. AGEs accumulate progressively in the vasculature with ageing, thus reducing the elasticity of myocardium and large arteries. The aorta stiffens and the result is systolic hypertension

[3]. By binding to RAGE (specific AGE receptors) on endothelial cells, AGEs induce a signaling cascade involving nuclear factor kappa beta (NF-kB) that increases the production of reactive oxygen species [4]. Oxidative stress is important in the pathogenesis of hypertension [5].

The formation of AGEs cross-links changes the molecules of long-lived structures by intermolecular cross-linking and side-chain modifications, in this way changing their antigenicity [6]. The immune system registers these modified own structures as non-self and reacts with an antibody production. Glycated proteins form common immunological epitopes, which results in the formation of populations of anti-AGE antibodies (AGE Abs). It has been established that AGEs have an antigenic similarity, regardless of the protein, on which they are formed [7]. Anti-AGE antibodies are found in the serum of healthy human subjects as a part of the homeostatic mechanism which clears altered structures via *in situ* destruction, or via opsonization. Excessive accumulation of AGEs with age and in pathology appears to correlate with elevated levels of AGE Abs [6].

AGEs accumulate in the vessel wall, where they may perturb cell structure and function. AGEs have been implicated in both microvascular and macrovascular complications of diabetes. In the vasculature, the formation of atherosclerotic plaques involves AGEs cross-links between endothelial proteins and oxidized LDL cholesterol, which itself is likely to be modified by glycosylation into advanced lipoxidation products (ALEs) [8]. AGEs may modify the extra cellular matrix (ECM), modify the action of hormones, cytokines, and free radicals by engagin cell surface receptors; thus influencing the function of intracellular proteins [9-12].

Recent studies on the role of trace elements in the pathogenesis of several diseases have been gaining importance. Zinc, copper and iron play a crucial role in the oxidant/antioxidant mechanism, the imbalance of which leads to increased susceptibility to oxidative tissue damage, thereby leading to pathological changes in conditions such as diabetes mellitus, cancer, hypertension, etc. [13]. While trace amounts of these elements are needed physiologically, at increased levels they are harmful. Increased concentrations of iron and copper generate hydroxyl radicals from the superoxide anion, which in turn damages proteins, lipids and DNA [14].

AGE Abs and their connection with oxidative stress markers have not as yet been studied in hypertension. Thus, the present study was designed to check the hypothesis that increased levels of AGE Abs, and elevated levels of iron (Fe) and copper (Cu) as markers for oxidative stress, are in positive correlation with increasing age and hypertension. We therefore investigated the presence of antibodies against AGEs, Fe and Cu in sera of spontaneously hypertensive rats (SHR) and their normotensive controls – Wistar Kyoto Rats (WKY) at 2, 4 and 8 months of age.

Materials and Methods

Subjects

Male Wistar Kyoto Rats (WKY, n=24) and male Spontaneously Hypertensive Rats (SHR, n=22) were used. Animals were born and raised under conventional conditions in the animal facility of the Medical University of Pleven and were allowed free access to tap water and a standard laboratory chow. Animals were housed and kept under a normal 12h light/dark cycle at 22 º± 2ºC. They were divided into 6 groups: 2mSHR (SHR, 2 months of age, n=8); 4mSHR (SHR, 4 months of age, n= 7); 8mSHR (SHR, 8 months of age, n=7); 2mWKY (WKY, 2 months of age, n=7); 4mSHR (WKY, 4 months of age, n= 7); 8mWKY (WKY, 8 months of age, n=10). Our experimental design was approved by the Animal Care and Use of Laboratory Animals section of the Ethical Committee of the University, based on the principles described in the Guide for the Care and Use of Laboratory Animals [15].

Blood Collection

At the end of the 2nd, 4th and 8th month, following overnight fasting, the abdominal cavity of the rats was opened under pentobarbitone sodium anesthesia (26 mg/kg body weight, i.p.). Blood was collected from the bifurcation of the aorta and was put at 37˚ C to clot. After separation, 0.02% NaN_3 was added to each serum sample and stored at -20˚C prior to use.

Glycation of KLH for antigen

Keyhole Limpets Hemocyanin (KLH) (Sigma, 20mg/ml) was glycated *in vitro* with 3.33 M glucose in 0.4 M phosphate buffer, pH 7.5 with preservative 0.04% sodium azide, at 37˚C, for 12 weeks. The formation of advanced glycated end products of KLH (AGE-KLH) was quantified by

measuring the fluorescence at 360/440 nm excitation/emission. The AGE-KLH this obtained was used as antigen in direct ELISA.

Measurement of AGE Abs

A direct ELISA method was used to detect AGE Abs in rat sera as follows: Microtiter 96 well plates (Greiner Microlon) were coated with 500 ng per well of AGE-KLH and dissolved in bicarbonate buffer (pH 9.6). The plates were incubated for 2 hours at room temperature and then overnight at 4˚ C to allow for complete binding. Then the plates were washed three times with phosphate buffered saline, containing 0.05% Tween 20 (PBS-Tween), before blocking with 0.1% bovine serum albumin (BSA; Sigma Aldrich) and incubating at 37˚ C for one hour. The next step was addition of 100 µl of tested rat sera diluted 1:5 in PBS-Tween. Then peroxidate conjugated anti-rat IgG (Sigma), diluted 1:2500 in 5% human serum albumin (Maimex, Bulgaria) was added for 1 hour at 37° C. After each step wells were washed three times with PBS-Tween. Ortho-phenilenediamine (0.8 mg/ml) dissolved in 0.05 M citrate buffer, pH 5.0, plus 0.01% H_2O_2 was used as the colorimetric substrate. The reaction was stopped with 50 µl 8N H_2SO_4. The optical density (OD) of the samples was measured at 492 nm on an automatic micro-ELISA plate reader. Each sample was analysed three times as described above.

Methods for determination of Cu and Fe

Cu and Fe were determined in serum by routine clinical methods - endpoint colorimetric test from Giesse Diagnostics, and ferene photometric test (Horiba ABX, France), respectively.

Blood pressure determinations

Systolic blood pressure (SBP) was determined by tail cuff plethysmography (Blood Pressure Recorder, Ugo Basile, Italy) as described previously [16]. Conscious rats were placed on a heated pad in a temperature-controlled quiet room. After a 15-min rest with the tail placed inside a tail cuff, the cuff was inflated three to four times to condition the animal to the procedure. Three consecutive measurements were then taken for statistical analyses.

Statistical Analysis

The SHR groups were compared to the control WKY groups by one-way analysis of variance (ANOVA) and LSD test (Least Significant Difference) using the SPSS v.13 software. Data are expressed as mean ± SEM. Differences between SHR and WKY controls were considered significant for *P* values less than 0.05.

Results

The findings for AGE Abs showed no significant differences between SHR and WKY at 2 months (Fig. 1). In the 4mSHR group, the levels of AGE Abs were significantly higher than in 4mWKY (P=0.001). Significant differences were observed between antibody levels in 4mSHR, as compared to 2mSHR (P<0.001) and in 8mSHR, when compared to 4mSHR (P<0.001). In WKY rats there was no significant difference between 2mWKY and 4mWKY groups. Only WKY at 2 and 8 months differed significantly (P=0.017). In the 8 month old rats, the level of AGE Abs in SHR was significantly higher than in WKY (P <0.001). The levels of AGE Abs in SHR and WKY correlated positively with age ($r = 0.636$, $p = 0.005$), ($r = 0.420$, $p = 0.015$).

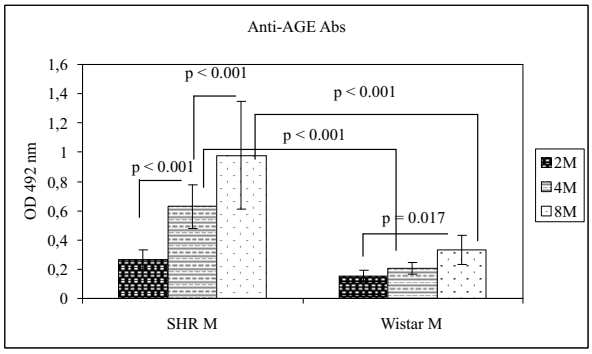

Figure 1. Levels of anti-AGE antibodies in SHR at different age and their age-matched normotensive controls (WKY). Data are reported as mean ± SEM

The data for serum concentration of Fe in all investigated groups are summarized in Fig.2. The 8mSHR group had a significantly increased concentration of serum Fe, as compared to the 2mSHR ($P = 0.013$) and the 4mSHR ($P = 0.011$) groups. In WKY, the serum Fe concentration was increased in the 8mWKY, as compared to the 2mWKY ($P = 0.002$) and the 4mWKY ($P = 0.023$) groups. There were no significant differences between SHR and age-matched WKY. A significant correlation between the serum concentration of Fe and age was observed for the SHR animals ($r = 0.274$, $p = 0.022$).

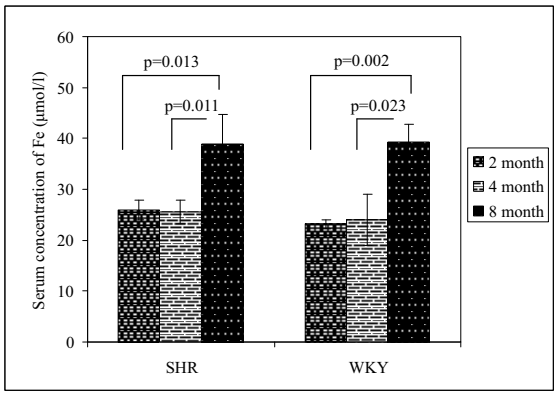

Figure 2. Serum concentration of Fe in SHR and WKY. Data are reported as mean ± SEM

The highest serum Cu concentration was found in SHR at 8 mounts, as compared to animals at 4 and 2 months of age ($P < 0$ .05). There was no significant differences between the WKY at different ages, but the serum Cu concentration in 8mSHR was increased significantly in comparison with 8mWKY (Fig. 3). The serum Cu concentration was found to correlate significantly with age for the SHR animals ($r = 0.314, p = 0.009$).

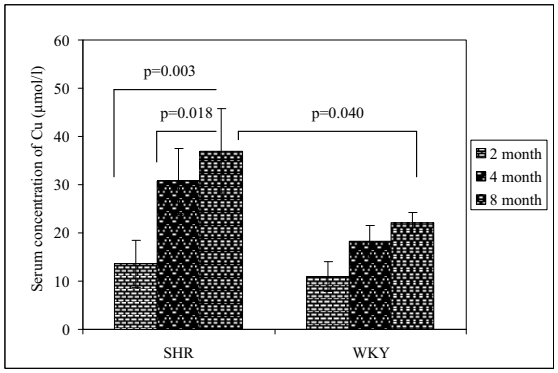

Figure 3. Serum concentration of Cu in SHR and WKY. Data are reported as mean ± SEM

Table 1 summarizes the data on the systolic blood pressure (SBP) of SHR and WKY. At the beginning of the experiment, systolic blood pressure was significantly increased in SHR compared with WKY rats. SHR showed a progressively increasing systolic blood pressure with age, reaching significance in the 8 month group ($p<0.001$), compared to SBP of animals from younger groups. There were no significant differences in the WKY groups at different ages.

Table 1. Systolic blood pressure in SHR and WKY rats

Groups	Systolic blood pressure (mm Hg)		
	Month 2	Month 4	Month 8
SHR	($n = 8$)	($n = 7$)	($n = 7$)
	162.1 ± 2.31	188.17 ± 3.65[a]	191.8 ± 2.65[b]
WKY	($n = 7$)	($n = 7$)	($n = 10$)
	115.23 ± 3.32	125.56 ± 2.37	127.49 ± 1.79

Data are presented as mean ± SEM. [a] $P < 0.05$, [b] $P < 0.001$; [a] - significant difference between 2mSHR and 4mSHR; [b] - significant difference between 2mSHR and 8mSHR.

Discussion

Hypertension is characterized by insulin resistance, and a number of studies have suggested that it plays a major role in its etiology [17]. The study of DeLano & Schmid-Schönbein [18] states emphatically that in hypertensive rats, proteases cleave extracellular portions of several protein receptors, such as the insulin receptor, so that insulin can no longer bind and facilitate normal metabolism of glucose. In insulin resistance, alterations in glucose and lipid turnover lead to the production of excess AGEs [19-20]. AGEs act directly, as well as via specific receptors. AGEs receptors (RAGE) are discovered on almost all cell types [21] and their activation induce intracellular signaling that increases oxidative stress and the production of pro-inflammatory and pro-sclerotic cytokines [22]. This leads to endothelial dysfunction, inflammation and oxidative stress. All these changes are major features of hypertension.

We did not find significant differences in AGE Abs levels between SHR and WKY rats at 2 months of age. At about two months of age, alterations occur in the arterial walls and hypertension develops in the SHR rats. The increased elastin content in the aortic wall of SHR becomes expressed as an increased number and/or greater thickness of elastic laminae [23]. Increased elastin and collagen in the vessel wall [24] and vascular wall hypertrophy also play an important role in hypertension reducing tissue flexibility and elasticity [25]. During the early phases of hypertension development, wall

elasticity might even be augmented in the SHR rats but long-term hypertension decreases arterial elasticity [26]. These structural and functional changes alter the antigenicity and immunogenicity of vascular elements, thus provoking the immune system to react with production of different types of antibodies including AGE Abs.

In our study, AGE Abs were presented in all the investigated samples/sera and their levels were in positive correlation with increasing age of the animals. We observed significantly higher levels of AGE Abs in 8-month-old SHR and WKY rats, as compared to the other age groups.

Age-related changes in AGE Abs were found in SHR on different selenium diets [27].

AGE Abs in the 4- and 8-month-old SHR rats were significantly higher than in the WKY at the same age. The presence of AGE Abs in sera of WKY could be explained by the fact that non-enzymatic glycation of proteins had already started to stimulate antibody production. These antibodies are probably part of normal homeostasis of the organism and play regulatory roles. In hypertension or age-related conditions, when non-enzymatic glycation of proteins is increased, the capacity of normal homeostasis seems to be inefficient. This may contribute to the accumulation of AGEs, and stimulate the production of AGE Abs.

To address the connection between AGE Abs and the oxidative status of the investigated animals, we tested the serum levels of Fe and Cu as markers for oxidative stress. Transition metals such as copper, zinc and iron are cofactors for intra- and extracellular oxidases, oxygenases and dismutases. These are preventive antioxidants which eliminate species that are involved in radical chain processes [28]. We found increased serum Fe and Cu concentrations in SHR and WKY rats with ageing. "Free" metal ions are good catalysts, and this can lead to biological damage. Thus free copper and iron accelerate autoxidation reactions, and both can react with H_2O_2. Iron is a redox active metal that can catalyze the formation of highly reactive hydroxyl radicals from H_2O_2 and decompose lipid peroxides to peroxyl and alkoxyl radicals, which favor the propagation of lipid oxidation. The toxicity of iron is related to its involvement in producing oxidant damage [29-33]. Copper is a redox-active metal that is able to catalyze the formation of hydroxyl radicals via a Haber-Weiss or Fenton-like reaction [34-36]. In addition, copper can be toxic by directly binding to sulfhydryl groups in proteins, which results in enzyme inactivation or altered protein conformation [37]. At cellular level, the pathologic effects that are associated with the accumulation of excess copper are consistent with oxidative damage of lipids, proteins and nucleic acids [38]. Copper supplementation is associated with inflammation and increased markers of oxidative stress in adults [39]. High concentration of copper and iron in sera cause distinct oxidative effects. Oxidation damaged cell structures are more susceptible to the influence of free glucose and non-enzymatic formation of early and late products of glycation.

Conclusions

The role of AGE Abs in the mechanism of hypertention is not clear. Insulin resistance and increased oxidative stress are features associated with hypertension. One direct consequence of insulin resistance is accumulation of AGEs in all structures, particularly in vasculature. Both increased AGEs and oxidative stress lead to endothelial dysfunction, which in turn is the basis for decreased production of NO and the corresponding contribution of decreased vasodilatation to increased hypertension. The results of our study confirm that AGE Abs, and Fe and Cu as markers for oxidative stress, are in positive correlation with increasing age and hypertension. Further investigations are necessary to establish the role of AGE Abs and their impact on pathogenic mechanisms of hypertension and ageing.

Acknowledgments

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