Ethanol beverages containing polyphenols decrease nuclear factor kappa-B activation in mononuclear cells and circulating MCP-1 concentrations in healthy volunteers during a fat-enriched diet

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Abstract

Aims: Different epidemiological studies have demonstrated that some ethanol containing beverages intake could be associated with a reduction of cardiovascular mortality, effect attributed in part to its antioxidant properties. Nuclear factor-kappa B (NF-kappaB) is a redox sensitive transcription factor implicated in the pathogenesis of atherosclerosis. We have examined the effect of four different ethanol containing beverages on the activation of NF-kappaB in peripheral blood mononuclear cells (PBMC) and circulating concentrations of monocyte chemoattractant protein-1 (MCP-1) in healthy volunteers receiving a fat-enriched diet.

Methods and results: Sixteen volunteers received 16 g/m2 of ethanol in form of red wine, spirits (vodka, rum, and brandy) or no ethanol intake along with a fat-enriched diet during 5 days and all of them took all alcohols at different periods. NF-kappaB activation (electrophoretic mobility shift assay) and circulating MCP-1 levels (ELISA) were examined in blood samples taken before and after 5 days of ethanol intake. Subjects receiving a fat-enriched diet had increased NF-kappaB activation in PBMC at day 5. Furthermore, MCP-1 levels were increased in plasma at day 5. Red wine intake and some ethanol beverages containing polyphenols (brandy and rum) prevented NF-kappaB activation and decreased MCP-1 release.

Conclusion: Consumption of moderate amounts of alcoholic drinks containing polyphenols decreases NF-kappaB activation in PBMCs and MCP-1 plasma levels during a fat-enriched diet. Our results provide additional evidence of the anti-inflammatory effects of some ethanol containing beverages, further supporting the idea that its moderate consumption may help to reduce overall cardiovascular mortality.

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1. Introduction

Previous epidemiological studies have found an association of moderate ethanol containing beverages intake with a reduced risk of cardiovascular disease [1–3]. These observations have been proposed to explain the French paradox: the coexistence of relative high fat intake and the lower rate of

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cardiovascular disease in France [4]. Although several studies have attributed this beneficial effect to the high consumption of red wine, other studies have suggested that different ethanol containing beverages, not specifically red wine, reduce also the risk of cardiovascular disease [5]. Different classes of antioxidants are present in ethanol containing beverages. The administration of some of them such as catechin or quercetin exerts an inhibitory effect on the development of aortic lesions in atherosclerotic Apo E knockout mice [6]. In this regard, nuclear factor kappa B (NF-kappaB) is a redox-sensitive transcription factor implicated in the transmission of different signals from the cytoplasm to the nucleus of numerous cell types [7]. This transcription factor is found as a trimer consisting of p50, p65 and IkB subunits in the cytosol. The release of IkB from the trimer results in the migration of the p50/p65 heterodimer to the nucleus [8], where after binding to specific sequences activates genes involved in the immune, inflammatory or acute phase response such as cytokines [monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8)], adhesion molecules [vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1)] and procoagulant proteins [tissue factor (TF), plasminogen activator inhibitor 1 (PAI-1)] [9]. Different molecules have been found to induce NF-kappaB activation, including oxidized-LDL and cytokines such as interleukin-1, lymphotixin and tumor necrosis factor-α [10]. Other factors inhibit NF-kappaB activation, including glucocorticoids, antioxidants [11] and lipid-lowering drugs such as statins [12]. Different studies have reported the participation of NF-kappaB in the pathogenesis of atherosclerosis. In this sense, NF-kappaB is present in the nuclei of endothelial cells, monocytes/macrophages and participates in the dysregulation of vascular smooth muscle cells in atherosclerotic plaques [13].

Monocyte chemoattractant protein-1 plays an important role in the recruitment and activation of monocytes and thus in the development of atherosclerosis. Enhanced MCP-1 expression has been detected in macrophages, endothelial cells, and vascular smooth muscle cells in the atherosclerotic plaque [14]. Activation of macrophages by MCP-1 also appears to be involved in the vulnerability of the plaque. Indeed, circulating MCP-1 levels are elevated in patients with acute myocardial infarction and in those with unstable angina, but not in patients with stable angina [15]. Production of MCP-1 and macrophage accumulation are also observed after coronary angioplasty or grafting [16], indicating that MCP-1 expression may be related not only to the instability of atheromatous plaques but also in the formation of restenotic lesions.

In a previous work it was shown that an acute intake of fat enriched diet caused activation of NF-kappaB in circulating human peripheral mononuclear cells after 9 h [17]. This activation was prevented by adding to the diet a moderate amount of red wine, but not vodka, an alcoholic beverage lacking the antioxidant polyphenols of red wine. In this work we have analyzed the effect of a fat-enriched diet on NF-kappaB activation and circulating MCP-1 concentration in healthy volunteers, in a period of 5 days of follow-up. Additionally, we have studied whether alcohol intake in the form of a polyphenol rich alcoholic drink (red wine), two alcoholic beverages with moderate contents of polyphenols (rum, brandy) and a polyphenol-free alcoholic beverage (vodka) could modulate NF-kappaB activity and plasma MCP-1 concentration during a fat-diet intake.

2. Materials and methods

2.1. Study subjects

Twenty potential participants (medical students or young doctors) were examined in order to exclude any pathological disorder, confirmed by blood test, including a normal lipid profile. Those who had plasma cholesterol levels >220 mg/dl, plasma triglycerides levels >200 mg/dl, or were hypertensive or smokers were excluded. Weight and height of subjects were measured and body surface index was calculated by the Dubois formula. Finally, eight males and eight females, aged 22–29 years, were included in this study. All of them gave a written informed consent for the study.

The study was approved by the Hospital’s Ethics Committee, according to the institutional and the Good Clinical Practice guidelines.

2.2. Study design

We have performed a cross-over design in five different weeks in which equal amounts of alcohol present in different ethanol containing beverages were analyzed. In the first week, all participants were randomized and distributed in five groups. Subjects received the same oral fat-enriched diet and different amounts of the different beverages tested: red wine, vodka, brandy, or rum (see below) in order to maintain constant the same amount of alcohol in each of the five phases. In a fifth observation (control phase) no alcoholic drink was given, adding sugar to compensate the amount of the alcohol calories lacking. In the five situations, the caloric intake was maintained constant. All participants went throughout the five phases, and in each case, phases were separated by at least 2 weeks. In the meantime, participants returned to their usual dietary habits, refraining from the consumption of any alcoholic beverage. Blood samples were obtained from participants after a 10-h fasting (day 0) and at 1 and 5 days after the beginning of each week of study.

2.3. Diet

Fat-enriched diet contained 1487 kcal/m² with 654 kcal/m² (44%) as fat. The distribution of fat was 22% as saturated, 12% as monounsaturated and 10% as polyunsaturated fat. It was prepared in the hospital with common solid and liquid...
foods and supplemented with cheese, chocolate and sugar-enriched drinks. The alcohol beverage was administered daily at lunch and dinner (60% and 40% of the amount, respectively). In all cases, alcohol represented an amount of 16 g/m². A calculated amount of sugar was added to their non-alcoholic drink in the phase of no alcohol to keep constant the total amount of calories in all five phases.

The content of alcohol was 12% in red wine, 37% in rum and 35% in brandy, and all of them were kept for 3 years in oak barrels. Vodka was tri-distilled and contained 40% of alcohol.

2.4. Measurement of antioxidant content in alcohol beverages

Total phenols, expressed as gallic acid equivalents, were determined using the Folin-Ciocalteu reagent as previously described [18].

2.5. Isolation of peripheral blood mononuclear cells (PBMCs)

The blood samples were diluted in phosphate buffered saline (PBS) 1:1 and cells were separated in 5 mL ficoll gradient (lymphocytes isolation solution, Rafer, Madrid, Spain) by centrifugation at 2000 × g for 30 min. PBMCs were collected, washed twice with cold PBS and resuspended in buffer A (see protein extraction). Approximately, 95% of the cells are mononuclear cells (flow cytometry, not shown).

2.6. Lipoprotein profile and MCP-1 determination

Venous blood samples were collected in EDTA. The plasma samples were stored at −80°C until analysis was performed. Lipid determinations were done using standard techniques. Briefly, lipid profile included: total cholesterol, HDL cholesterol, LDL cholesterol, very low density lipoprotein (VLDLc) cholesterol, total triglycerides and VLDL triglycerides (VLDLt). Lipoproteins were isolated by an ultracentrifugation method [19], and then cholesterol and triglycerides were assayed enzymatically in the subfractions obtained (HDL was obtained after treating UC infranatant with phosphotungstic acid-Mg, and LDL cholesterol was calculated as the difference between infranatant cholesterol and HDL cholesterol) using commercial enzymatic kits (Boehringer Mannheim, Mannheim, Germany) adapted to a RA-XT autoanalyzer (Bayer Diagnostics, Tarrytown, NY, USA). In all cases, procedures were adapted (sample/reagent ratio) to obtain a photometric precision equivalent to a cholesterol or triglycerides concentrations in the range of 150–300 mg/dL.

Plasma concentrations of soluble MCP-1 were determined in duplicate with commercially available enzyme-linked immuno-sorbent assay kits (R&D systems). One-hundred microliters of plasma samples were assayed in parallel to known MCP-1 recombinant concentrations. Each assay was calibrated using a MCP-1 standard curve. The minimum detectable level of soluble MCP-1 was 5 pg/mL. Intra-assay and inter-assay coefficients of variation were 5.4 and 5.2%, respectively.

2.7. Protein extraction and electrophoretic mobility shift assays

Proteins of PBMCs were extracted as described [17]. Briefly, PBMCs were collected, washed with cold PBS, and resuspended in 5 cell-pellet volumes in buffer A (in mmol/L: HEPES 10 pH 7.8, KCl 15, MgCl2 2, EDTA 0.1, DTT 1, and PMSF 1) and homogenized. Nuclei were centrifuged at 1000 × g for 10 min, and resuspended in 2 volumes buffer A. Then 3 mol/L KCl were added drop by drop to reach 0.39 mol/L KCl. Nuclear proteins were extracted for 1 h at 4°C and centrifuged at 100,000 × g for 30 min. Supernatant was dialyzed in buffer C (mmol/L: HEPES 50 (pH 7.8), KCL 50, PMSF 1, EDTA 0.1, DTT 1, and 10% glycerol), then cleared by centrifugation and stored at −80°C. Protein concentration was determined by the BCA method (Pierce, Rockford, IL, USA).

Gel shift assays were performed with a commercial kit according to the instructions of the manufacturer (Promega, Madison, WI, USA). Briefly, NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was (32P)-end-labeled by incubation with 10 U T4 polynucleotide kinase (Promega) in a reaction containing 10 μCi (γ-32P)ATP (3000 Ci/mmol) (Amersham), 70 mmol/L Tris–HCl, 10 mmol/L MgCl2, and 5 mmol/L DTT. The reaction was stopped by the addition of EDTA to a final concentration of 0.05 mol/L. Nuclear proteins (5 μg) were equilibrated in a binding buffer containing 4% glycerol, 1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris–HCl (pH 7.5), and 50 μg/mL poly(dI-dC) (Pharmacia LKB). When competition assays were performed, the cold probe was added to this buffer 10 min before the addition of the labeled probe. The labeled probe (0.35 pmol) was added to the reaction. The gel was dried and exposed to X-ray film.

Autoradiograph was subjected to densitometry with an Image Quant densitometric scanner (Molecular Dynamics, Sunnyvale, CA). Percentages were calculated in relation to the basal value in each subjects and conditions.

2.8. Statistical analysis

Results are expressed as mean ± S.E.M. (unless specified). Significance was established with GraphPAD InStat (GraphPAD Software). Analysis of variance (ANOVA) for repeated measures was used to analyze the differences in plasma lipids levels, NF-κappaB activation and MCP-1 concentrations. When statistically significant effects were demonstrated, Tukey’s post hoc comparison test was used to identify group differences. Differences were considered significant when p < 0.05.
3. Results

3.1. Fat-enriched diet and NF-kappaB activation

To examine whether fat-enriched diet could activate NF-kappaB, fasting blood samples of healthy volunteers were taken before and after 1 or 5 days of a fat-enriched diet. As shown in Fig. 1, electrophoretic mobility shift assay (EMSA) of nuclear proteins from peripheral blood mononuclear cells (PBMCs) showed a retarded band corresponding to NF-kappaB that was increased at 5 days (2.3 ± 0.3-fold versus day 0; \( p < 0.05 \)). In contrast, EMSA of AP-1 transcription factor did not show a retarded band (not shown), indicating a specific effect of fat-enriched diet on NF-kappaB.

At this time, we did not observe changes in the lipid profile of healthy volunteers induced by fat-enriched diet or beverage intake, probably due to the fact that the experimental period was not longer enough to elicit changes in fasting plasma lipids (control day 0 versus day 5 (units mg/dL): total cholesterol 163 ± 22 to 167 ± 22; triglycerides 81 ± 30 to 83 ± 29; LDL-C 103 ± 19 to 104 ± 17; VLDL-C 8 ± 5 to 7 ± 4; VLDL-t 34 to 33 ± 15; HDL-C 50 ± 12 to 50 ± 12; wine day 0 versus day 5: total cholesterol 155 ± 24 to 162 ± 21; triglycerides 71 ± 32 to 83 ± 23; LDL-C 97 ± 21 to 102 ± 18; VLDL-C 6 ± 4 to 7 ± 4; VLDL-t 34 to 33 ± 15; HDL-C 50 ± 12 to 50 ± 12; brandy day 0 versus day 5: total cholesterol 165 ± 25 to 169 ± 21; triglycerides 76 ± 21 to 80 ± 32; LDL-C 104 ± 20 to 104 ± 17; VLDL-C 8 ± 3 to 8 ± 7; VLDL-t 42 ± 18 to 40 ± 27; HDL-C 53 ± 10 to 53 ± 10; rum day 0 versus day 5: total cholesterol 171 ± 25 to 173 ± 21; triglycerides 80 ± 21 to 87 ± 24; LDL-C 98 ± 20 to 101 ± 17;
3.2. Alcohol intake and NF-kappaB activation

Firstly, we analyzed the polyphenolic content of the different alcohol beverages used in our study. The amounts of total polyphenols in the studied alcoholic beverages, expressed as equivalents of gallic acid, were the following: 2660 mg/L red wine, 357 mg/L rum, and 89 mg/L brandy. It is interesting to note that vodka is the only beverage without polyphenolic compounds.

To study the effect of these alcohol beverages on NF-kappaB binding activity, fasting blood samples were taken before and after 1 or 5 days of a fat-enriched diet with equal amounts of 4 different alcohol beverages (red wine, vodka, rum, or brandy). The amount of different beverages was adjusted to keep the same dose of alcohol in all observation periods. Ethanol beverages containing polyphenols (red wine, brandy, or rum) decreased NF-kappaB activation in a time-dependent manner (1.5 ± 0.2 red wine, 1.4 ± 0.3 brandy, 1.1 ± 0.2 rum; fold versus day 0, p < 0.05 versus control day 5 for all of them). In contrast, we observed that vodka intake did not modify the increment of NF-kappaB binding activity induced by the fat-enriched diet (Fig. 1), probably indicating that the effect of alcohol intake is related with the other non-alcoholic components present in these beverages.

3.3. Fat enriched-diet, alcohol intake and circulating MCP-1 levels

One of the proinflammatory chemokines induced by NF-kappaB activation is MCP-1. For this reason, we explored the effect of a fat-enriched diet and alcohol intake on circulating MCP-1 levels. Fasting blood samples were taken before and after 5 days of a fat-enriched diet. Fat-enriched diet induced an increment in plasma MCP-1 concentration at day 5 (79 ± 5 pg/mL versus 88 ± 4 pg/mL). The administration of different forms of alcohol beverages (red wine, brandy, or rum) along with a fat-enriched diet decreased circulating MCP-1 concentrations at day 5 (81 ± 8 pg/mL versus 65 ± 7 pg/mL, 89 ± 5 pg/mL versus 77 ± 5 pg/mL, 84 ± 9 pg/mL versus 74 ± 10 pg/mL, respectively). Red wine intake was superior to other alcohol beverages in decreasing circulating MCP-1 concentrations (Fig. 2). In contrast, vodka intake did not decrease circulating MCP-1 concentration at day 5 (78 ± 8 pg/mL versus 80 ± 12 pg/mL).

4. Discussion

In this paper we have analyzed the effect of different ethanol containing beverages intake on NF-kappaB activation and circulating MCP-1 concentrations in healthy subjects during a fat-enriched diet. NF-kappaB is a transcription factor implicated in various transcriptionally controlled processes such as inflammation and cell growth. Recent studies strongly suggest that NF-kappaB is involved in the pathogenesis of atherosclerosis. This transcription factor is activated in cells present in atherosclerotic lesions such as macrophages, endothelial cells and vascular smooth muscle cells, while no active NF-kappaB is detected in the same cells present in normal vessels [13]. Furthermore, NF-kappaB is mainly activated in the shoulder regions of carotid atherosclerotic plaques, an area characterized by a high inflammatory content and where the rupture of the atheroma usually takes place [20]. In addition, an increased expression of numerous genes known to be regulated by NF-kappaB have been found in the atherosclerotic lesion and NF-kappaB is markedly and selectively activated in PBMCs of patients with unstable angina pectoris [21]. On the other hand, hyperlipidemia has been shown to be a coronary risk factor. In this sense, NF-kappaB is activated in atherosclerotic plaques and PBMCs of rabbits fed with a hyperlipidemic diet [22]. Moreover, we demonstrated that postprandial lipaemia increases NF-kappaB activation in PBMCs of healthy volunteers [17] and, in the present work, we have demonstrated that a fat-enriched diet prolonged for 5 days can activate NF-kappaB in PBMCs of healthy volunteers, reinforcing the idea that diet manipulation can modulate the inflammatory state. However, we did not observe changes in lipid profile of healthy subjects after a fat-enriched diet, probably due to the fact that the collection of samples were taken during a fasting period and the short time of the study (1 week). Interestingly, we have previously demonstrated that this type of diet can modulate lipid values during postprandial lipaemia and induce NF-kappaB, probably through an increment of VLDL [17].

![Fig. 2. Changes in circulating MCP-1 after 5 days of fat-diet intake with or without ethanol beverages. Boxes represent 25th and 75th percentiles; line within boxes, median. Error bars mark 10th and 90th percentile. Values are expressed as change respect to day 0 (*p<0.05 vs. control).](image-url)
The protective effect of ethanol beverages has been primarily explained by an effect on blood lipids and platelets aggregation, resulting in a reduced coronary artery obstruction [23]. However, another explanation is that alcohol may protect against coronary heart disease through an anti-inflammatory mechanism [24]. In this regard, we have observed that the simultaneous consumption of moderate doses of some ethanol beverages containing polyphenols such as red wine, brandy or rum decreased NF-kappaB activation. However, another form of alcohol intake could not regulate NF-kappaB activity, indicating that some components of ethanol beverages should be implicated in this modulation and suggesting that alcohol per se cannot decrease NF-kappaB activation, at least in the period and conditions of our study. In this respect, all ethanol beverages tested in this work, except vodka, contain antioxidant compounds. It is known that oxidants induce NF-kappaB activation, while antioxidants, such as N-acetyl cysteine and pyrrolidine dithiocarbamate, can reduce NF-kappaB activity [11]. In this sense, recent results have demonstrated that red wine intake inhibited MCP-1 expression in cholesterol-fed rabbits, an effect partly attributed to its antioxidant compounds [25]. In addition, dealcoholized red wine reduced atherosclerosis in hypercholesterolemic rabbits [26] and in ApoE knockout mice [27], indicating that non-alcohol components of red wine have beneficial effects on vascular wall. In agreement, we have shown that ethanol beverages containing polyphenols decrease NF-kappaB activity and circulating MCP-1 concentrations, indicating that these effects might be related with the antioxidants present in these alcohol beverages. In this sense, we have previously demonstrated that two antioxidants present in red wine can reduced NF-kappaB elicited by VLDL in cultured monocytes [17]. Red wine was the only beverage that significantly diminished MCP-1 concentrations, indicating that beverages with a higher antioxidant content could have a major inhibitory effect on MCP-1 release. However, we have not observed any relation between polyphenols concentrations and reduction of NF-kappaB activation and MCP-1 levels. Although a lack of dose-effect is evident at the time of our study, it is possible that the dose-effect could be more apparent in a longer period of intervention. Is important to note that MCP-1 plasma levels have been recently associated with traditional risk factors for atherosclerosis in a large population study [28], supporting the potential role of MCP-1 as a biomarker of cardiovascular diseases. In addition, alcohol consumption or non-alcoholic components of alcohol beverages, mainly polyphenols, reduce endothelial adhesion molecule expression such as VCAM-1 and ICAM-1 [29], proteins which are also regulated by NF-kappaB.

Previous authors have reported different benefits depending of the alcohol beverage studied. In this regard, we have observed that only ethanol containing beverages with polyphenols have anti-inflammatory properties, while others have shown that moderate alcohol consumption was inversely associated with risk of cardiovascular disease independently of the type of beverage [5]. Furthermore, Estruch et al. have demonstrated that red wine and gin induced an anti-inflammatory response decreasing fibrinogen and IL-1α in healthy men [30]. However, in the same study, red wine intake also decreased other proinflammatory proteins such as C-reactive protein, VCAM-1, ICAM-1 and MCP-1, indicating that red wine intake may provide additional benefits. In addition, increment of HDL cholesterol has been associated with moderate alcohol intake independently of the type of alcohol beverage [31]. However, in our study, we have not observed changes in HDL concentrations, probably for the short period of the study. Reduction of other inflammatory markers have been associated with alcohol consumption. Mukamal et al. have demonstrated that alcohol intake diminished white blood cell count and factor VIIIc in older adults [32]. In agreement, moderate alcohol consumption has been associated with lower C-reactive protein concentrations, an effect that was independent of alcohol-related effects on lipids [33].

Finally, it is important to remind that the relation between alcohol consumption and clinical outcomes in human showed a J-shaped curve, indicating that the protective effect of beverages is only related with light/moderate drinking, whereas cardiovascular mortality is increased in heavy drinkers [34].

5. Conclusion

Some ethanol containing beverages diminished NF-kappaB activation and circulating MCP-1 concentrations during a fat-enriched diet in healthy volunteers, an effect probably related with the antioxidant compounds present in these beverages. Our results provide additional evidence that consumption of ethanol beverages containing polyphenols could have anti-inflammatory effects, supporting the idea that its light/moderate use may reduce cardiovascular mortality.

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References

rosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arte-