ADVANCED GLYcation END-PRODUCTS IN PATIENTS WITH CHRONIC ALCOHOL MISUSE

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Abstract — Aims: The aim of our study was to determine serum levels of advanced glycation end-products (AGE) in patients with chronic alcohol misuse and to examine their relationship to markers of nutrition and inflammation. Methods: The study group consisted of 23 heavy alcohol drinkers treated for chronic alcohol misuse and 22 healthy controls. Studied parameters included AGE (fluorescence, CML – carboxymethyllysine and pentosidine), lipids, glucose, albumin, leptin, prealbumin, C-reactive protein (CRP) and pregnancy-associated plasma protein A (PAPP-A). Results: AGE fluorescence was significantly higher in chronic alcoholic patients than in healthy subjects (4.3 ± 0.7 × 10^7 vs 3.7 ± 0.5 × 10^7 AU/g protein, P < 0.005), while CML was only slightly but not significantly elevated (569.1 ± 106.6 vs 545.5 ± 85.8 μg/l) and pentosidine levels did not differ (105.4 ± 29 vs 102.2 ± 23 nmol/l). In alcoholics, AGE correlate significantly negatively with leptin (r = −0.46, P < 0.05) and pentosidine with prealbumin (r = −0.43, P < 0.05), otherwise there was no relationship between AGE and other biochemical parameters (glucose, cholesterol, albumin, CRP, PAPP-A). Conclusion: Our findings suggest a more complex relationship among advanced glycation, oxidative stress and metabolism of ethanol and their link to nutrition and nutrition-associated parameters. AGE as a result of oxidative stress might be similarly linked to increased cardiovascular risk of heavy alcohol drinkers, as are malnutrition and inflammation; however, further studies are needed to confirm this hypothesis.

INTRODUCTION

Alcohol consumption is associated with cardiovascular morbidity and mortality in a dose-dependent manner. Moderate alcohol intake has protective effects by decreasing coronary heart disease mortality, while excessive alcohol misuse has detrimental effects on the cardiovascular system leading, for example, to cardiomyopathy, coronary heart disease or hypertension and haemorrhagic stroke (Klatsky et al., 1990). Cardiovascular complications are associated with structural changes of vessel walls and myocardium. Modification of biological structures may be influenced by oxidative and carbonyl stress as well as by the actual metabolic situation, inflammation and nutritional effects.

Advanced glycation (glycoxidation) end products (AGE), similar to other oxidative and carbonyl stress end products [advanced lipoperoxidation end products (ALE) and advanced oxidation protein products (AOPP)], take part in the pathogenesis of many chronic diseases and their complications, for example diabetes mellitus (Vlassara, 1997), cardiovascular complications (Zocalli et al., 2001, Kaneda et al., 2002) and chronic renal failure (Miyata et al., 2000). Advanced glycation end-products are represented by a heterogeneous group of compounds with characteristic pigmentation and fluorescence. Among them, the best known are pentosidine and carboxymethyllysine (CML), the latter, however, is a non-fluorescent compound. AGE modify proteins and thereby change their physical and chemical properties. Accumulation of AGE has several toxic effects.

Apart from direct damage to the structure of the extracellular matrix, they act via specific receptors, for example RAGE. AGE–RAGE interaction activates nuclear factor NF-κB, which is followed by stimulation of transcription of genes for cytokines and growth factors, increased expression of adhesive molecules, increased vascular permeability and further toxic effects (Yan et al., 1994; Bierhaus et al., 1998; Kislinger et al., 1999).

Formation of AGE is influenced by the metabolic situation and by nutritional factors. They can rise via non-enzymatic glycation, which is enhanced in hyperglycaemia and due to oxidative and carbonyl stress (Miyata et al., 2000). Altered liver function can result in accumulation of AGE in the organism due to the role of the liver in their detoxification (Sebeková et al., 2002). Similarly, decreased renal function causes their decreased excretion by the kidney (Makita et al., 1994). Acetaldehyde as a product of ethanol oxidation should prevent formation of AGE (Al-Abed et al., 1999), while oxidative stress accompanying alcohol misuse might enhance it (Sun et al., 2001; Zima et al., 2001).

The aim of our study was to determine serum levels of AGE in alcohol-dependent individuals and to examine their relationship to markers of nutrition and inflammation.

MATERIAL AND METHODS

Study group

The studied group consisted of 23 heavy alcohol drinkers (15 men, eight women, mean age 47 ± 10 years), treated for chronic alcohol misuse. All patients had a history of drinking more than 80 g ethanol per day for 13 ± 8 years, however, there was no acute alcohol intake in the examination period. The study was performed during treatment after at least a 1-month...
detoxification period, when normalization of liver enzymes was achieved. The majority of patients had liver steatosis, none of the studied group suffered from liver cirrhosis or alcoholic hepatitis in the history. All patients were otherwise healthy (no diabetes mellitus nor alteration of liver and renal function) and had no signs of acute infection. By the time of the study, they took neither ethanol nor antioxidants. 22 healthy controls were used for comparison (six men, 16 women, mean age 49 ± 12 years).

The study was approved by the local Institutional Ethical Committee. All subjects gave their informed consent prior to entering this study.

Blood collection occurred in the morning after overnight fasting. Blood was collected via puncture of the cubital vein and centrifuged at 1450 g, 4 °C for 10 min. Serum was stored at −80 °C and analysed within 3 months.

Analytical methods

**AGE assay.** AGE were estimated using a spectrofluorimetric method (excitation 350 nm, emission 435 nm) in serum diluted with phosphate buffer according to Henle (Henle et al., 1999) and Munch (Munch et al., 1997) (spectrofluorimeter Fluoromax-3; Jobin Yvon Horiba, USA) and are expressed in arbitrary units (AU) and in AU/g protein.

**CML assay.** Carboxymethyllysine was determined immunochemically. After digestion of the serum samples with Proteinase K and inactivation of the protease at 80 °C, AGE–CML was determined with ELISA technique using the CML-specific monoclonal antibody 4G9 and calibration with 6-(N-carboxymethylamino)caproic acid (Roche Diagnostics, Penzberg, Germany). Results are expressed in µg/l and in µg/g protein.

**Pentosidine assay.** Pentosidine was determined in acidic hydrolysates by reversed-phase high-performance liquid chromatography (HPLC; Schimadzu, C18) according to Spáček (Spáček and Adam, 2002). We monitored the emission signal at 385 nm upon excitation at 335 nm. The concentration of pentosidine is expressed in nmol/l and nmol/g protein.

**Other parameters.** Leptin and carbohydrate-deficient (CD) transferrin were assessed with standard ELISA kits (leptin, Biovendor, Czech Republic; CD transferrin, Axis, Norway). Pregnancy-associated plasma protein A (PAPP-A), a new marker associated with cardiovascular risk, inflammation and oxidative stress (Bayes-Genis et al., 2001) was determined using the supersensitive immunochemical method TRACE (time resolved amplified cryptate emission) (Kryptor, Brahms, Germany). Other biochemical parameters (glucose, cholesterol and its fractions, triacylglycerols, total protein, albumin, prealbumin, liver enzymes, bilirubin, creatinine, urea, C-reactive protein, haemoglobin) were measured with routine clinical chemistry methods.

Statistics

Results are expressed as means ± standard deviations. The serum concentration of leptin is due to high inter-individual variability described with median and interquartile range. Unpaired t-test, Mann–Whitney U-test (for leptin) and correlation coefficient r (Pearson, Spearman) were used for statistical evaluation. All results were considered as statistically significant at P < 0.05.

RESULTS

Serum concentrations of AGE in alcoholic patients and healthy subjects are shown in Table 1. Table 2 depicts other biochemical parameters in both studied groups.

Advanced glycation end-products were significantly higher in chronic alcoholics than in healthy subjects, while CML was only slightly but not significantly elevated and pentosidine levels did not differ.

In alcoholic patients, AGE correlate significantly negatively with leptin (r = −0.46, P < 0.05; Fig. 1) and pentosidine with prealbumin (r = −0.43, P < 0.05), otherwise there was no relationship between AGE and other biochemical parameters (cholesterol, albumin, C-reactive protein, PAPP-A).

### Table 1. Advanced glycation end-products (AGE) in alcoholic patients and in healthy subjects

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Alcoholic patients</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE (AU)</td>
<td>3.1 ± 0.5 × 10^5*</td>
<td>2.7 ± 0.4 × 10^5</td>
</tr>
<tr>
<td>AGE (AU/g protein)</td>
<td>4.3 ± 0.7 × 10^3**</td>
<td>3.7 ± 0.5 × 10^3</td>
</tr>
<tr>
<td>CML (µg/l)</td>
<td>569.1 ± 106.6</td>
<td>545.5 ± 85.8</td>
</tr>
<tr>
<td>CML (µg/g protein)</td>
<td>7.9 ± 1.3</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>Pentosidine (nmol/l)</td>
<td>105.4 ± 29.0</td>
<td>102.2 ± 23.0</td>
</tr>
<tr>
<td>Pentosidine (ng/g protein)</td>
<td>1.48 ± 0.43</td>
<td>1.39 ± 0.31</td>
</tr>
</tbody>
</table>

All results are expressed as mean ± standard deviation. *P < 0.05, **P < 0.005 alcoholic patients versus healthy subjects.

### Table 2. Biochemical parameters in alcoholic patients and in healthy subjects

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Alcoholic patients</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.9 ± 0.8</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.6 ± 1.0</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.5 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.5 ± 0.8</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/l)</td>
<td>1.3 ± 0.5</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>71.6 ± 3.5</td>
<td>73.7 ± 5.2</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>43.7 ± 2.1</td>
<td>42.8 ± 2.1</td>
</tr>
<tr>
<td>Prealbumin (g/l)</td>
<td>0.22 ± 0.06</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>Aspartate aminotransferase (µkat/l)</td>
<td>0.35 ± 0.25</td>
<td>0.44 ± 0.15</td>
</tr>
<tr>
<td>Alkaline phosphatase (µkat/l)</td>
<td>1.28 ± 0.31</td>
<td>1.13 ± 0.30</td>
</tr>
<tr>
<td>γ-glutamyl transferase (µkat/l)</td>
<td>0.31 ± 0.25</td>
<td>0.42 ± 0.35</td>
</tr>
<tr>
<td>Carbohydrate deficient transferrin (%)</td>
<td>2.1 ± 0.9</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>7.9 ± 2.8</td>
<td>9.3 ± 2.6</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>83.5 ± 10.2</td>
<td>86.7 ± 10.0</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>4.2 ± 1.2</td>
<td>4.8 ± 1.3</td>
</tr>
<tr>
<td>C-reactive protein (g/l)</td>
<td>1.8 ± 1.6</td>
<td>1.8 ± 2.1</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>136 ± 9</td>
<td>138 ± 11.0</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>7.13 (3.86–18.68)</td>
<td>12.93 (8.74–21.48)</td>
</tr>
<tr>
<td>Pregnancy-associated plasma protein A (mIU/l)</td>
<td>11.2 ± 2.3</td>
<td>9.8 ± 3.2</td>
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</tbody>
</table>

Results are expressed as mean ± standard deviation. Serum concentration of leptin is due to high inter-individual variability described with median and interquartile range.
Our results show elevation of some AGE in patients with chronic alcohol misuse and their relationship to nutritional parameters.

**DISCUSSION**

Advanced glycoxidation products are associated with cardiovascular complications, as they accumulate typically in atherosclerotic plaques (Miyata et al., 2000) and are linked to the alteration in the heart geometry (Zocalli et al., 2001).

Concerning ethanol and AGE, beneficial effects have been pointed out so far. In *in-vitro* experiments, acetaldehyde, as a product of alcohol dehydrogenase and the microsomal ethanol oxidizing system reaction, has been shown to block formation of AGE (Al-Abed et al., 1999). Similarly, in a study on rats with diabetes mellitus, ethanol was shown to influence the late phase of glycation (observed as decrease of AGE-haemoglobin without any influence on glycated haemoglobin HbA1c) (Al-Abed et al., 1999). The results of Al-Abed et al.’s study were in line with previous findings describing decreased cardiovascular risk in subjects with moderate alcohol intake.

On the other hand, metabolism of ethanol has been found to be associated with oxidative stress (Sun et al., 2001; Zima et al., 2001), which might enhance formation of AGE (glycoxidation). Alcohol may accelerate oxidative stress directly or indirectly, which can increase cell death, modification of biological structures and tissue damage. Free radicals are formed in chain reactions, with the contribution of cytochromes, as a result of mitochondrial damage, and due to decreased antioxidant defence mechanisms (Sun et al., 2001).

It is probably the dose that decides which effect of alcohol will be predominant. In our study in heavy drinkers with chronic alcohol misuse, we found elevation of fluorescent AGE and a tendency to slightly higher CML levels (a non-fluorescent compound). Lack of significant differences in CML (and pentosidine) levels might be as a result of their low concentration in plasma in both groups and the small number of patients studied. Predominantly fluorescent glycoxidation end products are detected with the fluorescent method. The bulk is made up of crosslinks; pentosidine is in this range only a minor product. (Munch et al., 1997). However, other compounds might also contribute partially to the fluorescence in the examined range, for example acetaldehyde-protein modification (Tuma et al., 1996) or lipoperoxidation products. Elevation of fluorescent compounds in serum (probably accompanied by their accumulation in tissues, thus contributing to their damage) in alcoholics is present even if they stop drinking. This finding could partly explain the enhanced tissue damage and higher cardiovascular risk of ex-drinkers.

Association of AGE with inflammation is not at doubt and has been demonstrated in several *in-vitro* studies. Clinical studies, however, give controversial results, probably due to the complexity of the reactions occurring *in vivo*. We failed to demonstrate any relationship of AGE with markers related to inflammation (CRP and PAPP-A), probably for the reason that our patients were stable.

Several studies have shown the influence of nutrition on serum/plasma levels of AGE. Šebeková, for example, has found higher AGE-levels in long-term vegetarians (Šebeková et al., 2001) due to fructose-induced AGE formation (Krajcovicova-Kudlackova et al., 2002). Elevation of AGE in alcoholic patients might also be explained by the deficiency of vitamins. Vitamin B derivatives pyridoxamine (Khalifah et al., 1999) and benfothiamine (Stracke et al., 2001) and vitamins A, C and E as antioxidants (Miyata et al., 2000) can prevent or diminish formation of AGE.

In addition to the association with nutrition, AGE show a relationship to nutritional parameters. We have observed a negative correlation between AGE and leptin and a negative correlation between pentosidine and prealbumin in chronic alcoholics. The view on leptin levels in alcoholic patients remains controversial, as some investigators describe...
decreased levels (Rojdmark et al., 2001; Santolaria et al., 2003) while others have found an increase (Nicolas et al., 2001; Kiefer et al., 2002). Nicolas describes serum leptin levels as increased in a dose-dependent manner in chronic alcoholism, regardless of nutritional status or the presence of compensated liver disease (Nicolas et al., 2001). Kiefer shows a possible association between alcohol intake, elevation of tumour necrosis factor alpha (TNF-α) and consequent increased secretion of leptin (Kiefer et al., 2002). Similarly, AGE can stimulate the secretion of cytokines including TNF-α via specific receptors (Bierhaus et al., 1998). On the other hand, heavy drinking is usually associated with malnutrition, cachexia and, eventually, acute phase reaction. Despite the positive association of leptin with proinflammatory cytokines in sepsis, for example (Arnalich et al., 1999), in patients with chronic ailments (chronic renal failure) leptin was described as a negative acute-phase protein that correlates negatively with C-reactive protein and positively with albumin (Don et al., 2001) and its low levels were reported in cachectic chronic heart failure patients also (Filippatos et al., 2000). The slight elevation of AGE as a consequence of oxidative stress, tendency to lower prealbumin as marker of malnutrition (and its negative correlation with pentosidin) and variable changes in leptin might reflect this.

Our findings suggest a more complex relationship among advanced glycation, oxidative stress and metabolism of ethanol and their link to nutrition and nutrition-associated parameters. Similar to malnutrition and inflammation, AGE as a result of oxidative stress might be linked to the increased cardiovascular risk of heavy alcohol drinkers; however, further studies are needed to confirm this hypothesis.

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REFERENCES


