Lipoprotein-Associated Phosphatidylethanol Increases the Plasma Concentration of Vascular Endothelial Growth Factor

Marja K. Liisanantti, Minna L. Hannuksela, Maria E. Rämet, Markku J. Savolainen

Objective—To study whether qualitative changes in high-density lipoprotein (HDL) phospholipids mediate part of the beneficial effects of alcohol on atherosclerosis, we investigated whether phosphatidylethanol (PEth) in HDL particles affects the secretion of vascular endothelial growth factor (VEGF) from endothelial cells.

Methods and Results—PEth increased the secretion of VEGF into the culture medium of EA.hy 926 endothelial cells. The mitogen-activated protein kinase (MAPK) phosphorylation increased by 3.3-fold and protein kinase C (PKC) by 2.2-fold by PEth-containing HDL. Moreover, we showed that intravenous injection of PEth incorporated into HDL particles increased plasma concentration of VEGF by 2.4-fold in rats in vivo. Similar effect was observed when the rats were injected with HDL particles isolated from alcohol drinkers.

Conclusions—HDL particles containing PEth affect endothelial cells by MAPK and PKC signaling. This may mediate the effects of ethanol on the arterial wall by increasing VEGF secretion from endothelial vascular cells. That may explain, at least in part, the beneficial effect of moderate alcohol consumption on atherosclerosis. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: alcohol ■ endothelial cells ■ vascular endothelial growth factor ■ lipoproteins ■ phospholipids

As with plasma high-density lipoprotein (HDL) cholesterol concentration, HDL phospholipid concentration also correlates inversely with the severity of coronary artery disease. In addition, the ability of reconstituted HDL particles to inhibit endothelial cell adhesion molecule expression depends on their phospholipid composition, which also emphasizes the importance of phospholipids for the putative antiatherogenic functions of HDL particles.

Previous epidemiological studies have shown that even light or moderate alcohol consumption has protective effects against coronary heart disease. A large part of the protective effects could be mediated by an increase in HDL cholesterol, but the exact mechanisms are not known. HDL may play a crucial role in reverse cholesterol transport. Some data suggest, however, that alcohol does not enhance reverse cholesterol transport despite its increasing effect on the plasma HDL cholesterol concentration.

HDL may also have other functions unrelated to its role in reverse cholesterol transport, for example, HDL particles seem to be able to regulate the expression or abundance of various growth factors, endothelial nitric oxide synthase, cytokines, and adhesion molecules. Alcohol drinkers have elevated serum levels of adhesion molecules, but the mechanism of increase is not known.

The fact that even light alcohol consumption of 1 drink twice per week protects against atherosclerosis compared with total abstinence is difficult to explain, because such a low level of drinking does not affect the HDL cholesterol concentration. A plausible explanation for the effect of moderate drinking may be provided by our new hypothesis suggesting that the sustained action of occasional drinking is caused by the involvement of lipoproteins as carriers of phosphatidylethanol (PEth).

PEth, structurally an ethanol adduct of phosphatidic acid (a lipid second messenger), is formed in the presence of ethanol and affects cellular metabolism and function. Ethanol itself has been shown to induce in vitro angiogenesis in the cultured endothelial cells by the activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). The present research demonstrates that PEth markedly increases the secretion of vascular endothelial growth factor (VEGF) in cultured endothelial cells and increases its plasma concentration in experimental animals in vivo. This effect of lipoprotein-associated PEth is mediated mainly by MAPK.

Methods

Please see online Methods section at http://atvb.ahajournals.org for further details.

Subjects

Male alcoholic subjects (n=17) referred by general practitioners for withdrawal therapy to the Alcoholism Treatment Unit of Oulu and...
healthy nonalcoholic men (n=17) recruited from among the local paper mill employees were recruited for blood tests. Venous blood samples were taken from subjects after an overnight fast. The clinical characteristics of the study subjects are shown in Table I (available online at http://atvb.ahajournals.org).

**Animals**

Anesthetized male Sprague-Dawley rats were injected intravenously with native and PEth-modified HDL particles. Retro-orbital blood (0.3 mL) was collected into EDTA-containing tubes for VEGF determinations.

**Isolation and Labeling of Lipoproteins**

Lipoproteins were isolated from plasma by sequential ultracentrifugation on the basis of their density, as described.26–28 PEth was labeled, 29 extracted, 30 and separated by thin-layer chromatography (TLC). Iodination of the PEth–HDL was performed using IODO Beads Iodination Reagent (Perbio Science).

**Chemical Analyses**

Plasma and lipoprotein compositions were determined by kits of Roche Diagnostics GmbH (cholesterol, triglycerides), Wako Chemicals GmbH (phospholipids), and Bio-Rad Laboratories (proteins).

**Cell Culture Experiments**

Human endothelial EA.hy 926 cells were grown under standard conditions and incubated for different time points in the serum-free medium containing ethanol, phosphatidylethanolamine (PC), PEth, HDL. PEth–HDL, or alcoholic HDL.

**Determination of PEth–HDL Cell Association and Selective PEth Uptake**

EA.hy 926 cells were incubated for 5 hours with [125I]PEth–HDL and [14C]-labeled PEth-HDL, and the cell association of PEth–HDL and selective PEth uptake was determined, as described previously, detailed by Acton and Rigotti.31

**Immunooassays**

The VEGF concentration was measured from the human and rat plasma and cell culture medium by kits of R&D Systems.

**Western Blot Analysis of PKC and p44/42 MAPK**

Please see online Methods section at http://atvb.ahajournals.org for further details.

**Results**

**Characteristics of the Study Subjects**

Age, body mass index (BMI), and daily alcohol intake data for the subjects are shown in Table I. Age and BMI did not differ between the groups, nor did VLDL cholesterol, plasma total triglyceride, or serum bilirubin concentration. The alcoholics had a 19% lower total cholesterol concentration (P = 0.002) and a 39% lower LDL cholesterol concentration (P = 0.0001) than the controls. The HDL cholesterol concentration was 28% higher in the group of alcoholics than in controls (P = 0.062).

**VEGF Concentration in the Plasma of the Study Subjects**

To examine the hypothesis that alcohol drinking is capable of affecting the VEGF secretion or concentration in circulation, we collected plasma samples from controls and alcoholics. The plasma VEGF concentration was 39% higher in the alcoholics (74 ± 37 pg/mL, n = 17) than in the controls (Figure 1a) (53 ± 23 pg/mL, n = 17, P = 0.056), although there was pronounced overlapping.

**PEth Increases the Plasma VEGF Concentration in Rats**

To prove that PEth present in lipoprotein particles increases the secretion of VEGF in vivo, the effects of PEth-modified HDL on plasma VEGF concentration were determined in vivo in rats. One group of rats (PBS) was injected intravenously with PBS buffer, whereas another group (control HDL) received injections of human HDL isolated from control subjects. The third group of rats (alcoholic HDL) was injected with HDL isolated from the plasma samples of

![Figure 1. Plasma concentration of VEGF is high in alcoholic subjects and is increased after injection of alcoholic HDL or PEth-modified HDL into rats. a, The mean plasma VEGF values (n=17) are marked with horizontal bars. ■ indicates alcoholic subjects; ○, controls. b, The rats were injected with PBS (□ indicates PBS, n=9), control HDL (● indicates control HDL, n=9), alcoholic HDL (○ indicates alcoholic HDL, n=8), or PEth–HDL (● indicates PEth–HDL, n=9).](http://atvb.ahajournals.org/)

**Statistical Analysis**

The results are expressed as means and standard deviation (SD). The statistical significance of the differences between the means was assessed by independent samples 2-tailed Student t test or 1-way analysis of variance (ANOVA, Tukey test), as appropriate. P = 0.05 was considered significant.
Selective PEth uptake† 156 (15)* 0.62 (0.04)*
PEth–HDL particle uptake 54 (4) 0.22 (0.02)
PEth–HDL binding 49 (14) 0.20 (0.06)

The data represent the means (SD) from 2 experiments performed in triplicate wells. The statistical significance of the differences between the means was assessed by Student t test.

† The selective uptake of PEth from the HDL particles was calculated as the amount of HDL protein.

alcoholics, and the fourth group (PEth–HDL) received intravenous injections of control HDL containing PEth added in vitro. Alcoholic HDL increased the plasma VEGF concentration 2-fold (from 41 ± 5 to 79 ± 8 pg/mL, P < 0.003) compared with the control rats (PBS) 2 hours after the injection (Figure 1b). PEth–HDL increased the VEGF concentration by 144% (from 41 ± 5 to 100 ± 19 pg/mL) compared with the control rats (PBS) 3 hours after the injection (P < 0.0001).

Transfer of PEth From HDL Into Endothelial Cells

To find out if PEth is transferred from HDL particles into the endothelial cells, we used radiolabeled PEth–HDL particles. 125I-PEth–HDL was used to follow the fate of the protein component and 13C-PEth–HDL was used to determine the fate of the phospholipid component. To determine PEth–HDL—cell association (binding and internalization) and selective PEth uptake, we compared the fates of the phospholipid and protein components (Table). Heparin was used to separate the binding of HDL to a cell surface from the amount of internalized HDL. Lipoprotein binding was performed at 37°C and the cell-surface HDL release was determined at 4°C. Selective PEth uptake from HDL particle to the endothelial cells was more efficient (0.62% of the radioactivity) than the uptake of intact PEth–HDL particles (0.22% of the radioactivity) (P < 0.004).

Secretion of VEGF From Endothelial Cells

VEGF is an angiogenic growth factor and potentially also an endogenous vascular protective factor. Previous studies have shown that PEth formation in mononuclear cells is accompanied by responses in cell function. We hypothesized that PEth, either formed inside cells or associated with lipoproteins taken-up by cells, enhances VEGF secretion from cells. To test this hypothesis, we treated EA.hy 926 endothelial cells with different HDL particles and PEth liposomes. The VEGF concentration in the cell culture medium was 14% higher in the presence of alcoholic HDL (2.4 ± 0.4 pg/mL) than in the presence of control HDL (2.1 ± 0.2 pg/mL) (Figure 2a, P < 0.05). The VEGF concentration in the cell culture medium was not affected by the presence of ethanol or HDL alone at the concentration of 0.2 mg/mL compared with nontreated cells (Figure 2b). By contrast, the VEGF concentration in the medium increased significantly (from 2.4 ± 1.3 to 3.6 ± 0.4 pg/mL, P < 0.04) when both HDL and ethanol were added together into the culture medium (Figure 2b). The same effect was produced by PEth–HDL particles, which increased the VEGF concentration by 2.4-fold as compared with the control cells (from 2.4 ± 1.3 to 5.7 ± 1.2 pg/mL, P < 0.002) (Figure 2b). The addition of ethanol into the medium did not further enhance the effect of PEth–HDL (Figure 2b). To prove that the increasing effect of VEGF secretion is caused by PEth per se, another set of experiments with phospholipid liposomes was performed (Figure 2c). The VEGF concentration in the cell culture medium was markedly increased in the presence of PEth (from 2.1 ± 0.9 to 20.7 ± 1.5 pg/mL, P < 0.0002) (Figure 2c), whereas PC increased the VEGF concentration only from 2.1 ± 0.9 to 5.2 ± 3.0 pg/mL (P < 0.05).

Figure 2. VEGF secretion increased from EA.hy 926 cells by alcoholic HDL and PEth. a, Alcoholic HDL (0.2 mg/mL) increased the VEGF secretion more than control HDL (0.2 mg/mL) (n = 12). b, PEth–HDL increased the VEGF concentration in the absence (white columns) and presence (black columns) of 100 mmol/L ethanol and HDL only in the presence of ethanol (n = 8). c, PC (0.5 mg/mL) increased slightly and PEth (0.5 mg/mL) increased markedly the VEGF concentration (n = 4). *P < 0.05, **P < 0.002, ***P < 0.0002.
PEth Stimulates MAPK Kinase Activity in EA.hy 926 Cells

Ethanol induces in vitro angiogenesis by stimulating the MAPK activity in EA.hy 926 cells. Therefore, the effect of PEth–HDL on the MAPK phosphorylation was investigated. As shown in Figure 3, incubation of serum-starved EA.hy 926 cells in medium containing PEth–HDL for 10 minutes resulted in a 3.6-fold (P<0.006) increase in the MAPK (Erk1/Erk2) phosphorylation compared with control cells treated with PBS. HDL increased the Erk1/Erk2 phosphorylation by 3.3-fold (P<0.03) compared with control cells treated with PBS. The increase in the Erk1/Erk2 phosphorylation remained significantly elevated above control (PBS) levels when the cells were incubated with HDL for up to 15 minutes. PEth–HDL had a more extended effect on the Erk1/Erk2 phosphorylation than HDL alone. The phosphorylation induced by PEth–HDL remained elevated for up to 24 hours compared with control cells. PD098059 inhibited PEth–HDL-mediated stimulation of Erk1/Erk2 phosphorylation by 84% (Figure 4a) (P<0.004).

PEth Stimulates PKC Activity in EA.hy 926 Cells

Because ethanol stimulates PKC activity in endothelial cells, the effect of PEth–HDL on the PKC phosphorylation was investigated. As shown in Figure 5, incubation of serum-starved EA.hy 926 cells in medium containing PEth–HDL for 10 minutes resulted in a 2.2-fold (P<0.05) increase in the PKC phosphorylation compared with control cells treated with PBS. HDL increased the phosphorylation by 2-fold (P<0.05) compared with control cells treated with PBS. PEth–HDL-mediated stimulation of the PKC phosphorylation was slightly reduced by a PKC inhibitor Go6983, but the result was not statistically significant (Figure 4a).

PEth-Induced VEGF Secretion Is Inhibited by PKC and MAPK Inhibitors

Incubation of serum-starved EA.hy 926 cells for 15 minutes in a medium containing PEth–HDL increased VEGF secretion by 5-fold compared with cells treated with PBS (Figure 4b) (P<0.004). To ascertain whether the PEth-induced VEGF secretion is mediated through PKC and MAPK, the effect of inhibiting PKC and MAPK on this phenomenon was investigated. The maximal effect of the inhibitors was seen at the 15-minute time point (Figure 4b). The increase in VEGF secretion induced by PEth–HDL was inhibited by 40% in the presence of the PKC inhibitor, Go6983, and by 25% in the presence of the MAPK inhibitor, PD098059.

Discussion

PEth is formed in mammalian organs in the presence of ethanol and it is also present in the blood of subjects who...
have been drinking alcohol.\textsuperscript{20,33–35} PEth is formed especially in the lungs and the intestine, but also in vascular endothelial cells.\textsuperscript{36–38} The present study suggests that PEth affects vascular endothelial cells and may mediate at least part of the cardioprotective effects of ethanol. As a proof of principle, the present study demonstrates that lipoprotein-associated PEth increases the production of VEGF in endothelial cell cultures. VEGF was chosen as an end point marker because its function is considered beneficial in the later phases of atherosclerosis because of its ability to inhibit neointima formation.\textsuperscript{32} In addition, moderate levels of ethanol induce the expression and secretion of VEGF in cultured vascular smooth muscle cells and chick embryo chorioallantoic membranes\textsuperscript{39,40} and ethanol, at least at high concentrations, induces the in vitro angiogenesis through PKC and MAPK signaling pathways in endothelial cells.\textsuperscript{55} The present cell culture and animal experiments suggest that the increased plasma VEGF concentrations observed in alcohol drinkers may be caused by ethanol-induced qualitative alterations in the HDL particles of alcohol drinkers, and that this effect may be mediated by PEth through the MAPK and PKC signaling pathways.

The stimulation of the MAPK activity, as measured as an increase in the phosphorylation of Thr202/Tyr204 residues of Erk1/Erk2, was prolonged by PEth–HDL compared with the stimulation caused by native HDL. In addition, the partial inhibition of PEth-induced VEGF secretion by specific MAPK inhibitor PD098059 indicates that the MAPK pathway could be one of the signaling routes between PEth and VEGF secretion in endothelial cells. This is in accordance to a recent report in hepatocytes.\textsuperscript{40} The PEth-induced stimulation of the phosphorylation of PKC and the partial inhibition by G6983 of the PEth-induced VEGF secretion from the endothelial cells indicate that the PKC pathway could also contribute to the PEth-induced VEGF secretion in endothelial cells. Moreover, this study gives a plausible explanation for the ethanol-induced increase in plasma VEGF level by demonstrating in an animal model that the addition of an ethanol-induced abnormal lipid, PEth, into HDL particles is also capable of elevating plasma VEGF levels. However, we cannot exclude the contribution of other compounds present in HDL isolated from plasma of alcohol drinkers, despite the apparent similarity in the VEGF response in vivo.

The present study provides evidence that PEth–HDL may interfere with the vascular endothelium in a different manner than native HDL. This qualitative difference leads into increased concentration of VEGF in plasma in vivo and also in endothelial cells, which line the blood vessel and are in close contact with smooth muscle cells and macrophages. All of these cells may sense changes in the environment when the composition and concentration of lipoproteins are altered, eg, by ethanol.

The mechanism of the cardioprotective effect of moderate alcohol drinking has been elusive. For most hours of the day, ethanol is not present in the body of a social drinker. Therefore, the direct effects of ethanol on vascular endothelial cells might be only temporary if they are confined to the period of ethanol abundance, even though PEth may be metabolized more slowly than PC. The relatively long half-life of lipoproteins may prolong the presence of PEth in the body from a few hours to several days even in an occasional drinker.\textsuperscript{59} This kind of “memory molecule” might help to explain the beneficial effects of light or moderate drinking on cardiovascular diseases. The effect of PEth may last even longer, because erythrocytes may act as a reservoir of PEth,\textsuperscript{41,42} and because this abnormal phospholipid is transported between lipoproteins.\textsuperscript{23} Lipoproteins may transport PEth from erythrocytes to vascular wall cells.\textsuperscript{43} More sensitive methods are needed for the measurement of PEth content in lipoprotein fractions, however. This work is currently underway in our laboratory.

In conclusion, ethanol not only alters the quantity of lipoproteins or their components but also may cause incorporation of abnormal derivatives of lipids and proteins into lipoproteins, and may therefore have marked and sustained effects on all vascular cells.\textsuperscript{43} Further studies are needed to elucidate more detailed mechanisms of how PEth regulates the VEGF function and secretion in endothelial and in other vascular cells.

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References


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Data supplement

Methods

Subjects
A series of 17 male alcoholics referred by general practitioners for withdrawal therapy to the Alcoholism Treatment Unit of Oulu were recruited for blood tests. This unit treats ambulatory patients who seek assistance for terminating their dependence on alcohol and have no signs or symptoms of other diseases. Healthy non-alcoholic men (n = 17) recruited from among the local paper mill employees served as control subjects. Venous blood samples were taken from both the alcoholics and the controls after an overnight fast. The intake of alcohol was measured by an extensive interview concerning the amount of beer, wine, and strong alcoholic beverages consumed during the previous two weeks. The mean alcohol intake with a range was calculated and expressed in grams of pure alcohol per day. The study was approved by the Ethical Committee of the University of Oulu. The clinical characteristics of the study subjects are shown in Table I.

Animals
Nine-week old male Sprague-Dawley rats (330 ± 27 grams in body weight; mean ± SD, n = 35) were housed in a controlled environment with free access to food and water. The experiments were approved by the Animal Care and Use Committee, University of Oulu. The rats were anesthetized with fentanyl-fluanisone and midazolam before injecting them intravenously with HDL particles isolated by ultracentrifugation and dialyzed in phosphate-buffered saline (PBS), pH 7.4. HDL particles were isolated from the plasma of control subjects and alcoholics as described previously. These HDL fractions (0.70 µmol cholesterol per 100 g body weight) or control HDL supplemented with phosphatidylethanol (PEth) were injected into the tail vein. Retro-orbital blood (0.3 ml) was collected into EDTA-containing tubes at the indicated times.
Isolation of lipoproteins

Blood samples were obtained after an overnight fast from control subjects and alcoholics, and plasma was separated by centrifugation. Lipoproteins were isolated from plasma by sequential ultracentrifugation on the basis of their density as described earlier.\textsuperscript{1-3} 1,2-Dioleoyl-sn-glycero-3-phosphoethanol (sodium salt) (Avanti Polar Lipids) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) were incorporated into human HDL particles (5\% of total lipoprotein phospholipids). Phospholipid-modified HDL particles were first centrifugated at a density of 1.006 g/ml in order to remove the unincorporated phospholipids from the HDL fraction, and then at a density of 1.21 g/ml. All isolated lipoproteins were dialyzed against PBS, pH 7.4 before use.

Lipoprotein labeling procedure

PEth was labeled by the phospholipase D-catalyzed transphosphatidylation reaction using 1,2-di[1-\textsuperscript{14}C]oleoyl-sn-glycero-3-phosphocholine (100 mCi/mmol, Amersham Biosciences) as a labeled substrate.\textsuperscript{4} Phospholipids were extracted as described by Meacci et al\textsuperscript{5} and separated by thin-layer chromatography (TLC) in ethylacetate:iso-octane:acetic acid (81:45:18). The [14C]labeled PEth-containing spot was transferred to a clean tube, extracted as previously, dissolved in ethanol, and incorporated to HDL as described earlier. Iodination of the PEth-HDL (5 mg protein) was performed using IODO Beads Iodination Reagent (Perbio Science) and 1 mCi of Na\textsuperscript{125}I (17.5 Ci/mg, NEN Life Science Products Inc.). The labeling reaction was carried out in PBS, pH 7.4 buffer according to the manufacturer’s instructions. Unincorporated \textsuperscript{125}I\textsubscript{2} and excess Na\textsuperscript{125}I were removed from the iodinated HDL by a PD10 desalting column (Amersham Biosciences). Labeled PEth-HDL particles were stored at 4°C and used within one week.
Chemical analyses

The concentrations of cholesterol, triglycerides, and phospholipids in the plasma and the lipoprotein fractions were determined by enzymatic colorimetric methods using a Kone Specific Selective Chemistry Analyser (Kone Oy) and kits of Roche Diagnostics GmbH (cholesterol, triglycerides) and Wako Chemicals GmbH (phospholipids). The protein concentrations were determined by Lowry method using a kit (Bio-Rad Laboratories).

Cell culture experiments

Human endothelial EA.hy 926 cells (kindly provided by Dr. Cora-Jean S. Edgell, University of North Carolina, NC, USA) were cultured in DMEM high glucose supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and HAT supplement. The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air. For the VEGF determinations, the cells were seeded at a concentration of 1-2 x 10^6 cells/ml on plates (Ø 10 cm), grown for three days, washed twice with PBS, and incubated for 24 hours in the serum-free medium containing 20-400 mM ethanol, 200 µg/ml phosphatidylcholine (PC), 200 µg/ml PEth, 200 µg/ml HDL, 200 µg/ml PEth-HDL or 200 µg/ml alcoholic HDL. For VEGF determinations, samples of cell culture medium were collected at indicated time points, concentrated by an Ultrafree-4 centrifugal filter unit (Millipore Corporation, Bedford, Massachusetts, USA), and stored at −70 °C.

Determination of PEth-HDL cell association and selective PEth uptake

To determine cell association (heparin-non-releasable binding and internalization) of HDL and selective HDL-PEth uptake, EA.hy 926 cells were grown to confluence in 6-well plates, and incubated with 25 µg of PEth-HDL protein labeled with ¹²⁵I in the protein moiety and with [¹⁴C]PEth in the lipid moiety in separate incubations as described earlier for 5 hours. The amount
of specific PEth-HDL binding, holoparticle uptake and selective PEth uptake were determined by the difference between the total radioactivity and the amount of nonspecific radioactivity in the presence of excess unlabeled lipoprotein as described detailed by Acton and Rigotti.8

**Immuoassays**

The VEGF concentration was measured from the human plasma and cell culture medium (4-fold concentrated) by a Quantikine human VEGF kit (R&D Systems Inc.). The VEGF concentration in rat plasma was measured by a Quantikine M VEGF kit (R&D Systems).

**Western Blot Analysis of PKC and p44/42 MAPK**

Serum-starved EA.hy 926 cells were incubated with 0.2 mg/ml HDL, 0.2 mg/ml 0.5 % PEth-HDL, 1 µM Gö6983 (a PKC inhibitor, Calbiochem-Novabiochem), or 10 µM PD098059 (a Erk1/Erk2 MAPK inhibitor, Sigma) for the indicated amounts of time. After treatment, cells were lysed with lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2 % w/v SDS, 10 % glycerol, 50 mM DTT, 0.01 % bromphenol blue). Aliquotes of cell lysates (20 µl) were loaded onto 10 % polyacrylamide gels for electrophoresis. After electrophoresis proteins were transferred to PVDF membranes (Millipore), and incubated with the following antibodies: Phospho-PKC (pan) (Cell Signaling Technology), PKC (Ab-1) (Oncogene Research Products), Phospho-p44/42 Map Kinase (Thr202/Tyr204) (Cell Signaling Technology), MAP Kinase-2 (Erk-2) (Clone 1B3B9) (Sigma), and β-actin (Clone AC-15) (Sigma). Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and ECL Western blotting detection reagents (Amersham Biosciences) were used to for detection, and the signals were analyzed by Kodak 1D Imaging System (Eastman Kodak Company).
Statistical analysis

The results are expressed as means and standard deviation (SD). The alcohol intake and plasma triglyceride values were not normally distributed and were therefore log-transformed before the statistical analysis. The statistical significance of the differences between the means was assessed by independent samples’ 2-tailed Student’s t-test or one-way analysis of variance (ANOVA, Tukey’s test), as appropriate. $P$ values were considered significant at 0.05.
References


Table I Characteristics of the study subjects

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<td>BMI (kg/m^2)</td>
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<td>Alcohol intake (g/day)</td>
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<td>20 (0-63) ‡</td>
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<td>Total cholesterol (mmol/l)</td>
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<td>5.71 (0.84) ‡</td>
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<td>LDL cholesterol (mmol/l)</td>
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<td>HDL cholesterol (mmol/l)</td>
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<td>1.51 (0.47) *</td>
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<td>Plasma triglycerides (mmol/l)</td>
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<td>1.28 (0.52)</td>
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<tr>
<td>γ-glutamyltransferase (mmol/l)</td>
<td>232 (345)</td>
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<td>Aspartate aminotransferase (U/l)</td>
<td>78 (62)</td>
<td>32 (13) †</td>
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The data are presented as means (SD). The ethanol consumption is given as mean with a range. The statistical significance of the differences between the means was assessed by Student’s t-test. * P = 0.061, † P < 0.04, ‡ P < 0.009.