ALCOHOL MISUSE INCREASES SERUM ANTIBODIES TO OXIDIZED LDL AND C-REACTIVE PROTEIN
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Abstract — Aims: To clarify the relationship of alcohol consumption with serum antibodies to oxidized low-density lipoprotein (oxLDL) and the inflammation marker C-reactive protein (CRP). Methods: The study population consisted of 280 men with evidence of alcohol misuse by having self-reported alcohol consumption values over 280 g absolute ethanol per week and 250 age-matched moderate drinkers from a population of Finnish men participating in the FINRISK survey study. Serum samples were analysed for antibodies to oxLDL, C-reactive protein (CRP), total cholesterol, HDL-cholesterol, triglycerides, carbohydrate-deficient transferrin (CDT) and gamma-glutamyl transferase (GGT). The characteristics of the top and bottom half of the alcohol misusers, in regard to weekly alcohol consumption, were compared with the controls. Results: Serum antibody titres to oxLDL were higher in the top half and the levels of CRP, HDL-cholesterol, triglycerides, GGT and CDT were elevated in both the top half and the bottom half of the alcohol misusers, compared to controls. Conclusion: We propose that alcohol misuse may result in increased inflammation leading to oxidation of LDL.

INTRODUCTION
Epidemiological studies have demonstrated a U-shaped curve relating alcohol consumption and the incidence of coronary heart disease (CHD), including an inverse association at lower intake levels. It has been shown that reduced CHD mortality is associated to moderate alcohol consumption and increased mortality with heavy drinking (Friedman and Kimball, 1986). One of the most well known, plausible mechanisms leading to the initial inverse association and its lower risk of CHD is an increase in high-density lipoprotein (HDL) produced by regular ethanol consumption (Barboriak et al., 1979).

Alcohol intake may also influence CHD risk through modulation of inflammatory parameters. Moderate alcohol intake is associated with decreased C-reactive protein (CRP) and leukocyte count, but there is an inverse association with heavy drinking (Imhof et al., 2001). Beneficial effects of, especially, red wine on CHD risk have been attributed to polyphenols because of their antioxidant activity (Frankel et al., 1993). This postulated mechanism involves quenching of free radicals leading to decreased oxidative damage of LDL, hence reducing its potential for causing atherosclerosis. However, it is also possible that a predominant pro-oxidant effect of alcohol itself may outweigh any antioxidant effect of beverage polyphenols (de Rijke et al., 1996; van Golde et al., 1999). Oxidized LDL (oxLDL) is immunogenic and induces the formation of autoantibodies, which may be detected in human serum (Yla-Herttuala et al., 1994). The possible association between alcohol intake and serum antibodies to oxLDL has not been studied thoroughly.

We used a well-characterized population of Finnish men with documented alcohol misuse and controls from the FINRISK study to clarify the relationship of alcohol consumption with serum antibodies to oxLDL. The inflammatory marker C-reactive protein (CRP) was also studied.

SUBJECTS AND METHODS

Subjects
The National Public Health Institute of Finland has performed large cross-sectional population surveys, the FINRISK studies, examining the risk factors of coronary artery disease every 5 years beginning in 1972 (Puska et al., 1995). The present study is a substudy of the 1997 FINRISK survey, which had a total sample size of 11 500 (6000 men, 5500 women). The study was conducted in five geographic areas. The participation rate was 71% among men and 76% among women.

The survey included a self-administered questionnaire that was sent to the subjects in advance, including 165 questions about previous and existing diseases, which participants returned to the survey site. The participants’ height, weight and blood pressure were measured using standard procedures, and a venous blood specimen was taken. All subjects had fasted at least 4 h. Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared (m²). The variable ‘number of smokes per day’ was computed by adding up the numbers of filter cigarettes, non-filter cigarettes, self-made cigarettes, pipe-fulls, and cigars smoked on average per day for all subjects who reported having smoked within 1 month prior to the survey.

Quantitative estimation of alcohol intake was carried out by using 12 structured questions to determine the amount and frequency of drinking. The measure for average weekly intake included the following drinks: beer, cider, liquor, long drinks
and wine. The total mean consumption of all alcoholic drinks was used, expressed as grams pure ethanol per week. Quantitative frequency of drinking was calculated on the basis of alcohol consumption during the last week and during the last 2 months, and expressed as g/week.

The study was conducted according to the Helsinki Declaration on Human Experimentation 1975 and was approved by the Ethical Committee of Primary Health Care Clinics in Finland. All participants gave informed consent to the scientific use of the data and samples collected in the study.

Analytical methods

Aliquots of sera were removed and stored at −70°C until analysis in a freezer that was not in daily use. Serum CRP was determined by using the N High Sensitivity CRP assay as recommended by the manufacturer (Dade Behring, Marburg, Germany). Immunonephelometry was performed using BN™ Systems (Dade Behring). The concentrations of the samples were determined versus dilutions of standards of known concentrations.

Autoantibodies against oxLDL were determined as described earlier (Kalela et al., 2002). In short, antigens for this assay included the following: (1) native LDL (natLDL) prepared from the pooled plasma of three donors and protected against oxidation by 0.27 mmol/l EDTA and 20 μmol/l butylated hydroxytoluene (BHT) in phosphate buffered saline (PBS); and (2) oxLDL obtained after 24-h oxidation of the natLDL with 2 μmol/l CuSO₄. For the enzyme-linked immunosorbent assay, half of the wells on a polystyrene plate (Nunc, Roskilde, Denmark) were coated with 50 μl native and the other half with 50 μl copper-oxidized LDL antigen (both at a concentration of 5 μg/ml) in PBS for 16 h at 4°C. After removal of the unbound antigen and washing of the wells, the remaining non-specific binding sites were saturated using 2% human serum albumin in PBS containing 20 μmol/l BHT and 0.27 mmol/l EDTA for 2 h at 4°C. After washing, 50 μl of the serum samples (diluted 1:30) were added to wells coated with natLDL and oxLDL and incubated overnight at 4°C. After incubation the wells were aspirated and washed six times before peroxidase conjugated goat anti-human-IgG monoclonal antibody (Organon, USA no. 55220 Cappel), diluted 1:4000 in buffer (0.27 mmol/l EDTA, 20 μmol/l BHT, 1% HSA, 0.05% Tween in PBS), was added to each well for 4 h at 4°C. After incubation and washing, 50 μl freshly made substrate (0.4 mg/ml o-phenylenediamine (Sigma) and 0.0435% H₂O₂, in 100 mmol/l citrate buffer, pH 5.0) was added and incubated for exactly 4 min at room temperature. The enzyme reaction was terminated by adding 50 μl 2 mol/l H₂SO₄. The optical density (OD) was measured at 492 nm using a microplate reader (Wallac 1420 Victor; Wallac Oy, Turku, Finland). The results were expressed as the mean OD values from duplicate determinations. Autoantibody titre against oxLDL was calculated by subtracting the binding of autoantibodies to natLDL from that to copper-oxidized LDL. This approach reduces the possibility of getting false-positive values due to cross-reactivity with both LDL epitopes.

Total cholesterol and triglycerides were determined from fresh serum samples by an enzymatic method (Roche Diagnostics, Hoffmann-La Roche). HDL cholesterol was measured with the same enzymatic method after precipitation of LDL and VLDL with polyethylene glycol. The blood samples were analysed in the laboratory of the National Public Health Institute in Helsinki, Finland.

Carbohydrate-deficient transferrin (CDT) concentrations in serum samples were analysed by a double antibody kit (CDTect™, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) according to the manufacturer’s instructions. The test is based on anion exchange chromatography and radio-immunoassay, and has a detection limit of 1 U/l and a measuring range of 5–300 U/l. The activity of serum gamma-glutamyl transferase (GGT) was determined according to the recommendations of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1976).

Statistics

The statistical evaluation was performed on a microcomputer using Statistica for Windows version 5.1 (Statsoft, Tulsa, OK, USA). Values of the two groups of alcohol misusers and controls were compared by ANOVA. In case of a significant interaction, the LSD post-hoc test was used. Associations between variables were tested with Spearman’s rank order correlation coefficients. P-values less than 0.05 were considered to be statistically significant. All results are expressed as mean ± SD. Normality of distributions was tested by Kolmogorov–Smirnov goodness of fit test. If the distribution was skewed, parameters were normalized by log transformation.

RESULTS

The calculated quantitative frequency of drinking was used to select an experimental group of 280 men with evidence of alcohol misuse (having values over 280 g absolute ethanol per week; corresponding to 23 standard 12 g drinks/week) and 250 age-matched controls with values under 280 g/week. The men were randomly selected from the total sample population, evenly from different regions. After exclusion of men with CRP values of 10 mg/l or more, indicating acute inflammation, and samples with technical difficulties at analysis, 258 subjects remained in the alcohol misuse group and 236 in the control group. The alcohol misusers were divided into top and bottom halves in regard to weekly alcohol consumption, and compared to the controls.

Table 1 shows the background characteristics, lipids, and inflammatory markers of controls and for men with two degrees of alcohol misuse. Both CDT and GGT are markers of alcohol consumption. The mean values of CDT and GGT were significantly elevated in the bottom top halves of the alcohol misusers compared to controls (P < 0.001 for all), confirming the classification of alcohol consumption found with the structured questionnaire. Compared to controls, the bottom half and top halves of the alcohol misusers had higher BMI (P < 0.05 for both), higher serum levels of HDL-cholesterol (P < 0.01 and P < 0.001, respectively) and higher triglycerides (P < 0.01 and P < 0.05, respectively). Serum total cholesterol level was elevated only in the bottom half of the alcohol misusers (P < 0.01), and smoking was increased only in the top half of the alcohol misusers (P < 0.001), compared to controls. The inflammatory marker CRP was also increased in the bottom and top halves of the alcohol misusers,
Table 1 Background characteristics, lipids, and inflammatory markers of controls and for men with two degrees of alcohol misuse

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls Alcohol &lt; 280 g/week Mean (SD)</th>
<th>Alcohol misuse of 280–383 g/week Mean (SD)</th>
<th>Alcohol misuse of &gt;383 g/week Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>46.6 (11.6)</td>
<td>48.2 (11.9)</td>
<td>46.2 (11.3)</td>
</tr>
<tr>
<td>Alcohol consumption (g/week)</td>
<td>78.4 (71.5)</td>
<td>326.5 (31.6)**</td>
<td>589.3 (259.7)**†</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.4 (3.5)</td>
<td>27.3 (3.9)*</td>
<td>27.4 (4.7)*</td>
</tr>
<tr>
<td>Smoking (smokes/day)</td>
<td>5.1 (11.5)</td>
<td>8.3 (11.4)</td>
<td>11.4 (13.2)**†</td>
</tr>
<tr>
<td>CDT (IU/l)</td>
<td>14.2 (4.6)</td>
<td>19.0 (9.0)**</td>
<td>22.5 (13.1)**†</td>
</tr>
<tr>
<td>GGT (IU/l)</td>
<td>38.0 (31.2)</td>
<td>81.9 (98.4)**</td>
<td>109.9 (242.3)**</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.50 (1.02)</td>
<td>5.87 (1.20)**</td>
<td>5.68 (1.19)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.26 (0.28)</td>
<td>1.38 (0.40)**</td>
<td>1.43 (0.42)**</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.163 (0.069)</td>
<td>0.170 (0.067)</td>
<td>0.183 (0.070)**</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.49 (1.84)</td>
<td>1.88 (1.90)**</td>
<td>2.01 (2.35)**</td>
</tr>
</tbody>
</table>

Statistical significance was determined by ANOVA. CDT, carbohydrate deficient transferin; GGT, gamma glutamyl transferase; OD, optical density; oxLDL ab, autoantibodies to oxidized LDL–autoantibodies to native LDL. *P < 0.05, **P < 0.01, ***P < 0.001 versus controls, †P < 0.05 versus alcohol misuse of 280–383 g/week group.

discussed compared to controls (P < 0.01 and P < 0.05, respectively). Antibody titres to oxLDL were increased in the top half of the alcohol misusers (P < 0.01). The differences for CRP and antibodies to oxLDL remained significant even after adjusting for BMI, smoking, total cholesterol, HDL-cholesterol and triglycerides. Statistically significant differences between the top and bottom halves of the alcohol misusers were found only in smoking and CDT (P < 0.05 for both).

When the subjects with alcohol misuse were pooled and compared to controls, serum antibody titres to oxLDL and levels of the inflammatory marker CRP remained higher in the alcohol misusers than in the controls (0.176 ± 0.069 vs 0.163 ± 0.067 OD units, P < 0.05 and 1.94 ± 2.14 vs 1.49 ± 1.84 mg/l, P < 0.05) (data not shown).

Within the control group, there was a positive correlation between smoking and antibodies to oxLDL (P = 0.003), but no significant correlation for smoking and CRP. No correlations between smoking and antibodies to oxLDL or CRP were found in the alcohol misusers. In the control group, there were only 16 men who were teetotalers. Their mean serum antibody titre to oxLDL was 0.146 ± 0.086 OD units and CRP 0.98 ± 0.96 mg/l, while the corresponding values for the remaining controls were 0.163 ± 0.067 OD units and 1.53 ± 1.87 mg/l, respectively (P = NS between teetotalers and the rest of the control group for both).

**DISCUSSION**

The main finding of the present study is increased titres of oxLDL antibodies in sera of alcohol misusers compared to controls. Oxidative modification of LDL makes it more likely to cause atherosclerosis than its native form (Steinberg et al., 1989) and oxLDL autoantibodies have been suggested to predict myocardial infarction (Puurunen et al., 1994). Previous evidence suggests that excessive ethanol consumption may result in increased oxidative stress with the formation of lipid peroxides and free radicals (van Golde et al., 1999). Our findings are in concert with an earlier study (Vitlala et al., 2000), which reported elevated antibody titres against oxLDL in alcoholic liver disease patients compared to nondrinking controls. In contrast, a recent study has reported an inverse relationship between serum oxLDL antibody titre and daily wine intake in a population of older adults (aged 65–94 years) in Italy (Di Bari et al., 2003). The mean consumption of wine in that study was 176 ml/day, corresponding to 154 g/week absolute ethanol. In our study, the mean age of the subjects was under 50 years, and the controls consumed less than 280 g/week (mean 78 g/week) of absolute ethanol from mixed beverages. Compared to these controls, serum oxLDL antibody titre was elevated only in alcohol misusers, with a consumption of over 380 g/week (mean 589 g/week). It may therefore be that the Italian culture of alcohol consumption by older adults, described by Di Bari et al., represents beneficial cardiovascular antioxidant effects of fairly moderate wine consumption, whereas our population of alcohol misusers approaches the consumption levels where the effect is pro-oxidant.

The postulated mechanisms for the U-shaped correlation between alcohol consumption and the incidence of coronary heart disease mortality (Friedman and Kimball, 1986) also involve other effects on the inflammatory response. In fact, a U-shaped association between alcohol intake and CRP and leukocyte count has also been reported (Imhof et al., 2001). CRP predicts cardiovascular events both in patients with stable angina pectoris and in apparently healthy persons (Thompson et al., 1995; Ridker et al., 1997), through possibly reflecting a local inflammatory state in atherosclerosis. The principal source of CRP has been assumed to be the liver, but it has also been shown that arterial tissue itself produces CRP (Yasojima et al., 2001). In atherosclerotic plaques the major producers are smooth muscle-like cells and macrophages. Our findings indicate that CRP levels begin to increase in men with a weekly ethanol consumption more than considered to represent social drinking. In fact, a recent report (Albert et al., 2003) has revealed that moderate alcohol consumption was associated with lower CRP concentrations than no or occasional alcohol intake, an effect that was independent of alcohol-related effects on lipids. For subjects having no alcohol or less than 1 drink/month, they reported CRP levels of 2.60 mg/l, which is substantially higher than for the social...
drinker controls in our study. Likewise, for subjects taking one to two drinks daily, median CRP levels were 1.80 mg/l (Albert et al., 2003), which is a value in between the CRP levels of our social drinker controls and alcohol misusers. The proportion of teetotalers in our control population was too small to draw conclusions on serum CRP levels and titres of oxLDL antibodies. However, our results are in agreement with earlier findings to suggest that there is indeed increased inflammation with alcohol misuse.

In conclusion, our results suggest that alcohol misuse has a pro-inflammatory effect, reflected by an increase in serum CRP level. We propose that through this effect, excessive ethanol consumption may result in increased oxidative stress leading to oxidation of LDL.

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REFERENCES


