Red Wine Intake Prevents Nuclear Factor-κB Activation in Peripheral Blood Mononuclear Cells of Healthy Volunteers During Postprandial Lipemia

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Background—Several epidemiological studies have demonstrated the beneficial effect of red wine intake in reducing total and cardiovascular mortality. This effect has been attributed in part to its antioxidant properties. Because the monocytes/macrophages and the nuclear transcription factor κB (NF-κB) are implicated in the pathogenesis of atherosclerotic lesions, we examined the effect of red wine intake on the activation of NF-κB in peripheral blood mononuclear cells.

Methods and Results—Sixteen healthy volunteers were studied 3 times each: after a moderate dose, a low dose, and no wine with a fat-enriched breakfast. Lipid profile and NF-κB activation (electrophoretic mobility shift assay) were examined in blood samples taken before and 3, 6, and 9 hours after wine intake. In addition, mononuclear cells were incubated with VLDL in the presence of some antioxidants (quercetin and α-tocopherol succinate) contained in red wine to study their effects on NF-κB activation. Subjects receiving a fat-enriched breakfast had increased NF-κB activation in peripheral blood mononuclear cells coinciding with the augmentation in total triglycerides and chylomicrons. Red wine intake prevented NF-κB activity even though it induced a certain increase in serum lipids, particularly VLDL, that did not increase after the fat ingestion alone. However, another form of alcohol intake (vodka) did not modify the NF-κB activation provided by postprandial lipemia. In cultured mononuclear cells, isolated human VLDL caused NF-κB activation in a time-dependent manner that did not occur in the presence of the red wine antioxidants quercetin and α-tocopherol.

Conclusions—Our results provide a new potential mechanism to explain the beneficial effects of red wine intake in the reduction of cardiovascular mortality. (Circulation. 2000;102:1020-1026.)

Key Words: antioxidants ■ blood cells ■ molecular biology ■ atherosclerosis

Many epidemiological studies have shown that moderate red wine intake decreases cardiovascular mortality and incidence of ischemic stroke.1-3 Different classes of antioxidants present in red wine, such as quercetin, catechin, and α-tocopherol succinate,4 are able to modify the lipid peroxidation of LDL.5 Dietary supplementation with red wine has been associated with increased resistance of plasma LDL to oxidation and an increment in human plasma antioxidant capacity.6,7 The potent antioxidant activity of phenolic substances, particularly in red wine, has been proposed as an explanation of the "French paradox,"8 ie, the coexistence of a high-fat diet with a low incidence of coronary heart disease.9 A recent study demonstrated that red wine, catechin, or quercetin consumption exerts an inhibitory effect on the development of aortic lesions in atherosclerotic apolipoprotein E–knockout mice.10

Nuclear factor (NF)-κB is a redox-sensitive transcription factor that is involved in the transmission of various signals from the cytoplasm to the nucleus of numerous cell types.11 It is found as a trimer consisting of p50, p65, and IκB subunits in the cytosol. The release of IκB from the trimer results in the migration of the p50/p65 heterodimer to the nucleus and the subsequent DNA binding.12 This process activates genes involved in the immune, inflammatory, or acute-phase response, such as cytokines (monocyte chemoattractant protein-1, interleukin-8), adhesion molecules, and procoagulant proteins (tissue factor, plasminogen activator inhibitor 1). A variety of stimuli have been found to induce NF-κB activation, including phorbol myristate acetate, oxidized LDL, and cytokines such as tumor necrosis factor-α, lymphotixin, and interleukin-1,13 whereas other agents, including antioxidants,14 statins,15 and glucocorticoids, inhibit NF-κB.
activation. Recent data strongly suggest that NF-κB could be involved in the pathogenesis of atherosclerosis. NF-κB is present in the human atherosclerotic lesions in the nuclei of macrophages and endothelial cells and participates in dysregulation of vascular smooth muscle cells in human atherosclerosis. Conversely, accumulating evidence suggests that postprandial lipemia is strongly associated with a risk of development of atherosclerotic lesions. In this study, we have shown that a fat-enriched breakfast increases triglycerides and chylomicrons, whereas the simultaneous consumption of red wine was associated with an increment of total triglycerides, chylomicrons, and VLDL triglycerides. Postprandial lipemia was correlated with an increment of NF-κB activation in peripheral blood mononuclear cells (PBMCs) that was prevented by red wine intake. An intake of another form of alcohol, vodka, did not prevent the activation of this transcription factor provided by postprandial lipemia. Because VLDLs were the only lipoproteins that were augmented with red wine intake but not after the fat ingestion alone, we tested the effect of VLDLs on NF-κB activation. VLDLs elicited an increase in NF-κB activation in human mononuclear Thp-1 cells that was prevented by coinbucation with quercetin and α-tocopherol succinate, 2 antioxidants contained in red wine. Because NF-κB regulates many genes involved in the pathogenesis of coronary artery disease, these results provide a new explanation of the potential beneficial effects of moderate consumption of red wine in human beings.

Methods

Study Subjects

Eight men and 8 women, 22 to 33 years old, were included in this study. Participants (medical students or young doctors) were examined to exclude any pathological disorder confirmed by blood test, including an abnormal lipid profile. Those who had plasma cholesterol levels >220 mg/dl or plasma triglyceride 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. All participants gave written informed consent for the study.

Study Design

On 3 different days, all participants received the same fatty breakfast. On each occasion, a different dose of red wine was given: moderate intake, low intake, and no intake (see below). In the 3 situations, the caloric intake was maintained constant, with sugar added when the wine consumption was low or none. The 3 phases in each case were separated by 2 to 4 weeks. In addition, in a new group, 11 of 16 volunteers received vodka at 200g for 30 minutes. PBMCs were collected, washed twice with cold PBS, and resuspended in buffer A (see Protein Extraction). Approximately 95% of the cells were mononuclear cells (flow cytometry, not shown).

Diet

The standard fat-enriched breakfast contained 602 kcal/m2, with 342 kcal/m2 (56.8%) as fat. It was prepared with common solid and liquid foods: white bread, ham, margarine, coffee, and whole milk. The red wine was aged in the barrel for 3 years. In men, wine represented an extra 140 kcal/m2 (20 g/m2) in the moderate dose and 84 kcal/m2 (12 g/m2) in the low dose. According to National Cholesterol Education Program recommendations, women received a lower dose (40% reduction), 84 kcal/m2 (12 g/m2) and 50 kcal/m2 (7.2 g/m2), respectively. As a whole, male participants received a total of 742 kcal/m2 and female participants 686 kcal/m2. A calculated amount of sugar was added to the milk in the case of no wine or a low dose of wine to keep the total amount of calories constant. In all cases, saccharine was allowed for sweetness of the liquid intake.

Lipoprotein Profile and VLDL Isolation

Lipoprotein profile was measured by standard techniques, and VLDLs were isolated from pooled plasma from healthy blood bank donors. Potential endotoxin contamination of lipoproteins was monitored with the chromogenic limulus amoeobocyte lysate assay (Bio-Whitaker) using Escherichia coli endotoxin supplied with the kit for the standard curve. Samples with endotoxin >2.5 pg/ml of protein were discarded.

Cell Culture

Thp-1 cells (human monocytic cell line) were obtained from the American Type Culture Collection (TIB 202) (ATCC) and cultured in RPMI medium (BioWhitaker) containing penicillin (100 U/mL), streptomycin (100 μg/mL), and glucose (2 mmol/L) with 10% FBS (BioWhitaker). Quercetin and α-tocopherol succinate were obtained from Sigma Chemical Co and DMSO from Merck. Quercetin was dissolved in DMSO and α-tocopherol succinate in ethanol.

Isolation of PBMCs

The blood samples were diluted 1:1 in PBS, and cells were separated in 5 mL Ficoll gradient (lymphocyte isolation solution, Rafer) by centrifugation at 2000g for 30 minutes. PBMCs were collected, washed twice with cold PBS, and resuspended in buffer A (see Protein Extraction). Approximately 95% of the cells were mononuclear cells (flow cytometry, not shown).

Protein Extraction and Electrophoretic Mobility Shift Assays

Proteins of Thp-1 and PBMCs were extracted as described. Briefly, Thp-1 cells were made quiescent for 24 hours in 0.5% FBS medium, and then 4×10^6 cells were incubated with the stimuli for different periods of time. Peripheral blood mononuclear or Thp-1 cells were collected, washed with cold PBS, and resuspended in 5 cell-pellet volumes buffer A (in mmol/L: HEPES 10 [pH 7.8], KCl 15, MgCl2, 2 EDTA 0.1, DTT 1, and PMSF 1) and homogenized. Nuclear proteins were extracted for 10 minutes and resuspended in 2 volumes buffer A. Then 3 mol/L KCl was added drop by drop to reach 0.39 mol/L KCl. Nuclear proteins were extracted for 1 hour at 4°C and centrifuged at 100 000g for 30 minutes. 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 bichinchoninic assay method (Pierce).

Gel shift assays were performed with a commercial kit according to the instructions of the manufacturer (Promega). Briefly, NF-κB consensus oligonucleotide (5’-AGTTGAGGGGACTTTCC-3’) was end-labeled with ^32P by incubation with 10 U T4 polynucleotide kinase (Promega) in a reaction containing 10 μCi (^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 minutes before the addition of the labeled probe. The labeled probe (0.35 pmol) was added to the reaction. For supershift assays, 1 μg anti-p65 (Santa Cruz Biotechnology Inc) or anti-p50 (Chemikon) antibodies were added and incubated for 1 hour. Nuclear extracts of HeLa cells were used as positive control. The gel was dried and exposed to X-ray film. The autoradiograph was subjected to densitometry with an Image Quant densitometric scanner (Molecular Dynamics). Percentages were calculated in relation to the basal value in each subject and situation.
Statistical Analysis

Results are expressed as mean±SD (unless specified). Significance was established with GraphPad InStat (GraphPAD Software). Student’s t test and 2-way ANOVA were used to compare the data. Differences were considered significant when P<0.05.

Results

Red Wine and Postprandial Lipemia

To test the effect of red wine on postprandial lipemia, blood samples of healthy volunteers were collected before and 3, 6, and 9 hours after a fat-enriched breakfast with or without red wine intake. Lipid profile revealed an increment of total triglycerides and chylomicrons after a fat-enriched breakfast (Figure 1), and red wine intake was associated with a further augmentation in total triglycerides at 3 hours (P<0.05, no wine versus moderate dose) and at 6 and 9 hours (P<0.05, moderate vs no wine and low dose). Chylomicrons triglycerides. Red wine consumption increases chylomicrons at 3 hours (P<0.05, no wine vs moderate dose). VLDL triglycerides. VLDL increases after red wine intake at 3 hours (P<0.05, no wine vs low and moderate dose) and 6 hours (+P<0.05, no wine and low vs moderate dose). Results are mean of all volunteers.

Postprandial Lipemia and NF-κB

To examine whether postprandial lipemia could activate NF-κB, blood samples of healthy volunteers were taken before and 3, 6, and 9 hours after a fat-enriched breakfast. As shown in Figure 2, electrophoretic mobility shift assay (EMSA) of nuclear proteins from PBMCs showed a retarded band that was increased at 6 and 9 hours (2.5- and 3-fold versus basal; P<0.05). This band was displaced with a 100 molar excess of unlabeled NF-κB oligonucleotide (Figure 3), whereas no displacement was observed with the unlabeled and unrelated oligonucleotide AP-1. A supershifted band was revealed by incubation of nuclear extracts with anti-p50 or anti-p65 antibodies but not with anti–c-Rel antibody. These results indicated that the heterodimer p50/p65 was implicated in the activation of NF-κB in PBMCs.

Red Wine Intake and NF-κB Activation

To study the effects of red wine intake on NF-κB activity, blood samples of volunteers were taken before and 3, 6, and 9 hours after a fat-enriched breakfast with 2 different doses of red wine; PBMCs were extracted; and nuclear proteins were obtained. As shown in Figure 2, red wine intake prevented NF-κB activation at 6 and 9 hours (P<0.05, no wine versus low and moderate dose).

Vodka Intake and NF-κB Activation

To test whether alcohol intake could prevent NF-κB activation, blood samples of volunteers were taken before and after
a fat-enriched breakfast with another form of alcohol intake (vodka) in an amount equal to that of the low dose of red wine. Lipid profiles did not reveal significant changes in relation to the low dose of red wine. However, we observed (Figure 2) that vodka intake did not modify the increment of NF-κB activation provided by postprandial lipemia (\( P<0.05 \) vodka versus low and moderate dose).

**VLDL, Wine Antioxidants, and NF-κB Activation**

VLDL is the only lipoprotein that increases in serum after red wine intake but not after the fat ingestion alone. In this sense, we examined the effect of native VLDL on NF-κB activity in the human mononuclear cell line Thp-1. Those cells were made quiescent for 24 hours in 0.5% FBS, and then 4\( \times \)10^6 cells were incubated in the presence of 10 μg/mL human VLDL (a concentration similar to that found in human plasma). Cells were collected at 0, 3, 6, and 9 hours, and nuclear proteins were extracted. As shown in Figure 4, EMSA revealed a retarded band in the nuclear extracts from PBMCs. The intensity of the retarded band, indicated by an arrow, was markedly increased at 6 and 9 hours of incubation with VLDL (3.5- and 5-fold, respectively; \( P<0.05 \) versus basal).

Because these results suggested that red wine was able to intercept lipid-mediated NF-κB activation, we next studied the effect of some wine antioxidants on Thp-1 cells treated with VLDL. The treatment of Thp-1 with VLDL (10 μg/mL) and the antioxidants contained in red wine, such as quercetin and \( \alpha \)-tocopherol succinate (100 μmol/L), inhibited VLDL activation of NF-κB (Figure 4). Neither ethanol nor DMSO had any effect on NF-κB activation (not shown).

**Discussion**

In this article, we analyze the effect of red wine intake on postprandial lipemia and NF-κB activation after a fat-enriched breakfast. NF-κB is a ubiquitous transcription factor that participates in various transcriptionally controlled processes, such as cytokine responsiveness, inflammation, and cell growth control. Recent studies strongly suggest that the inducible NF-κB is involved in the pathogenesis of athero-
We observed that a fat-enriched breakfast caused a postprandial lipemia, particularly related to triglyceride-rich lipoproteins, and that red wine intake increased this postprandial lipemia. In addition, a fat-enriched breakfast increased NF-κB activation in a time-dependent manner in PBMCs as a result of the translocation of p50 and p65 subunits. Interestingly, the simultaneous consumption of red wine in moderate amounts avoided the activation of NF-κB despite the increment in chylomicrons and VLDL and total triglycerides. However, another form of alcohol intake (vodka) did not prevent the NF-κB activation, suggesting that alcohol could not regulate the activation of NF-κB. It has just been demonstrated that red wine that is aged in the barrel exerts a vasodilatory effect within a concentration rate that could be reached in vivo by moderate wine consumption, and moderate alcohol intake has been associated with a significant decrease in total mortality. Furthermore, monocytes are involved in the progression of atherosclerosis and are potent activators of blood coagulation through their ability to synthesize procoagulant factors (plasminogen activator inhibitor-1, tissue factor) that are regulated by NF-κB. Conversely, it is known that oxidants increase NF-κB activation, whereas such antioxidants as pyrrolidine dithiocarbamate and N-acetyl cysteine inhibit NF-κB activation. Because of the redox regulation of NF-κB, it is possible that the antioxidants contained in red wine were the cause of the inhibition of NF-κB activation. In this sense, Feng et al demonstrated that red wine intake inhibited monocyte chemoattractant protein-1 expression in cholesterol-fed rabbits, a protein regulated by NF-κB, and this effect might be partly attributed to its antioxidant effects. In addition, catechin and vitamin E prevent the development of fatty streak in hypercholesteremic hamsters and attenuate early lesion development in rabbits. Moreover, red wine and nonalcoholic wine products can prevent plaque formation in hypercholesterolemic rabbits despite significant increases in LDL. Also, red wine polyphenols inhibit proliferation of vascular smooth muscle cells and reduce the susceptibility of low-density lipopro-
teins to oxidation in vitro\textsuperscript{33} and in vivo.\textsuperscript{34} However, in patients at high risk for cardiovascular events, treatment only with vitamin E had no apparent effect on cardiovascular outcomes,\textsuperscript{35} at least during the years of this study.

Conversely, VLDL was the only lipoprotein that increased with red wine intake but not after the fat ingestion alone in our study. Because of its potential in vivo effect on the phenomenon described, we also studied the effect of VLDL on NF-κB activation in cultured mononuclear cells because they have been implicated in the early events of the pathogenesis of atherosclerosis. The treatment of Thp-1 cells with native VLDL increased NF-κB activation in a time-dependent manner. Furthermore, other authors have observed that native VLDLs induce NF-κB activation in endothelial cells\textsuperscript{36} and that oxidized VLDLs also induce the activation of this transcription factor in PC12 cells.\textsuperscript{37} These results could provide an explanation of NF-κB activation by postprandial lipemia. In addition, 2 antioxidant compounds present in red wine, quercetin and α-tocopherol succinate, inhibited this activation, and ethanol did not modify NF-κB activation induced by VLDL (not shown). In this sense, α-tocopherol succinate, a vitamin E derivative, inhibits NF-κB activation in Thp-1 cells induced by lipopolysaccharide,\textsuperscript{38} and quercetin inhibits interleukin-8 and monocyte chemotactant protein-1 expression in synoviocytes.\textsuperscript{39} However, Wadsworth et al\textsuperscript{40} reported that quercetin and resveratrol did not inhibit lipopolysaccharide-induced activation of NF-κB in RAW 264.7 cells. It is possible that different cells, activators of NF-κB, and incubation conditions of antioxidants could provide different results. Suzuki et al\textsuperscript{41} demonstrated that antioxidants did not block NF-κB activation induced by some activators of this transcription factor. These results correlate with the data obtained in vivo and support the hypothesis that the beneficial effects of red wine are a result of its antioxidant capacity.

In conclusion, red wine intake, but not another form of alcohol beverage intake (vodka), prevents NF-κB activation in PBMCs elicited in healthy volunteers by postprandial lipemia. Because NF-κB activation is involved in the pathogenesis of atherosclerotic lesions, the inhibitory effect of red wine on NF-κB activation provides a further explanation of the beneficial effects of red wine intake in cardiovascular disease.

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