Differential Effects of Glucose and Alcohol on Reactive Oxygen Species Generation and Intranuclear Nuclear Factor-κB in Mononuclear Cells

Sandeep Dhindsa, Devjit Tripathy, Priya Mohanty, Husam Ghanim, Tufail Syed, Ahmad Aljada, and Paresh Dandona

It has previously been shown that oral intake of 300 calories of glucose (75 g), lipid, or protein increases reactive oxygen species (ROS) generation by polymorphonuclear cells (PMNL) and mononuclear cells (MNCs). We investigated the effects of 75 g glucose on proinflammatory transcription factor, nuclear factor-κB (NFκB), in mononuclear cells. To further investigate whether the effects of macronutrient-induced oxidative stress are due to consumption of calories or are nutrient specific, we investigated the effects of acute oral challenge of equicaloric amounts of alcohol (300 calories) on ROS generation and NF-κB activation in MNCs and PMNL and compared them with those of glucose and water (control). Sixteen normal healthy adult volunteers were given either vodka (10 subjects), glucose solution (10 subjects), or 300 mL water (7 subjects). Vodka and glucose drinks were equivalent to 300 calories. We measured ROS generation and intranuclear NF-κB activation by PMNL cells and MNCs at 1 hour, 2 hours, and 3 hours following ingestion. ROS generation by both MNC and PMNL increased significantly ($P < .05$ for MNC and $P < .01$ for PMNL) following intake of glucose solution, but did not change significantly following alcohol or water. NF-κB binding activity in MNC nuclear extracts also increased ($P < .001$) following ingestion of glucose solution, but did not change after the administration of alcohol or water. We conclude that (1) 75 g oral glucose increases NF-κB binding activity in MNCs. (2) While 75 g glucose (300 calories) induces an increase in ROS generation and intranuclear NF-κB, equicaloric amounts of alcohol did not produce these effects.

WE HAVE PREVIOUSLY shown that oral intake of 75 g glucose (300 calories) induces an oxidative state by increasing reactive oxygen species (ROS) generation by polymorphonuclear (PMNL) cells and mononuclear cells (MNCs).[1] Oral intake of cream or protein also causes a significant increase in ROS.[2] A 48-hour fast in normal subjects results in a reduction of ROS generation and oxidative damage to proteins, amino acids, and lipids.[3] The state of obesity is associated with low-grade inflammation,[4] and oxidative stress and 4 weeks of energy (calorie) restriction (4,184 kJ/d) in the obese results in a marked reduction of ROS generation and oxidative damage to proteins, amino acids, and lipids.[5] Similarly long-term calorie restriction results in a decrease of plasma tumor necrosis factor-α (TNF-α) concentration, which is known to be elevated in the obese.[6] Thus, macronutrient intake is a major determinant of ROS generation and oxidative stress.

Nuclear factor-κB (NF-κB), a heterodimer of p65 (Rel A) and p50 proteins is a proinflammatory transcription factor located in the cytosol, where it is bound to inhibitor κB (IκB). Inflammatory signals, such as endotoxin and proinflammatory cytokines, cause phosphorylation and ubiquitination of IκB, thus liberating and activating NF-κB. This allows NF-κB to translocate to the nucleus and to activate transcription of genes that are involved in the inflammatory response, such as proinflammatory cytokines, and adhesion molecules. NF-κB is modulated by the redox status in the cell and ROS are known to activate NF-κB.[7-11] Because glucose intake activates ROS generation, we decided to investigate the effect of 75 g glucose intake on NF-κB binding activity in MNCs.

Excessive consumption of calories results in obesity; and obesity is a known to be a proinflammatory state. However, it is not clear whether the oxidative stress that follows macronutrient intake is due to consumption of calories or is specific to the type of macronutrient ingested.

Alcohol is consumed in sufficient amounts all over the world to be considered a significant contributor to the caloric intake in humans. We, therefore, investigated whether the intake of 2 U alcohol (300 calories) has a different effect on oxidative stress and inflammatory mechanisms as compared with the effects of an intake of glucose equivalent in energy to 2 U alcohol. We also investigated the effects of ingestion of equivalent volume of water as a control arm in the study.

SUBJECTS AND METHODS

Twenty-seven normal, healthy, adult volunteers (ages 23 to 36 years; mean 28 ± 1.8) were recruited into our study. All were nonsmokers, normotensive, had a normal lipid profile, normal renal and liver function tests, and were not on any medications. All patients gave their written, informed consent. Institutional Review Board of our hospital approved the study protocol.

Ten subjects (7 men and 3 women; body mass index [BMI]: 23 ± 1) participated in the alcohol study and 10 subjects (8 men and 2 women; BMI: 23 ± 1) in the glucose study. Four men and 3 women participated in the water challenge study (BMI: 22.7 ± 1.2). Subjects arrived in the morning to our Clinical Research Center after having fasted overnight. A baseline blood sample was taken. Subjects were then given alcohol, glucose solution, or water.

Alcohol was given as vodka, 2 mL/kg body weight (corresponding to 0.8 mg/kg ethanol). This is roughly equivalent to 2 regular drinks of alcohol, corresponding to 300 calories of alcohol. The drink was mixed with 300 mL water. The dose of alcohol was consumed evenly over 10 minutes. Blood samples were then drawn hourly for the next 3 hours.

Glucose solution was given as 75 g glucose dissolved in 300 mL

From the Division of Endocrinology, Diabetes, and Metabolism, State University of New York at Buffalo and Kaleida Health, Buffalo, NY.

Submitted March 10, 2003; accepted October 30, 2003.

Address reprint requests to Paresh Dandona, BSc, MBBS, DPhil, FRCP, Director, Diabetes-Endocrinology Center of Western NY, Chief of Endocrinology, State University of New York at Buffalo, 3 Gates Circle, Buffalo, NY 14209.

0026-0495/04/5303-0014$30.00/0
doi:10.1016/j.metabol.2003.10.013

water (Glucola) to drink over 5 minutes. Blood samples were then drawn hourly for the next 3 hours.

A total of 300 mL water was given to the subjects in the water challenge arm of the study, and blood samples were then drawn hourly for the next 3 hours.

MNC and PMNL Isolation

Blood samples were collected in Na-EDTA as an anticoagulant. A total of 3.5 mL of the anticoagulated blood sample was carefully layered over 3.5 mL of the PMN isolation medium (Robbins Scientific, Sunnyvale, CA). Samples were centrifuged at 400 × *g* in a swing out rotor for 30 minutes at 18°C. At the end of the centrifugation, 2 bands separate out at the top of the red blood cell (RBC) pellet. The top band consists of MNC, while the lower consists of PMNL. The MNC band was harvested with a Pasteur pipette, washed with Hank's balanced salt solution (HBSS), and reconstituted to a concentration of 4×10^5 cells/mL in HBSS. This method provides yields greater than 95% pure MNC suspension.

Measurement of ROS Generation

Respiratory burst activity of PMNLs and MNCs was measured by detection of superoxide radical via chemiluminescence.[12,13] A total of 500 μL PMNLs or MNCs (2×10^5 cells) was delivered into a Lumiaggregometer (CHRONO-LOG Corp, Havertown, PA) plastic flat-bottom cuvette to which a spin bar was added. Fifteen microliters of 10 mmol/L luminol was then added, followed by 1 μL of 10 mmol/L formylmethionylleucinylphenylalanine (FMLP). Chemiluminescence was recorded for 15 minutes (a protracted record after 15 minutes did not alter the relative amounts of chemiluminescence produced by various cell samples). Our method, developed independently, is similar to that published by Tosi and Hamedani.[14] The interassay coefficient of variation (CV) for this assay is 6%. We have further established that in our assay system, there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and catalase: superoxide dismutase inhibited chemiluminescence by 82% at 10 μg/mL, whereas catalase inhibited chemiluminescence by 47% at 40 μg/mL. Chemiluminescence is also inhibited by diphenyleneiodonium chloride (data not shown), a specific inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the enzyme responsible for the production of superoxide radicals.[15] Our assay system is exquisitely sensitive to diphenyleneiodonium chloride at nanomolar concentrations.

NF-κB Electrophoretic Mobility Shift Assay

NF-κB gel retardation assay was performed as previously described.[16,17] DNA-binding protein extracts were prepared from MNC by the method described by Andrews et al.[18] Total protein concentrations were determined using bicinchoninic acid (BCA) protein assay (Pierce, Rockland, IL). NF-κB gel retardation assay was performed using NF-κB binding protein detection kit (Life Technologies, Long Island, NY). Briefly, the double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the NF-κB binding site was radiolabeled with γ-P^{32} by T4 kinase. Then, 5 μg of the nuclear extract was mixed with the incubation buffer, and the mixture was preincubated at 4°C for 15 minutes. Labeled oligonucleotide (60,000 cpm) was added, and the mixture was incubated at room temperature for 20 minutes. Samples were then applied to wells of 6% nondenaturing polyacrylamide gel. The gel was dried under vacuum and exposed to x-ray film. Densitometry was performed using Bio-Rad (Hercules, CA) molecular analyst software. Specifity of the band was tested by supershifting the band with antibody against p65 subunit, p50 subunit, and p75 (c-Rel) of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA).

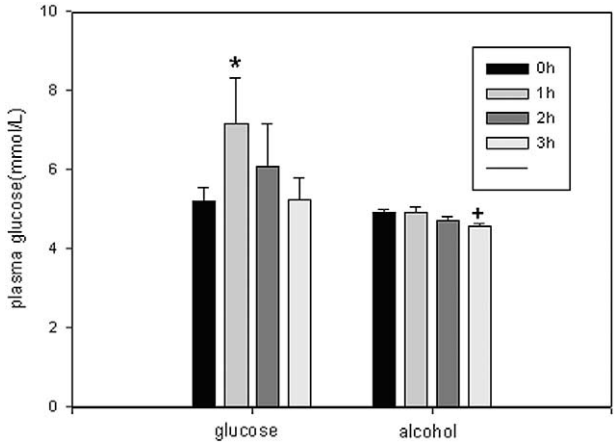

Fig 1. Plasma glucose concentrations (mmol/L) at 0, 1, 2, and 3 hours following intake of glucose or alcohol. *$P < .05$ *v* zero hour.

Insulin, Glucose, and Alcohol Measurement

Plasma insulin was measured from fasting plasma samples using an enzyme-linked immunosorbent assay kit (Diagnostics Systems Laboratories, Webster, TX). Plasma glucose was measured in whole blood by a Hemocue glucose analyzer (Hemocue, Mission Viejo, CA). Serum alcohol levels were measured by alcohol reagent (ALC)-induced change in NADH absorbance (Synchron LX system, Brea, CA).

Statistical Analysis

Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). All data are expressed as mean ± SEM. All data on ROS generation and NF-κB were normalized to a baseline of 100% in view of the interindividual variability and are expressed accordingly as percent of the baseline. Analysis was performed with 1-factor analysis of variance (ANOVA). Dunnett's method was used for comparison against the baseline. Paired *t* test was used to compare the change in glucose levels and insulin values. $P < .05$ was considered significant.

RESULTS

Plasma Variables

The mean plasma alcohol concentration in the alcohol group was 0 at zero hour, 111 ± 7.8 mg/dL (24.1 ± 1.7 mmol/L) at 1 hour, 96 ± 8.9 mg/dL (20.8 ± 1.9 mmol/L) at 2 hours, and 79 ± 11.4 mg/dL (17.1 ± 2.5 mmol/L) at 3 hours after alcohol intake. Glucose and insulin concentrations tended to decrease following alcohol, and the decrease was significant at 3 hours (4.89 ± 0.11 mmol/L at zero hour *v* 4.56 ± 0.08 mmol/L at 3 hours for glucose ($P < .02$); 71.65 ± 11 pmol/L at zero hour *v* 54.47 ± 11.57 pmol/L at 3 hours for insulin; $P < .001$). Glucose and insulin concentrations increased significantly at 1 hour following glucose (Figs 1 and 2). There was no change in insulin or glucose concentrations following water challenge (data not shown).

ROS Generation

ROS generation by both MNC and PMNL increased significantly following intake of glucose solution and remained higher than baseline until the end of 3 hours ($P < .05$ for MNC

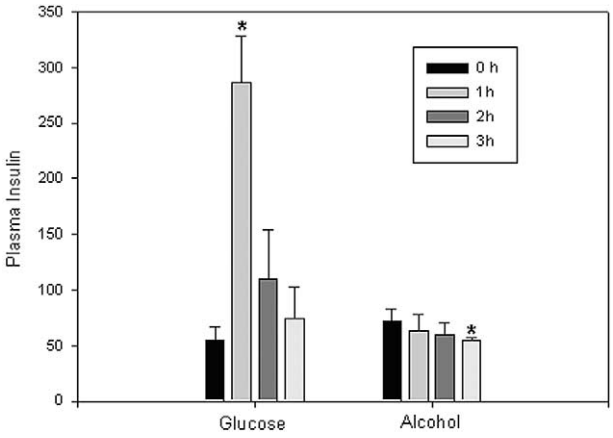

Fig 2. Plasma insulin concentrations (pmol/L) at 0, 1, 2, and 3 hours following intake of glucose or alcohol. *$P < .001$ as compared with baseline (zero hour).

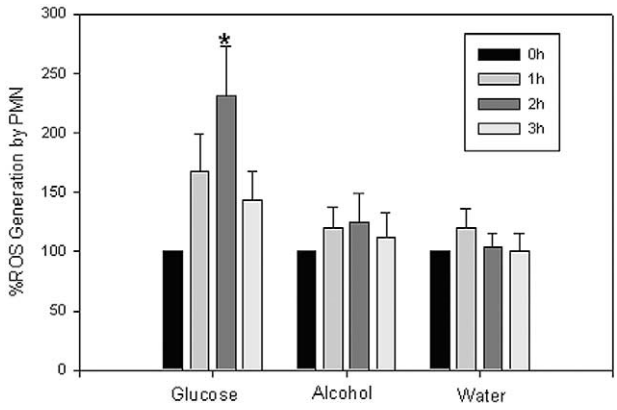

Fig 4. ROS generation by PMN at 0, 1, 2, and 3 hours following intake of glucose, alcohol, or water. Results are expressed as % increase over baseline. Baseline reading (= 100%): 144 ± 43 mV for glucose; 185 ± 57 mV for alcohol; 195 ± 84 mV for water; *$P < .01$ as compared with baseline (zero hour).

and $P < .01$ for PMNL). This is consistent with our previously published results.[1] ROS generation did not change significantly following the administration of alcohol or water (Figs 3 and 4).

Intranuclear NF-κB Binding Activity

NF-κB binding activity in MNC nuclear extracts increased following ingestion of glucose solution ($P < .001$). Following alcohol challenge, NF-κB binding activity was quite variable, but did not change significantly ($P = .241$). There was no change in NF-κB binding after the administration of water (Fig 5).

DISCUSSION

Our data show clearly that the intake of 75 g glucose (300 calories) causes an increase in ROS generation by MNC and PMNL cells. These observations are consistent with our previous demonstration that glucose, lipid, or protein challenge at an energy equivalent of 300 calories causes a significant increase in ROS generation: a 140% increase for glucose,[1] 100% for lipid, and 80% for protein over the basal levels.[2] Both glucose and lipids also induce lipid peroxidation. We also show, for the first time in humans in vivo, that the intake of glucose induces

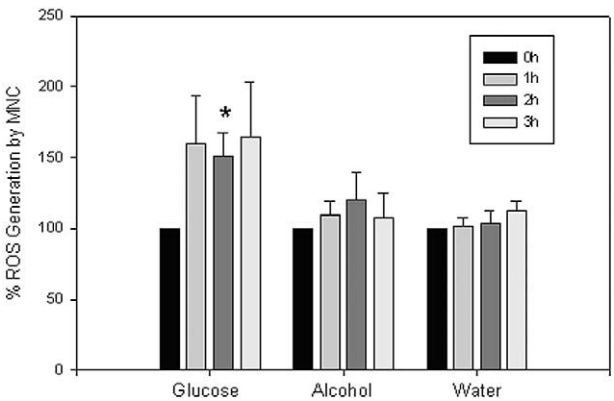

Fig 3. ROS generation by MNC at 0, 1, 2, and 3 hours following intake of glucose, alcohol, or water. Results are expressed as % increase over baseline. Baseline reading (= 100%): 130.2 ± 29 mV for glucose; 186 ± 44.9 mV for alcohol; 166.7 ± 16.7 mV for water; *$P < .05$ as compared with baseline (zero hour).

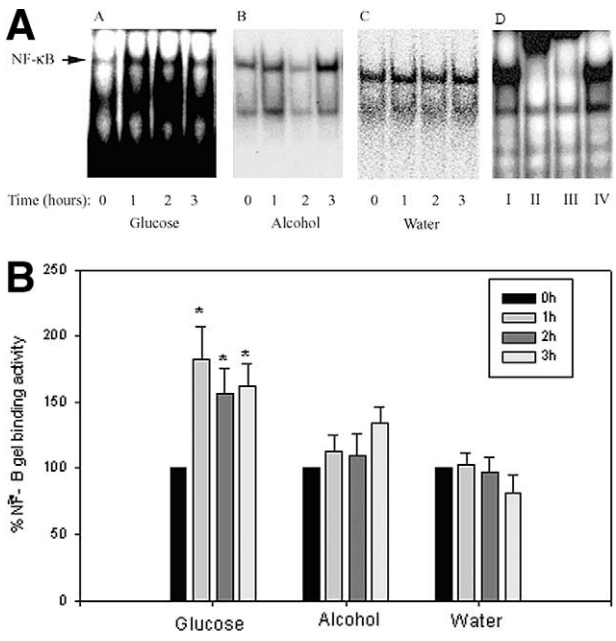

Fig 5. (A) A representative electrophoretic mobility shift assay (EMSA) gel showing NF-κB binding activity levels in MNC nuclear protein extracts following (A) glucose, (B) alcohol, and (C) water intake. The sequence-specificity of the protein-DNA interactions was determined by super-shift EMSA. (D) Lane (I) shows NF-κB binding activity in nuclear extract for 1 of the subjects; lane (II) super-shift using an antibody against p50 subunit of NF-κB; lane (III) against p65 subunit of NF-κB; lane (IV) p75 (c-Rel) of NF-κB. (B) Densitometric analysis of NF-κB binding activities. All values were normalized to 100% for baseline time point and the following values were expressed as percent of basal level. The results are presented as mean ± SE (*$P < .001$ as compared with baseline [zero hour]).

an increase in intranuclear NF-κB binding activity in monocytes.

The increase in ROS generation and NF-κB following macronutrient intake is relevant to the fact that obesity is characterized by increased oxidative stress and inflammation, which are partially reversed by weight loss even over a relatively short period of 4 weeks.[4,5] Thus, obesity may reflect a cumulative pro-oxidant and proinflammatory effect of chronic excessive intake of macronutrients. Excessive intake of calories results in obesity; and obesity is a known risk factor for atherosclerosis. Because atherosclerosis is considered to be an inflammatory condition, activation of NF-κB following intake of glucose suggests a causative role for glucose and calorie intake in the development of inflammation, and possibly, atherosclerosis.

An equal amount of energy (300 calories) consumed as alcohol, however, did not cause an increase in ROS generation by PMNL or MNC, nor did it change intranuclear NF-κB binding in MNCs. Thus, while the major dietary macronutrients cause oxidative stress and even inflammation, equicaloric amounts of alcohol do not either activate ROS generation in leukocytes or induce an increase in intranuclear NF-κB. This is of importance because our data demonstrate for the first time that macronutrient-induced oxidative stress and inflammation is not calorie intake–dependent, but is dependent upon specific macronutrients. In the context of obesity and inflammation, it is relevant that there is at least one food, "alcohol," which does not induce either ROS generation or inflammatory activity.

Because NF-κB binding is modulated by the redox status in the cell and ROS are known to activate it,[7-11] it is possible that the absence of an increase in ROS generation following alcohol is responsible for the absence of an increase in NF-κB.

It is also of interest that modest alcohol intake has been shown to be associated with diminished cardiovascular mortality in several studies.[19-22] Although our studies do not demonstrate an inhibitory effect of alcohol on either oxidative stress or inflammation acutely in MNC, it does not cause a stimulation of these processes either, despite the energy intake inherently associated with alcohol ingestion. Further investigations are necessary to determine whether alcohol has other subtle effects on inflammatory processes and oxidative stress. One mechanism that we have previously shown to be relevant to cardiovascular disease is the inhibition of platelet aggregation and platelet thromboxane A2 synthesis by acute intake of alcohol.[23] Chronic alcohol intake is also associated with favorable changes in hemostatic and lipoprotein profile,[24] lower C-reactive protein (CRP) levels as compared with nondrinkers and heavy drinkers[25] and with platelet hypopaggregability.[26]

It has been shown in vitro that alcohol inhibits lipopolysaccharide-, interleukin (IL)-1–, and TNF-α–induced NF-κB activation in monocytes and does not result in induction of p65/p50 NF-κB heterodimer in unstimulated monocytes.[27,28] It is possible that alcohol may inhibit NF-κB in humans in vivo under conditions of proinflammatory stress, such as obesity.

Red wine has been shown (in doses containing 20 to 35 g ethanol) to inhibit postprandial increase of NF-κB in MNCs after a 602 kcal/m^2 meal.[29] This effect may be related to the flavonoid content of red wine.[30,31] Our experiments, on the other hand, avoid interference from other micronutrients.

Plasma glucose concentrations decreased significantly 3 hours after alcohol ingestion. Ethanol inhibits gluconeogenesis and is known to cause hypoglycemia, especially after an overnight fast or during states of malnutrition when liver glycogen stores are low.[32] Insulin concentrations also decreased significantly 3 hours after alcohol intake, and this is probably due to the decrease in plasma glucose.

In conclusion, 75 g glucose causes an increase in both ROS generation and intranuclear NF-κB binding in MNCs. Alcohol intake equivalent in calories to 75 g glucose does not have the effect of producing oxidative stress and inflammation as measured by NF-κB activation. This raises the possibility that oxidative stress and inflammation are related to the type of macronutrient ingested rather than the caloric intake per se. These observations may be relevant to our search for foods relatively safe in terms of generating oxidative stress and causing inflammation, which are potentially atherogenic.

REFERENCES

1. Mohanty P, Hamouda W, Garg R, et al: Glucose challenge stimulates reactive oxygen species (ROS) generation by leucocytes. J Clin Endocrinol Metab 85:2970-2973, 2000

2. Mohanty P, Ghanim H, Hamouda W, et al: Both lipid and protein intakes stimulate increased generation of reactive oxygen species by polymorphonuclear leukocytes and mononuclear cells. Am J Clin Nutr 75:767-772, 2002

3. Dandona P, Mohanty P, Hamouda W, et al: Inhibitory effect of a two day fast on reactive oxygen species (ROS) generation by leucocytes and plasma ortho-tyrosine and meta-tyrosine concentrations. J Clin Endocrinol Metab 86:2899-2902, 2001

4. Dandona P, Weinstock R, Thusu K, et al: Tumor necrosis factor-alpha in sera of obese patients: Fall with weight loss. J Clin Endocrinol Metab 83:2907-2910, 1998

5. Dandona P, Mohanty P, Ghanim H, et al: The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. J Clin Endocrinol Metab 86:355-362, 2001

6. Ziccardi P, Nappo F, Giugliano G, et al: Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. Circulation 105:804-809, 2002

7. Baeuerle PA, Baltimore D: NF-kappa B: Ten years after. Cell 87:13-20, 1996

8. McCord JM: The evolution of free radicals and oxidative stress. Am J Med 108:652-659, 2000

9. Baeuerle PA, Henkel T: Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141-179, 1994

10. Stein B, Baldwin AS Jr, Ballard DW, et al: Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J 12:3879-3891, 1993

11. Barnes PJ, Karin M: Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066-1071, 1997

12. Dandona P, Thusu K, Hafeez R, et al: Effect of hydrocortisone on oxygen free radical generation by mononuclear cells. Metabolism 47:788-791, 1998

13. Dandona P, Karne R, Ghanim H, et al: Carvedilol inhibits reactive oxygen species generation by leukocytes and oxidative damage to amino acids. Circulation 101:122-124, 2000

14. Tosi MF, Hamedani A: A rapid, specific assay for superoxide release from phagocytes in small volumes of whole blood. Am J Clin Pathol 97:566-573, 1992

15. Hancock JT, Jones OT: The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. Biochem J 242:103-107, 1987

16. Aljada A, Garg R, Ghanim H, et al: Nuclear factor-kappaB suppressive and inhibitor-kappaB stimulatory effects of troglitazone in obese patients with type 2 diabetes: Evidence of an antiinflammatory action? J Clin Endocrinol Metab 86:3250-3256, 2001

17. Aljada A, Ghanim H, Assian E, et al: Increased IkappaB expression and diminished nuclear NF-kappaB in human mononuclear cells following hydrocortisone injection. J Clin Endocrinol Metab 84:3386-3389, 1999

18. Andrews NC, Faller DV: A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499, 1991

19. Gaziano JM, Gaziano TA, Glynn RJ, et al: Light-to-moderate alcohol consumption and mortality in the Physicians' Health Study enrollment cohort. J Am Coll Cardiol 35:96-105, 2000

20. Romelsjo A, Leifman A: Association between alcohol consumption and mortality, myocardial infarction, and stroke in 25 year follow up of 49618 young Swedish men. BMJ 319:821-822, 1999

21. McElduff P, Dobson AJ: How much alcohol and how often? Population based case-control study of alcohol consumption and risk of a major coronary event. BMJ 314:1159-1164, 1997

22. Abramson JL, Williams SA, Krumholz HM, et al: Moderate alcohol consumption and risk of heart failure among older persons. JAMA 285:971-1977, 2001

23. Mikhailidis DP, Jeremy JY, Barradas MA, et al: Effect of ethanol on vascular prostacyclin (prostaglandin I2) synthesis, platelet aggregation, and platelet thromboxane release. BMJ 287:1495-1498, 1983

24. Rimm EB, Williams P, Fosher K, et al: Moderate alcohol intake and lower risk of coronary heart disease: Meta-analysis of effects on lipids and haemostatic factors. BMJ 319:1523-1528, 1999

25. Imhof A, Froehlich M, Brenner H, et al: Effect of alcohol consumption on systemic markers of inflammation. Lancet 357:763-767, 2001

26. Mikhailidis DP, Jenkins WJ, Barradas MA, et al: Platelet function defects in chronic alcoholism. BMJ 293:715-718, 1986

27. Mandrekar P, Catalano D, Szabo G: Inhibition of lipopolysaccharide-mediated NFkappaB activation by ethanol in human monocytes. Int Immunol 11:1781-1790, 1999

28. Mandrekar P, Catalano D, Szabo G: Alcohol-induced regulation of nuclear regulatory factor-kappa beta in human monocytes. Alcohol Clin Exp Res 21:988-994, 1997

29. Blanco-Colio LM, Valderrama M, Alvarez-Sala LA, et al: Red wine intake prevents nuclear factor-kappaB activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. Circulation 102:1020-1026, 2000

30. Serafini M, Maiani G, Ferro-Luzzi A: Alcohol-free red wine enhances plasma antioxidant capacity in humans. J Nutr 128:1003-1007, 1998

31. Kondo K, Matsumoto A, Kurata H, et al: Inhibition of oxidation of low-density lipoprotein with red wine. Lancet 344:1152, 1994

32. Wilson NM, Brown PM, Juul SM, et al: Glucose turnover and metabolic and hormonal changes in ethanol-induced hypoglycaemia. BMJ 282:849-853, 1981