# Light, But Not Heavy Alcohol Drinking, Stimulates Paraoxonase by Upregulating Liver mRNA in Rats and Humans

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**Paraoxonase 1 (PON) may contribute to the cardioprotective action of high-density lipoprotein (HDL) because it inhibits low-density lipoprotein (LDL) oxidation, a prerequisite for the onset of atherosclerosis. Because light drinking and heavy drinking have diametrically opposite effects on cardioprotection, we have determined the effects of ethanol dosage on rat serum PON activity and its hepatic expression. Furthermore, we have investigated PON activity and polymorphism in human light and heavy drinkers. Our results confirm that HDL-PON inhibited LDL oxidation, destroyed oxidized LDL, and inhibited its uptake by macrophages. Light ethanol feeding caused a 20% to 25% ($P < .05$) increase in PON activity in both serum and liver and a 59% ($P < .001$) increase in the level of liver PON mRNA compared with pair-fed control rats. In contrast, heavy ethanol feeding caused a 25% ($P < .05$) decrease in serum and liver PON activities with a 51% ($P < .01$) decrease in liver PON mRNA level. Light drinkers had a 395% ($P < .001$) higher, whereas heavy drinkers had a 45% ($P < .001$) lower serum PON activity compared with nondrinkers. Significantly, the number of homozygotes versus heterozygotes with respect to high or low activity PON phenotype was similar in all the groups. Therefore, we conclude that light drinking upregulates, whereas heavy drinking downregulates PON activity and its expression, irrespective of its genetic polymorphism.**


WHILE THE EFFECTS of chronic ethanol consumption on liver have been well studied and documented, its effect on the cardiovascular system is controversial. Heavier drinking is related to higher prevalence of cardiomyopathy,[1-3] hypertension,[4] hemorrhagic stroke,[5] and cardiac dysrhythmias.[6] However, lighter drinking in many population studies cited in recent reports[7-12] is linked to lower prevalence of coronary artery disease (CAD). In several other studies of cardiovascular mortalities,[13,14] abstainers and heavy drinkers are at higher risk than light drinkers.

Numerous cross-sectional and intervention studies,[15-17] including ours,[18] have documented an increase in plasma high-density lipoprotein (HDL) cholesterol concentration as a result of chronic light ethanol consumption. Correcting for smoking as another risk factor, it has been found that a strong negative correlation still exists between light drinking and incidence of CAD.[19-22] There are conflicting reports with regard to whether $HDL_2$ or $HDL_3$ subfraction is increased after light drinking.[23-26] In contrast, HDL level is markedly decreased in severe alcoholic liver diseases.[27] Further, heavy alcohol drinkers are not protected against CAD.[28] Our ongoing study has established that heavy ethanol consumption markedly decreased apolipoprotein (apo) E content of plasma HDL.[29] This has been confirmed by others.[30] We further showed that chronic ethanol-mediated apo E deficiency in HDL resulted in its impaired reverse cholesterol transport function in both rats and heavy alcoholics.[31,32]

It has been shown that low-density lipoprotein (LDL) oxidation is increased in chronic alcohol abusers.[33] Acetaldehyde (ALD), the first metabolite of ethanol, also modifies lysine residues of LDL[34] and the product rapidly disappears from the plasma.[35,36] Because modified LDL is taken up by macrophages via the scavenger pathway, it is reasonable to hypothesize that heavy alcoholics have greater risk of CAD than abstainers or light drinkers because of an ethanol-mediated increase in modified LDL levels.

Paraoxonase 1 (PON), a newly discovered serum enzyme intimately associated with HDL, has been shown to play a major role in the protective role of HDL against CAD.[37] Specifically, PON is believed to prevent the oxidation of LDL to oxidized LDL (oxLDL) and to destroy the oxLDL to biologically inactive products. It is a calcium-dependent HDL-associated ester hydrolase that catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates.[38] PON is a 43-kd protein tightly associated with apo A-I in HDL and has the highest activity in the liver and blood.[37] Serum PON concentration varies widely between different animal species and among humans.[38,39] Individuals with familial hypercholesterolemia and insulin-dependent diabetes mellitus have significantly lower levels of PON than do normal individuals.[40] A low level of HDL-associated PON is also correlated with susceptibility to myocardial infarction (MI), fish eye disease,[41] and tangier disease.[42] Most importantly, HDL-associated PON has been reported to also inhibit copper-induced lipid peroxidation in LDL.[43] A 3-week daily moderate alcohol consumption in middle-aged men showed a modest 6.9% to 9.3% increase in serum PON activity in a recent diet-controlled randomized study.[44]

PON1 exhibits polymorphism at 192 locus resulting in widely different levels of this enzyme in the human population.[45-47] Thus, PON1 R192 isoform hydrolyzes paraoxon with a high turnover rate, whereas PON1 Q192 has a low turnover rate. Consequently, the protective role of an individual's HDL against coronary heart disease is dependent on his/her PON1

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genotype that determines its capacity to protect LDL against oxidative modification. As a result, PON1 polymorphism can be used to predict an individual's susceptibility to cardiovascular disease. In view of these, in the present study, we have evaluated whether or not light or heavy alcohol exposure has specific effect on PON expression irrespective of its polymorphism.

In an attempt to understand the possible mode of action of ethanol after light and heavy drinking, we have studied the effects of different concentrations of ethanol feeding for 8 weeks on liver and serum PON activities, as well as liver PON mRNA levels in the rat. We have extended these studies to humans by correlating serum activity of PON with its polymorphism in alcohol abstainers, light drinkers, and heavy drinkers. In addition, we confirm the positive correlation between HDL-PON activity and HDL ability to (1) protect LDL from oxidation, (2) destroy the oxidized LDL, and (3) inhibit the uptake of oxidized LDL by macrophages.

## MATERIALS AND METHODS

### *Reagents*

Culture medium Dulbecco's modified Eagle's medium (DMEM) (hi-glucose), heat inactivated fetal bovine serum, glutamine, and antibiotics were obtained from Life Technologies (Rockville, MD). Heparin (10,000 USP U/mL) was obtained from Elkin-Sinn (Cherry Hill, NJ). Dextran sulfate (50 kd) was obtained from Genzyme (Cambridge, MA). Diet components were from ICN Biomedicals (Costa Mesa, CA) or from Dyets (Bethlehem, PA). All other reagents and chemicals were of analytical grade.

### *Animals and Feeding Regimen*

The humane use of animals was approved by the Institutional Animal Care and Use Committees of the Veterans Affairs and George Washington University medical centers. Male Wistar-Furth rats (body weight, 125 to 150 g) were procured from Charles River (Wilmington, MA). After a week of acclimatization, they were divided into 3 groups (6 rats per group) and fed for 8 weeks on the control, light alcohol, or heavy alcohol liquid isocaloric diet, respectively. The heavy alcohol diet was prepared essentially according to the method of Lieber and DeCarli[48] and had the following nutrient composition (in g/L): oil mixture (olive oil, cod-liver oil, corn oil in a ratio of 67:8:25) 126.75; ethanol, 50; casein, 47; and dextrin maltose 11.36. Thus 40% of the total energy of this diet was from fat, 36% from ethanol (the light alcohol-fed group received 10% of the total calories as ethanol), 20% from protein, and the rest from carbohydrates. These feeding regimens are expected to result in blood ethanol levels of 8.3 mmol/L (38.9 mg/dL) and 30 mmol/L (140 mg/dL).[48] The corresponding control diet contained equicaloric amounts of dextrin maltose in place of ethanol. The rats in the control group were pair-fed with the animals in the corresponding alcohol group. At the end of 8 weeks, all rats were killed by aortic exsanguination under pentobarbital anesthesia (50 mg/kg body weight). Serum samples from these rats were stored at −80°C until used for PON activity assay as described below. Livers were immediately frozen in liquid nitrogen and then stored at −80°C until use for mRNA extraction.

### *Assay of PON Activity*

Serum samples from various groups of both animal and human subjects were assayed for PON activity using paraoxon as the substrate according to an established method.[49] A known aliquot of each serum sample was incubated with 1 mmol/L paraoxon and 2 mmol/L $CaCl_2$ in 1 mL final volume of a reaction mixture containing 50 mmol/L Tris/HCl buffer, pH 8.0. The increase in absorbancy at 412 nm due to the formation of 4-nitrophenol was monitored for a period of 120 seconds in a Shimadzu-1601 UV/VIS recording spectrophotometer (Shimadzu, Columbia, MD). PON activity was calculated based on the molar extinction coefficient of 18,000 $M^{-1} \cdot cm^{-1}$. One unit of PON activity is defined as 1 nmol of 4-nitrophenol formed per minute per milligram protein.

### *Isolation of Plasma HDL*

HDL was isolated from human pooled plasma according to the precipitation methods of Gidez et al[50] and Burstein et al.[51] Briefly, 0.1 vol heparin-$MnCl_2$ solution (1.383% heparin, 1.0 mol/L $MnCl_2$) was added to 1.0 vol plasma and incubated for 30 minutes at room temperature, followed by centrifugation for 20 minutes at 600 × *g* at 4°C. The supernatant fraction (very–low-density lipoprotein [VLDL]/LDL-free plasma) was taken for precipitation of HDL by addition of 0.1 vol 7.8% (wt/vol) dextran sulfate (50 kd) and 0.1 volume 1.49 mol/L $MnCl_2$. After thorough mixing, VLDL/LDL-free plasma was left at room temperature for 30 minutes and then centrifuged as before. The HDL pellet thus obtained was solubilized in 10% (wt/vol) sodium citrate (pH 8.3) and dialyzed extensively against phosphate-buffered saline (PBS) (pH 7.4). Protein concentration was determined by the Bradford method,[52] with bovine serum albumin (BSA) as standard, and cholesterol concentration was determined according to Allain et al.[53]

### *Preparation of Native LDL*

LDL (density, 1.019 to 1.063 g/mL) was isolated from human pooled plasma by differential ultracentrifugation[54] and then extensively dialyzed against 0.9% (wt/vol) NaCl, which had been bubbled with helium to minimize the amount of dissolved oxygen in the buffer. The purity of each LDL preparation was verified by agarose gel electrophoresis and its lipid composition (data not shown). LDL was stored at 4°C in helium-flushed and light-protected container and used within 2 weeks after isolation.

### *LDL Oxidation Conditions*

LDL preparation was subjected to oxidation by 4-hydroxynonenal (HNE), ALD, or $CuSO_4$ (Cu). (1) HNE modification: this procedure was performed according to Hoff et al.[55] LDL (0.1 mg/mL protein) was incubated in the dark under helium with an aqueous solution of HNE (3 mmol/L final concentration) in PBS (pH 7.4) containing 0.3 mmol/L EDTA for 5 hours at 37°C. (2) ALD modification: LDL (0.1 mg/mL protein) was incubated in the dark under helium with an aqueous solution of ALD (0.1 mmol/L final concentration) in PBS (pH 7.4) for 5 hours at 37°C. (3) Cu oxidation: LDL (0.1 mg/mL protein) was incubated with 10 μmol/L Cu at 37°C for 16 hours. After modification, all oxLDL preparations were dialyzed against 0.9% (wt/vol) NaCl solution and stored in the dark at 4°C until use. An aliquot from each LDL preparation was analyzed on agarose gel electrophoresis to verify LDL modification.

### *Testing HDL Protective Effect Against Cu LDL Oxidation*

LDL (100 μg protein) was modified by Cu, as described above, in the presence of 100 μg or 200 μg HDL protein per milliliter or without HDL. After the incubation period, the extent of LDL oxidation in each reaction mixture was measured fluorimetrically[56] using a SpectroVision FD300 dual monochromator fluorescence detector (excitation: 360 nm; emission: 430 nm; Groton Technologies, Acton, MA).

### *Testing HDL Ability to Destroy OxLDL*

LDL was modified by HNE, ALD, or Cu as described above. Each preparation of oxLDL (100 μg protein/mL) was then incubated with

100 $\mu$g or 200 $\mu$g HDL protein per milliliter, or without HDL, for 16 hours at 37°C. After this incubation period, the ability of HDL to reduce the extent of LDL oxidation was measured spectrophotometrically at 234 nm 57 using a Shimadzu UV160 spectrophotometer.

### *Labeling of Native LDL and OxLDL with [$^3$H]Cholesteryl Oleate*

Labeling of nonmodified or oxidized LDL was performed according to Fielding.[58] Briefly, 2 to 5 $\mu$Ci [$^3$H]cholesteryl oleate (79 Ci/mmol) originally in toluene was evaporated to dryness using nitrogen gas. The dried cholesterol was resolubilized in 200 $\mu$L dimethyl sulfoxide (DMSO). This was followed by addition of 0.8 mL 0.9% (wt/vol) NaCl, 0.3 mmol/L EDTA solution drop wise with continuous mixing. After incubation at 37°C for 10 minutes, this solution was added to 3 vol LDL solution (1 mg LDL protein per 2 $\mu$Ci [$^3$H]cholesteryl oleate) in a drop wise manner with continuous mixing. The reaction mixture was again incubated at 37°C for 3 hours followed by extensive dialysis of the labeled LDL against 0.9% (wt/vol) NaCl, 0.3 mmol/L EDTA solution. Based on the radioactivity and cholesterol content of the labeled LDL sample, its specific activity was determined.

### *Cholesterol Uptake of OxLDL in the Presence of HDL*

Mouse macrophages J774.A obtained from ATCC (Manassas, VA) and maintained in our laboratory were used. Culture conditions were: 37°C, humidified incubator with an atmosphere of 5% $CO_2$, DMEM supplemented with 2 mmol/L glutamine, 10% (vol/vol) heat inactivated fetal bovine serum, 1 U/L insulin, 50 IU/mL penicillin, and 50 $\mu$g/mL streptomycin. Cells were plated 24 to 48 hours before uptake experiments in 6-well plates at a density of $1 \times 10^6$ cells per well with 4 mL medium. The experiment was started when cells were about 90% confluent. Each radioactively-labeled LDL species was preincubated for 16 hours at 37°C with indicated amount of HDL, then added to the cells (100 $\mu$g LDL protein in 4 mL medium, corresponding to approximately 100,000 dpm per well), and the uptake of labeled cholesterol was performed for 24 hours. At 24 hours, cell media were collected and counted for radioactivity. Percentage uptake was calculated by comparing the medium's radioactivity at zero time with the medium's radioactivity at 24 hours for each well. Each condition was done in triplicate.

### *Determination of Rat Liver PON mRNA Levels*

Total liver RNA was extracted from each animal in all feeding groups by using Tri-Reagent (Sigma, St Louis, MO). Liver samples (50 to 100 mg of tissue) were homogenized in 1 mL of the Tri-Reagent using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The homogenized samples were kept at room temperature for 5 minutes, followed by the addition of 0.2 mL chloroform, vigorous shaking for a few seconds, kept at room temperature for 15 minutes, and centrifugation at 12,000 $\times$ *g* for 15 to 20 minutes at 4°C. The resulting upper aqueous phase was carefully pipetted out into a sterile tube, and the RNA was precipitated by addition of 0.5 mL isopropanol and kept at room temperature for 5 to 10 minutes. The RNA was pelleted by centrifugation as above for 15 minutes. Finally, the precipitated RNA was washed in 70% ethanol, dried, and quantified spectrophotometrically after solubilizing in Formazol, RNA solubilizing agent (Sigma, St Louis, MO).

### *Northern Blotting*

RNA electrophoresis was performed using formaldehyde (2.2 mol/L) agarose (1%, wt/vol) gels and 1X MOPS buffer (0.1 mol/L 3-(N-morpholino) 2-propanesulfonic acid (MOPS) pH 7.0, 40 mmol/L sodium acetate, 5 mmol/L EDTA) and the RNA transferred overnight onto a nitrocellulose membrane in 10X SSC buffer (175.3 g NaCl, 88.2 g sodium citrate in 2 L water, pH 7.2) as described by Greenberg and Bender.[59]

### *Northern Hybridization*

A 1,100-bp PON1 cDNA probe (kindly provided by D. Shih, UCLA, Los Angeles, CA)[60] was used for Northern hybridization. A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) purchased from ATCC (ATCC No.81141) was used to normalize the Northern blots. Probes were labeled using a Random Prime Kit (Invitrogen, Carlsbad, CA) and $\alpha$ [$^{32}$P]-deoxycytidine triphosphate (dCTP), 3,000 Ci/mmol. Hybridization was performed according to the method of Church and Gilbert.[61] Prehybridization was performed in 10 mL buffer (0.5 mol/L $NaHPO_4$, 1 mmol/L EDTA, 7% sodium dodecyl sulfate (SDS), 1% crystalline BSA) at 62°C for 10 minutes followed by the addition of probes at $1 \times 10^6$ cpm/mL of the hybridization buffer. Hybridization was performed by incubating the membrane with the labeled cDNA probes at 62°C for 16 to 18 hours. Later, blots were washed under stringent conditions at room temperature in Wash solution-1 (40 mmol/L $NaHPO_4$, 1 mmol/L EDTA, 5% SDS) for 10 minutes followed by another wash using Wash solution-2 (40 mmol/L $NaHPO_4$, 1 mmol/L EDTA, 1% SDS) at 62°C for 15 to 30 minutes and air-dried. Next, the membrane was subjected to autoradiography followed by quantitation of the radioactive spots corresponding to PON mRNA and GAPDH mRNA using a Radioanalytic system (AMBIS, San Diego, CA).

### *Determination of PON Polymorphism*

The genotyping of human subjects with respect to PON polymorphism was assessed according to the methods of Humbert et al[62] and Garin et al.[63] Briefly, DNA was extracted from the buffy coat from the blood samples of study subjects using a salting-out DNA extraction kit (Eppendorf, Westbury, NY). Allele specific primers described by Humbert et al[62] to encompass the polymorphism affecting position 192 were used to amplify the polymorphic region of the gene. The polymerase chain reaction (PCR) reaction contained approximately 100 to 500 ng DNA template, 0.1 $\mu$mol/L of each primer, 0.2 mmol/L of the 4 deoxynucleotides triphosphate (dNTPs), 1 U Taq DNA polymerase, and 1.5 mmol/L $MgCl_2$. DNA was amplified in a PCR machine for 30 to 35 cycles. The PCR optimal temperature and times were: denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute, extension at 72°C for 1 minute with a final extension for 5 minutes. The nucleotide substitution corresponding to position 192 (Gln$\rightarrow$Arg) creates an AlwI restriction site. Allele Q (glutamine) corresponded to a 99-bp fragment, whereas allele R (arginine) corresponded to 65 and 34 bp fragments. The PCR product was restriction digested using AlwI (New England Biolabs, Beverly, MA) at 37°C for 3 hours. The digested products were analyzed on a 3.5% agarose gel cast and run in 1X Tris-borate-EDTA (TBE) buffer.

### *Human Studies*

The study subjects were nondrinking, light alcohol-, or heavy alcohol-consuming male volunteers (age, 25 to 59 years) at the Veterans Administration and the George Washington University medical centers. Informed consent to draw blood samples for this study was obtained from all the study subjects. The human component of this study was approved by the Institutional Review Boards (IRBs) of both the Veterans Administration and the George Washington University medical centers. The study groups included abstainers (less than 1 drink per day in the past 6 months or longer), light drinkers (1 to 3 drinks or 13 to 39 g alcohol per day in the past 6 months or longer), and heavy drinkers (more than 6 drinks or 80 g alcohol per day in the past 6 months or longer). All the subjects were clinically classified to be free of liver disease. Fasting blood was drawn into vacutainer tubes con-

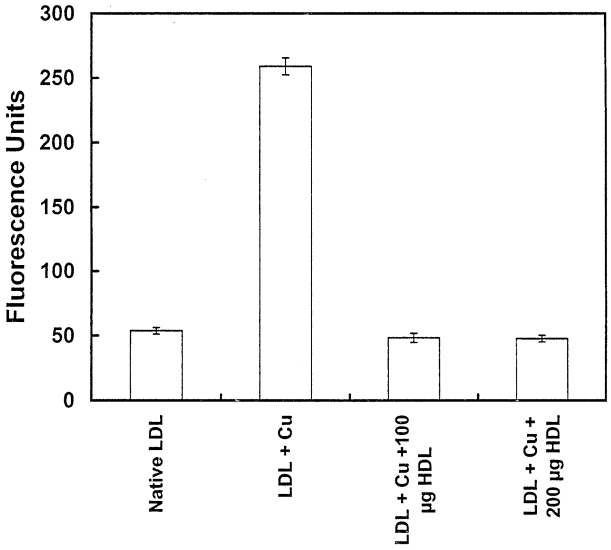


**Fig 1. Effect of HDL on LDL oxidation by Cu. Native LDL preparation (100 $\mu$g protein) was incubated with 10 $\mu$mol/L Cu in the absence or presence of indicated HDL amounts ($\mu$g protein) in a final volume of 1 mL, and the extent of LDL oxidation was measured fluorimetrically as described in Materials and Methods. Each value is the mean ± SE of 3 independent determinations.**

taining SST gel and clot activator (Becton Dickinson, Rutherford, NJ) for serum preparation.

### *Statistical Analysis*

The significance of the various effects was evaluated by a 1-way analysis of variance (ANOVA) followed by Tukey test.

## RESULTS

### *Protective Effect of HDL-PON on the Oxidative Modification of LDL*

Serum PON1 is tightly associated with HDL and inhibits oxidative modification of LDL. Thus, higher PON1 activity is thought to be beneficial in protecting against CAD, which is caused, in part, by high levels of oxLDL. Because the focus of this study is to understand the mechanism underlying the causal relationship between alcohol consumption and CAD, we have established an assay to measure LDL susceptibility to oxidation in various conditions. Thus, we performed copper sulfate-mediated LDL oxidation without or with 100 $\mu$g or 200 $\mu$g HDL protein. Results plotted in Fig 1 clearly show that HDL-PON prevented the oxidation of LDL by Cu.

### *Involvement of PON in the Protective Role of HDL*

To test whether this protective effect of HDL was due specifically to PON enzymatic activity, the following experiments were performed. Aliquots from the same freshly isolated HDL preparation were preincubated with or without 3 mmol/L EDTA at 37°C for 90 minutes (EDTA is known to inhibit PON activity because it is a $Ca^{++}$-dependent enzyme), followed by the removal of EDTA from the HDL preparation by extensive dialysis against PBS (pH 7.4). Similarly, aliquots of the same HDL preparation were preincubated with or without anti-PON antiserum (kindly provided by Dr Clement Furlong, Seattle, WA) at 37°C for 60 minutes. All of the HDL preparations were then tested for their ability to inhibit LDL oxidation by 10 $\mu$mol/L Cu. The results presented in Table 1 clearly show that the protective role of HDL on LDL oxidation is largely dependent on its enzymatic PON1 component.

**Table 1. Specificity of PON Contribution in HDL Protection Against LDL Oxidation**

| HDL Preincubation Condition | HDL PON Activity (U)* | % of LDL Oxidation† |
|---|---|---|
| No addition | 2.3 | 100 |
| +EDTA | 0.3 | 76 |
| + Anti-PON | 0.96 | 52 |

*One unit PON activity is defined as 1 nmol of 4-nitrophenol formed per minute per milligram HDL protein.

†LDL oxidation was performed in 10 $\mu$mol/L Cu for 16 hours at 37°C and measured by fluorescence. Percentage of inhibition was calculated by relating fluorescence value at 16 hours of LDL-Cu to fluorescence values at 16 hours of LDL-Cu with respective HDL preparations.

### *Effect of HDL on Destruction of OxLDL*

Because PON has the ability to transform lipid peroxidation products (ie, dienes) into nonreactive species, its activity was tested on already oxidized LDL. Thus, LDL dienes' content, as measured by spectrophotometry at 234 nm, should decrease after incubation with PON. In this experiment, LDL was oxidized with HNE, ALD, or Cu, then incubated with 0 $\mu$g, 100 $\mu$g, or 200 $\mu$g HDL protein. Results plotted in Fig 2 show a dramatic HDL concentration-dependent decrease of diene-con-

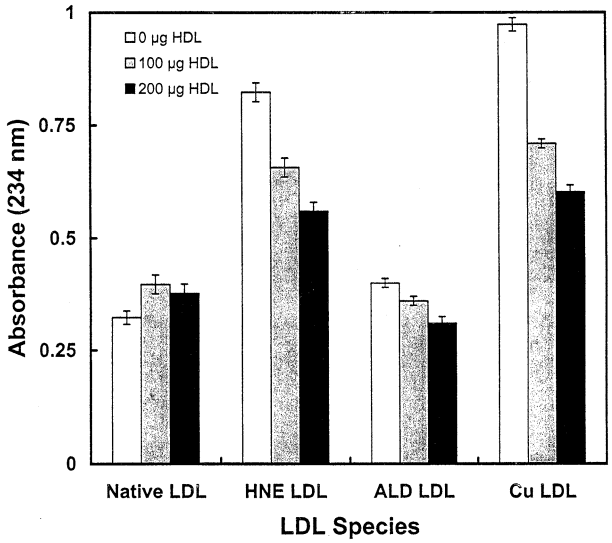


**Fig 2. Effect of HDL on the destruction of oxLDL. LDL (100 $\mu$g protein) was oxidized by HNE, ALD, or Cu as described in Materials and Methods, then incubated in the absence or presence of indicated HDL amounts ($\mu$g protein) in a final volume of 1 mL. The ability of indicated concentrations of HDL to destroy each oxLDL species was measured spectrophotometrically at 234 nm as described in Materials and Methods. Each value is the mean ± SE of 3 independent determinations.**

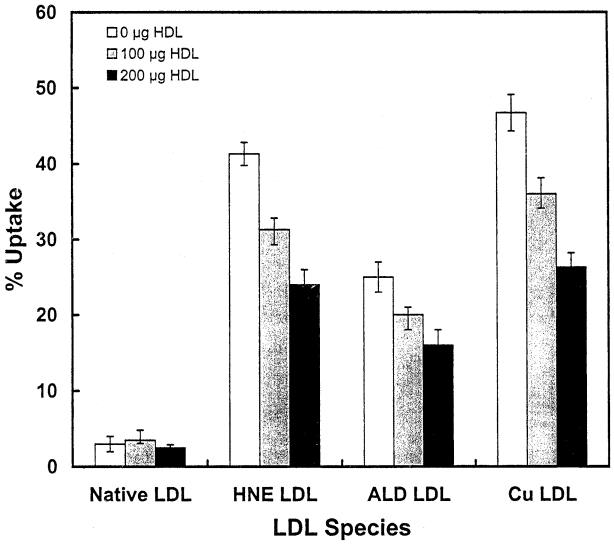


**Fig 3. Effect of pretreatment with HDL on the uptake of oxLDL by macrophage. Native LDL or LDL that was oxidized by HNE, ALD, or Cu was labeled with [$^3$H]cholesteryl oleate as described Materials and Methods. Each LDL species was then incubated in the absence or presence of indicated HDL amounts ($\mu$g HDL protein per 100 $\mu$g LDL protein) in a final volume of 1 mL before testing for uptake by the macrophage system as described in Materials and Methods. Each value is the mean ± SE of 3 independent determinations.**

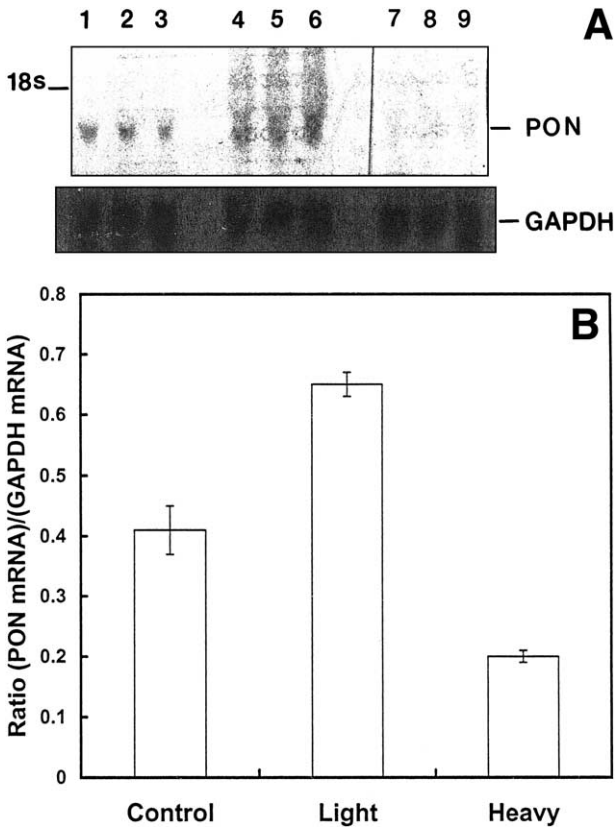


**Fig 4. Effect of chronic light or heavy ethanol feeding on liver PON mRNA expression. (A) Northern blot of rat liver mRNA hybridized to PON cDNA and housekeeping gene, GAPDH cDNA. Lanes indicate 20 $\mu$g total RNA from livers of control (lanes 1 to 3), light alcohol (lanes 4 to 6), and heavy alcohol (lanes 7 to 9) groups hybridized to PON cDNA and GAPDH cDNA. (B) PON mRNA levels relative to GAPDH mRNA levels in the control, light alcohol, and heavy alcohol groups. Each value is the mean ± SE of 3 independent determinations.**

taining oxLDL, regardless of which oxidant was used to prepare it. When HDL was preincubated with EDTA, no significant decrease of oxLDL was observed (data not shown).

*Effect of HDL-PON Pretreatment on the Uptake of OxLDL by Macrophage*

A consequence of the previous results (Fig 2) is that if PON decreases oxLDL level, it should also prevent its uptake by the macrophage scavenger receptor. Thus, in this experiment, the uptake of LDL or oxLDL (modified either with 4-HNE, ALD, or Cu) by the macrophage system was determined after incubation of the various LDL species with indicated concentrations of HDL, as described in Materials and Methods. The results shown in Fig 3 indicate that pretreatment of oxLDL with HDL resulted in a HDL concentration-dependent inhibition in the uptake of oxLDL by macrophages, regardless of which oxidant was used to prepare it.

*Effect of Alcohol on Serum and Liver PON Activity in Rats*

Serum and liver microsomal PON activity was determined in 3 groups of rats (6 rats per group) after 8 weeks of pair-feeding their respective isocaloric liquid diets containing 0%, 10%, and 36% of total dietary calories as ethanol, respectively. The results presented in Table 2 clearly show that feeding 10% ethanol-calories (light alcohol group) for 8 weeks significantly increased serum and liver PON activity by 20% and 25% ($P < .05$), respectively, while feeding 36% ethanol-calories (heavy alcohol group) for 8 weeks decreased PON activity by 25% ($P < .05$) in both serum and liver compared with the control group.

*Effect of Alcohol on PON Expression*

A representative blot with 3 RNA samples from each group is shown in Fig 4A. Quantitative analysis of these results is

**Table 2. Serum and Liver PON Activity in Control, Light, and Heavy Ethanol-fed Rats**

| Ethanol (% of diet calories) | Serum PON | | Liver PON | | *P* Value |
|---|---|---|---|---|---|
| | Units | % of Control | Units | % of Control | |
| 0 (control) | 6.4 ± 0.2 | 100 | 4.2 ± 0.1 | 100 | <.05 |
| 10 (light) | 7.7 ± 0.2 | 120 | 5.23 ± 0.15 | 125 | <.05 |
| 36 (heavy) | 4.8 ± 0.2 | 75 | 3.13 ± 0.2 | 75 | <.05 |

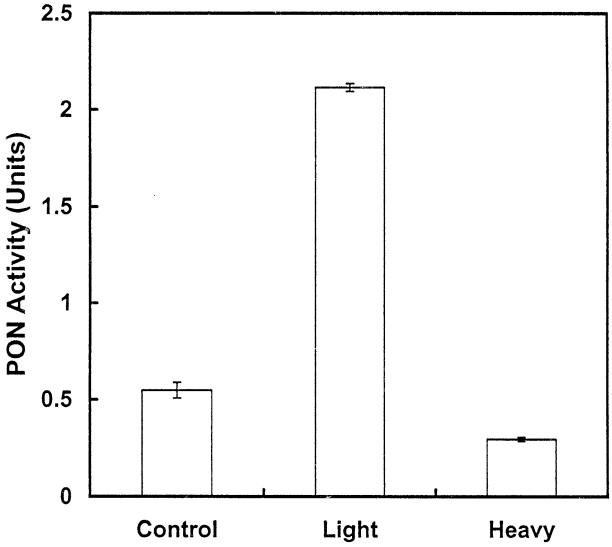


**Fig 5. Serum PON activity in human alcohol abstainers, light drinkers, and heavy drinkers. Serum PON activity was determined in the 3 groups of study subjects using paraoxon as the substrate as described in Materials and Methods. Each value is the mean ± SE.**

plotted in Fig 4B. The data from this experiment show that, compared with the control group, PON mRNA level relative to that of GAPDH mRNA increased by 59% ($P < .001$) in the light alcohol group, whereas it was decreased by 51% ($P < .01$) in the heavy alcohol group. There was no significant change with respect to the levels of GAPDH mRNA among the 3 groups.

*Studies in Humans*

*Serum PON activity in light and heavy drinkers compared with nondrinkers.* To test whether an increase in the activity of PON occurs in light drinkers, blood samples were collected from healthy volunteers (both male and female) who were nondrinkers, light drinkers (drinking 13 to 40 g ethanol per day, 1 to 3 drinks per day for at least 6 months or longer), and heavy drinkers (drinking ≥ 80 g ethanol per day or ≥ 6 drinks per day for 6 months or longer). Serum was prepared from all of the above blood samples, and PON activity in each sample was measured as described in Materials and Methods. It can be seen from the results presented in Fig 5 that, in contrast to a 395% ($P < .001$) increase in serum PON activity in light drinkers, there was a 45% ($P < .001$) decrease in its activity in heavy drinkers compared with nondrinkers.

*Genotyping based on differential substrate specificity.* Because it has been established that RR homozygotes (Arg-192) have high activity toward paraoxon while QQ homozygotes (Gln-192) have high activity toward diazoxon,[64] we measured the individual serum PON activity in the study groups using the 2 substrates. These results were confirmed by genotyping of DNA samples extracted from the lymphocytes of the study subjects for polymorphism at amino acid position 192 using allele-specific primers essentially as described by Humbert et al.[62] The results in Table 3 show that 67% to 75% of the study subjects are heterozygotes with respect to low or high activity PON phenotypes regardless of their drinking status.

## DISCUSSION

It is now well known that CAD is the principal cause of mortality and morbidity in developed countries, and several features of this metabolic disorder are due to the accumulation of oxLDL, the atherogenic lipoprotein, in the intimae of the arterial wall. On the other hand, HDL is antiatherogenic because of its ability to remove cholesterol from peripheral tissues to transport it to the liver for degradation, a process called reverse cholesterol transport. Another mechanism by which HDL acts as an antiatherogenic protein is by virtue of its ability to protect against LDL oxidation and to destroy oxLDL. Thus, PON can prevent foam cell formation caused by the uptake of oxLDL by macrophages. The accumulation of these foam cells in the intimae of arterial wall results in occlusion of the artery causing myocardial infarction.

Our present study confirms and extends the observations of previous investigators[49,60] that HDL is able to effectively inhibit the oxidation of native LDL by $CuSO_4$ (Fig 1). The fact that it was the PON component of HDL that was responsible for this protective effect is confirmed by marked inhibition of this protective effect by both EDTA and anti-PON1 antibody (Table 1). The lesser inhibition effect of anti-PON compared with that of EDTA could be due to either the lack of complete inhibition of PON1 activity by the anti-PON1 antibody or due to the existence of other PON isoenzyme activites that are not neutralized by anti-PON1 antibody. Moreover, we showed that the same HDL preparations not only destroyed the oxLDL generated by various oxidants, such as Cu, HNE, and ALD (Fig 2), but also prevented the uptake of oxLDL by macrophages (Fig 3). Thus, the antiatherogenic properties of HDL are explained by the ability of HDL to enhance the RCT process and by the ability of HDL-bound PON to prevent LDL oxidation and to destroy oxLDL, the atherogenic lipoprotein.

The possible beneficial effects of light alcohol drinking have been attributed to its ability to increase plasma levels of HDL,[7-12] the antiatherogenic lipoprotein. Significantly, our present study demonstrates for the first time that it could be due to the marked stimulation of serum PON activity in rats (Table 2). These results are strongly supported by our findings of a concomitant upregulation of PON mRNA (Fig 4) in the liver. In contrast, heavy alcohol feeding in rats significantly reduces both serum and liver PON activities (Table 2) with a corresponding downregulation of PON mRNA in the liver (Fig 4). Our studies in human subjects confirm our finding in rats showing the stimulatory and inhibitory effects of light drinking versus heavy drinking on serum PON activity (Fig 5). Our

**Table 3. Genotyping of Study Groups**

| Drinking Status | No. | No. of Subjects in Each Subgroup | | |
|---|---|---|---|---|
| | | RR | QQ | RQ |
| Nondrinkers | 12 | 2 | 2 | 8 |
| Light drinkers | 12 | 1 | 2 | 9 |
| Heavy drinkers | 6 | 1 | 1 | 4 |

results are consistent with recent 3-week crossover studies showing PON activity is increased in moderate drinkers[44] that was accompanied by increased apo AI and HDL cholesterol.[65]

Recent findings have shown that PON1 upstream polymorphism can regulate plasma PON1 level and activity.[66-68] In addition, Osaki et al[69] and Ota et al[70] showed that Sp1 binding site in the promoter region plays an important role in statin upregulation of PON1, and it has been suggested that the phosphorylation of the zinc finger region of Sp1 is required for its activation, possibly carried out by protein kinase C (PKC). Thus, assuming that upstream polymorphism is not a factor in our study, a speculative mechanism involving the modification of Sp1 binding properties, or its transcription, in an alcohol-dependent manner could be the cause of the observed effects reported herein. It should be noted that moderate alcohol exposure may only affect membrane fluidity, leading to more active membrane-bound PKC that would yield increased Sp1 binding, while heavy alcohol exposure may lead to reduced Sp1 binding, thus reducing PON1 transcription, and/or reduced PON1 mRNA stability.

In conclusion, the present study shows that chronic light alcohol drinking upregulates, whereas chronic heavy drinking downregulates PON expression, and that PON polymorphism is not a factor in that regulation. This may account for the known cardioprotective or harmful effects of chronic light versus heavy alcohol consumption on coronary heart disease.

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