Ethanol suppresses smooth muscle cell proliferation in the postprandial state: a new antiatherosclerotic mechanism of ethanol?

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ABSTRACT Epidemiologic studies suggest that moderate amounts of ethanol may reduce cardiovascular risk. The mechanisms of the alcohol-associated risk reduction are not known exactly. Vascular smooth muscle cell proliferation represents an important phenomenon in the pathogenesis of atherosclerosis. Recently, it was suggested that metabolic changes during the postprandial phase may be important in the pathogenesis of atherosclerosis. Therefore, we evaluated the effect of postprandial plasma with and without ethanol on the proliferation of rat vascular smooth muscle cells. Identical meals containing 1 g fat/kg body wt were given with and without ethanol (38 ± 0.5 g) to eight healthy young men. Blood was drawn hourly during an 8-h postprandial period; the plasma was separated and added to the cell cultures (0.3%, by vol). The proliferative response (DNA synthesis) of these cells was assessed by measuring the incorporation of [methyl-3 H]thymidine. The maximal blood ethanol concentration of 11.5 ± 0.6 mmol/L (x ± SEM) was attained within the first hour. The ingestion of the meal with ethanol led to a 20% reduction in the capacity of postprandial plasma to induce thymidine incorporation into smooth muscle cells compared with the meal without ethanol (P < 0.05). These results suggest that ethanol may reduce cardiovascular risk by modulating vascular muscle cell growth during the postprandial period. Considering the amount of time humans spend in the postprandial state during their lifetimes, these findings may be of great importance in the pathogenesis of atherosclerosis.

SUBJECTS AND METHODS

Subjects

Eight healthy young men were studied. Their mean (±SD) age was 24.4 ± 0.4 y, body weight was 75.6 ± 2.7 kg, and body mass index (in kg/m²) was 23.2 ± 0.7. Their body weight had been stable for the 3 mo before the study. The subjects had normal results from a physical examination and medical history, did not take any drugs, and had a usual ethanol intake of 40 ± 9 g/wk on the basis of a 1-wk dietary recall. All subjects were nonsmokers, except one man who smoked irregularly and who did not smoke during the study period. The study protocol was approved by the Ethics Committee of the Medical Faculty of the University Hospital Zürich and each man gave his written consent before entering the study.

Methods

After an overnight fast of ≥12 h, the men were studied for an 8-h postprandial period on 2 different days, separated by ≥1 wk. On each day the subjects received a test meal containing 1 g fat/kg body wt either with or without ethanol. The sequence of the experiments was in mixed random order. The energy content of the meal was 4137 ± 115 kJ and the proportion of protein, carbohydrates, and lipids of the meal was identical on both test days: 2% (5 ± 0 g), 30% (71 ± 1 g), and 68% (71 ± 3 g), respectively. The fat was given as butter and the carbohydrates as marmalade and zwieback (rusk; Migros Genossenschaftsbund, Zurich). The type of meal given was highly standardized and could be given repeatedly with a minimum of variability. The composition of our meal was the same as in other experiments in which postprandial phenomena were studied (6, 7).
imental day, the men received 0.5 g ethanol/kg body wt; the mean intake of ethanol was 38 ± 0.5 g as a 10% (by vol) water solution. The amount of alcohol given corresponded to about two glasses of wine (400 mL) or three cans of beer (900 mL). On the control day, ie, the day on which no ethanol was consumed, the same meal with an identical volume of normal tap water was given. The meal including the drink had to be ingested within 5 min whereas one-third of the drink was ingested before, one-third during, and one-third after the meal.

On the test day the subjects were admitted to the hospital at 0700 after an overnight fast of ≥12 h. They were placed in a semirecumbent position on a bed and a catheter was inserted into a cubital vein (Venflon; Becton Dickinson, Basel, Switzerland) and two baseline blood samples were drawn, each 15 min apart. The subjects then received their test meal. After the meal, blood was drawn every 30 min for the first 5 h and hourly thereafter. EDTA-treated plasma was immediately analyzed for lipid composition and ethanol. During the test, the subjects were mainly in a semirecumbent position on the bed and were allowed to read, study, or sleep. On both test days, every hour, 200 mL normal tap water was given to the subjects to maintain an adequate hydration state. During the interval between the 2 test days, the subjects were instructed to pursue their usual diet and physical activities. For three days before each admission the volunteers avoided intense physical activity and any alcohol or drug consumption.

Biochemical tests

Plasma triacylglycerols, fatty acids, total cholesterol, HDL cholesterol, HDL$_2$ and HDL$_3$ subfractions, insulin, C-peptide, glucose, and ethanol were measured in the central laboratory of the hospital by using standard techniques.

Determination of smooth muscle cell proliferation

Cell culture

The procedures for isolation and culture of SMCs from normal female rat aortic medial tissue explants (Sprague Dawley, TIF:RALF, 180–200 g; Ciba, Basel, Switzerland) were described in detail elsewhere (8). Cultured cells were fed every other day with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 U penicillin/L, 100 µg streptomycin/L, and 2 mmol L-glutamine/L.

For experiments, cultured SMCs from passages 10–12 were used. Transmission electron micrographs of our cultured vascular SMCs from passage 12 showed the typical synthetic phenotype. The cells were characterized by numerous mitochondria, extended profiles of rough endoplasmic reticulum, and peripheral bundles of actin filaments with typical dense bodies.

In addition, the same rat aortic cell cultures were tested for vimentin, desmin (primary antibodies and anti-mouse Ig FITC; Boehringer Mannheim, Mannheim, Germany), and α-actin (fluorescent phallotoxins; Molecular Probes, Inc, Eugene, OR) by immunohistochemical methods. Cells were grown on glass cover slips until near confluence and processed according to the manufacturer’s instructions. Each marker revealed an intense positive fluorescence staining of a filigree network of the respective filaments.

Measurement of DNA synthesis rate

The proliferative effect of EDTA-treated plasma (0.34 mol K$_3$EDTA/L) was examined according to Nemecek et al (9). Vascular SMCs were seeded in Petri dishes (30 mm diameter) and cultured in DMEM until confluent. The medium was then replaced by a starvation medium consisting of a mixture of DMEM and Ham’s F-10 medium (1:1, by vol) and 1% fetal calf serum. After another 24-h culture, cells were exposed to 0.3% (by vol) test plasma in the same medium. Because of a linear dose response relation between the plasma concentration (range: 0.1–1%) and thymidine incorporation in our model, 3 mL plasma/L cell culture medium was used, as described by Scott-Burden et al (10). After 19 h of incubation, 3 mCi [methyl-3 H]thymidine/L was added. The reaction was terminated 5 h later by aspirating the medium and subjecting the cultures to sequential washes with Dulbecco’s phosphate-buffered saline containing 1 mmol CaCl$_2$/L, 1 mmol MgCl$_2$/L, 10% trichloroacetic acid, and ethanol:diethyl ether (2:1, by vol). Acid-insoluble [3 H]thymidine was extracted into 1 mL of 0.5 mol NaOH/L; 0.2-mL portions of this solution were used for liquid scintillation counting and another 100 µL for determination of protein concentration.

Trypan blue exclusion tests before and after plasma stimulation showed no loss of viable cells during the incubation time. To exclude radiolabeled thymidine incorporation into DNA during DNA repair, the in situ end-labeling technique using terminal deoxynucleotidyl transferase was used (11). No positive fluorescence stainings in the cell nuclei were seen, indicating no precedent DNA fragmentation. Therefore, no radiolabeled thymidine was incorporated as a consequence of DNA repair.

Statistical analysis

All values are expressed as means ± SEMs, except where stated otherwise. The postprandial responses of the different variables were evaluated as absolute values at each time point and as the area under the curve (AUC) for each 1-h period and for the complete 8-h postprandial period (8-h AUC, ie, the sum of each 1-h AUC over the complete 8-h period), according to the trapezoidal rule. The calculations were normalized to the baseline (0 h) level. The results from the control day and the days when ethanol was ingested were compared by standard statistical methods, including the paired t test when applicable. All P values are two tailed. The programs JMP (SAS Institute Inc, Cary, NC) and STATVIEW (Abacus Concepts, Berkeley, CA) were used for the analyses.

RESULTS

SMC proliferation presented as AUCs for each 1-h period is shown in Figure 1. Compared with the control meal, the ethanol meal caused a reduction in SMC proliferation at each time point (ie, each hour). The 8-h AUC for SMC proliferation and triacylglycerol and fatty acid concentrations on the 2 test days is shown in Table 1. The maximal plasma triacylglycerol response occurred 3.2 ± 0.3 h after the ethanol meal and 4.5 ± 0.5 h after the control meal (P < 0.04). The maximal plasma triacylglycerol concentration for the corresponding days was 2.8 ± 0.6 and 3.7 ± 0.6 mmol/L, respectively (P < 0.02).

No ethanol was detected in the blood samples obtained at admission. The peak ethanol concentration after the ethanol meal was 11.5 ± 0.6 mmol/L and occurred within 1 h after ingestion; no more ethanol was detectable in the blood by 6 h after ingestion. There was no relation between the maximal ethanol concentration and SMC proliferation (ie, 8-h AUC) for each subject.
More than 20 y ago Zilversmit (2) postulated that atherosclerosis represents a postprandial phenomenon. During the postprandial period many proatherogenic phenomena do occur (12), some of which may be influenced theoretically by ethanol. Since Zilversmit postulated this hypothesis it has been shown that LDL cholesterol and especially modified LDL cholesterol are potent stimulators of SMC proliferation (13). However, in the present study the plasma cholesterol concentration did not change significantly postprandially.

Much evidence suggests that fatty acids and triacylglycerols may also play an important role in the control of cell growth and proliferation, particularly when they are modified as a result of lipid peroxidation (14–16). Also, in these studies triacylglycerol and fatty acid concentrations increased considerably during the postprandial phase: ethanol induced a greater increase in triacylglycerols than in fatty acids, probably because of the suppressive effect of ethanol on lipid oxidation (17). On the basis of our experiments the exact mechanism of the suppression of SMC proliferation could not be identified; however, several mechanisms are possible. It is conceivable that ethanol affects the structure, composition, or both, of the triacylglycerol molecules, rendering them less bioactive to SMCs. Another possibility would be a decrease in SMC proliferation as a result of decreased fatty acid concentrations. Effects related to metabolites of ethanol degradation should also be considered. Alcohol and acetaldehyde have been reported to slow the cell cycle in hamster ovary cells (18) and in other cell lines and fibroblasts (19–21). Preliminary findings from our laboratory showing a slight suppression of SMC proliferation after the addition of acetaldehyde are consistent with these results (unpublished observations). It is not known whether these phenomena also occur in vivo after alcohol ingestion.

Epidemiologic studies reported a cardioprotective effect of ethanol (3, 4). Some studies suggested that the protective effect may be greater when ethanol is consumed with meals (22). This study supports the latter epidemiologic observation because alcohol suppressed the postprandially increased stimulation of SMC proliferation. Considering the amount of time humans spend in the postprandial state during their lifetime, these findings may be of great importance in the pathogenesis of atherosclerosis.

In the present study the effect of postprandial plasma on vascular SMCs was assessed by using a rat model. SMCs mainly originating from rat aorta are used routinely in our laboratory and in others (23, 24). Whether these results are also applicable to human cell lines is, however, not known. Accordingly, in our preliminary experiments we studied selected biological aspects of vascular SMCs isolated either from rat aorta or human omen-

![FIGURE 1. Time course of smooth muscle cell [3H]thymidine incorporation after a fatty meal with and without (control) ethanol (0.5 g/kg body wt) during an 8-h postprandial period. Data (x ± SEM) are presented as areas under the curve for the corresponding 1-h period.](image)

However, changes in the SMC proliferation rate at each time point paralleled changes in triacylglycerol and fatty acid concentrations in the plasma. However, on the day that the ethanol meal was consumed, despite higher triacylglycerol concentrations, the capacity of the plasma to induce SMC proliferation was significantly lower (P = 0.03). Fatty acid concentrations decreased immediately after meal ingestion but then rose continuously until the end of the observation period. The maximal fatty acid concentration was 1725 ± 243 μmol/L after the ethanol meal and 1929 ± 671 μmol/L after the control meal (P = 0.70). Concentrations of total cholesterol, LDL cholesterol, total HDL cholesterol, and the HDL2 and HDL3 subfractions did not change significantly during the experimental period. Insulin, C-peptide, and glucose concentrations and the changes in concentrations during the postprandial period were not significantly different on the 2 test days.

**DISCUSSION**

This study showed that the stimulatory effect of postprandial plasma on SMC proliferation was reduced significantly by the concomitant ingestion of ethanol (Figure 1). The ingestion of 38 g ethanol (about two glasses of wine (400 mL) or three cans of beer (900 mL)] reduced the AUC of SMC proliferation by 20%.

**TABLE 1**

<table>
<thead>
<tr>
<th>Smooth muscle cell proliferation (cpm · h/μg cell protein)</th>
<th>Control meal</th>
<th>Alcohol meal</th>
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<tbody>
<tr>
<td>Triacylglycerols (mmol · h/L)</td>
<td>803 ± 137</td>
<td>1163 ± 208</td>
</tr>
<tr>
<td>Fatty acids (μmol · h/L)</td>
<td>6843 ± 731</td>
<td>5397 ± 889</td>
</tr>
</tbody>
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\^ x ± SEM, n = 8.

\y Significantly different from the control meal: \^ P = 0.032, \y P = 0.004.
tum majus. These cell lines were incubated with human LDL cholesterol that had been isolated as described previously (25). Human LDL cholesterol was found to increase thymidine incorporation in both cell types by 81% and 102%, respectively. In addition, stimulation with platelet-derived growth factor AB led to a 5–10-fold increase in thymidine incorporation in both cell types. In addition, nitric oxide synthesis upon LDL-cholesterol stimulation was similar in both cell types (26). These observations suggest that the results of the present study are also applicable to human vascular SMCs.

Our observations suggest that alcohol may favorably affect cardiovascular risk by modulating postprandial metabolism. However, these results should be interpreted with caution: 38 g ethanol corresponds to about two glasses of wine, an amount that is considered at the upper limit of the sensible drinking limit for men (27). Furthermore, the data should not be interpreted as promoting alcohol ingestion to reduce the risk of atherogenesis. At present, control of the well-known cardiovascular risk factors remains the cornerstone of the prevention of atherogenesis.

The analysis of DNA repair in vascular SMCs by P Groscurth (Institute of Anatomy, University of Zurich) is greatly acknowledged. We thank K Munz for preparing the electronmicrographs.

REFERENCES