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# Paraoxonase Activity Is Reduced by a Pro-atherosclerotic Diet in Rabbits

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**Serum paraoxonase (PON1) is believed to protect against the development of atherosclerosis because of its ability to retard the oxidation of low-density lipoprotein (LDL) by hydrolysing LDL-associated phospholipid and cholesteryl-ester hydroperoxides. We have examined the relationship between PON1 and atherosclerosis development in transgenic rabbits overexpressing human apolipoprotein (apo) A-I and nontransgenic littermates fed a pro-atherogenic diet. PON1 activity was higher in transgenic (4006.1 ± 716.7 nmol/min/ml) compared to control (3078.5 ± 623.3 nmol/min/ml) rabbits ($P < 0.01$) while high-density lipoprotein (HDL) cholesterol was 1.84 ± 0.54 mmol/L in transgenic rabbits and 0.57 ± 0.21 mmol/L in control rabbits ($P = 0.0001$). After feeding rabbits a high-cholesterol diet for 14 weeks HDL-cholesterol fell by 70% in both transgenic and control rabbits ($P < 0.001$ compared to week 0) PON1 activity fell by 50% in both groups of rabbits ($P < 0.01$ compared to week 0). The amount of thoracic aortic surface area covered by lesions was 29 ± 16% in the control group and 26 ± 15% in the transgenic group ($P$ = NS). A pro-atherosclerotic diet reduces PON1 which may exaggerate the effects of the diet on the development of atherosclerosis.** 

*Key Words:* **paraoxonase; atherosclerosis; diet; lipoproteins.**

Prospective epidemiological studies have consistently indicated that high levels of HDL protect against the development and progression of atherosclerosis [1, 2]. HDL is thought to directly limit the development of atherosclerosis. Repeated infusions of HDL into cholesterol fed rabbits resulted in less atherosclerosis when compared to controls [3]. Transgenic mice overexpressing human apo A-I have higher HDL and less aortic atherosclerosis than controls when they are maintained on an atherogenic diet [4]. The mechanism evoked to explain the protective effect of HDL in these studies is that it has a rate-limiting role in reverse-cholesterol transport [1–4].

In recent years, however, evidence has grown for an alternate mechanism for the protective effect of HDL: that it retards the oxidation of LDL, which is central to current theories of the initial and progression of atherosclerosis [5, 6]. Several studies have shown that HDL retards LDL-oxidation *in vitro* [7–10]. Klimov and colleagues [11] infused human HDL into cholesterol fed rabbits and significantly reduced the concentration of plasma LDL lipid-peroxidation products, indicating this mechanism may also operate *in vivo.* We were the first to show that the anti-oxidative affects of HDL were due to a mechanism which was enzymatic [10]. Several enzymes associated with HDL have been postulated to be responsible for the anti-oxidative activity of HDL [12]. However, the current weight of evidence would suggest that paraoxonase (PON1) is the major HDL component retarding LDL-oxidation. PON1 is capable of inhibiting LDL-oxidation *in vitro* [13–17] and HDL lacking PON1 (either from avian species which naturally lack PON1 or from PON1 gene knock-out mice) cannot prevent LDL-oxidation and can be pro-oxidative [18, 19]. This and other evidence [20] indicates that PON1 plays a pivotal role in the anti-oxidative/anti-inflammatory/anti-atherosclerotic properties of HDL.

The effects of overexpressing human apo A-I on PON1 and therefore, antioxidative protection against atherosclerosis in an animal model in which lipoprotein metabolism and atherosclerosis resemble those of humans have yet to be established. As has whether PON1 is altered by feeding an atherogenic diet in this

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model. We have therefore investigated the effects of human apo A-I gene overexpression and an atherogenic diet on PON1 and atherosclerosis in rabbits.

## METHODS

*Animal model.* New Zealand white rabbits transgenic for human apo A-I (line 8 as described previously [21] n = 92) and nontransgenic littermates, n = 99 (age 3 months, weight 3.2 ± 0.5 kg) were fed normal chow (NIH-09) supplemented with 0.5% cholesterol for 14 weeks. All of the diet, 150 g per day, was consumed daily. After 14 weeks on the high cholesterol diet, one subset of animals (18 trangenics and 16 controls) were continued on this diet for a further 16 weeks before being sacrificed and another subset (16 transgenics and 16 controls) were returned to a normal chow diet for a further 16 weeks.

Blood was taken after the animals had been fasted overnight at 0, and 14 weeks after which time the animals were killed (transgenics, n = 58; nontransgenics, n = 67) except in two experiments in which animals were returned to a normal chow diet for a further 16 weeks or continued on the atherogenic diet for a further 16 weeks before blood was sampled and they were sacrificed and one experiment (13 transgenics and 18 controls) in which blood samples were obtained at 0, 2, 6, 10, and 14 weeks. Blood was divided into tubes containing either no additive or 1 mg/ml EDTA, 50 mg/L gentamicin and 0.1% sodium azide. Serum and plasma were prepared by low speed centrifugation. Serum (for PON1 analysis) was stored at −20°C before analysis. Plasma (for lipid and lipoprotein analysis) was kept at 4°C before analysis (<1 week).

*Plasma lipid and apolipoprotein analysis.* Plasma total cholesterol, triglyceride, HDL-cholesterol non-HDL-cholesterol and phospholipid and human apo A-I were quantified as described previously [22]. Lipoprotein profiles were obtained by gel filtration chromatography using a Pharmacia FPLC system fitted with a Superose 6 column. Column fractions were assayed for cholesterol content and PON1 activity.

*PON1 activity and mass.* Serum PON1 activity towards paraoxon was determined as described in detail previously [23]. Serum PON1 concentration (mass) was determined by ELISA using anti-human PON1 antibodies which we have previously shown cross-react with rabbit PON1 [18]. Rabbit PON1 was quantified by reference to our human serum standard [23].

*Evaluation of atherosclerosis lesion development.* Lesion development was assessed in the thoracic aorta from the aortic valves to the diaphragm (above the coeliac artery) and in the abdominal aorta from the diaphragm to the iliac bifurcation. Morphometric analysis of the percentage of total aorta covered by lipid deposits, stained by Oil Red O were determined by computerised planimetry as described in detail previously [22].

*Statistical analysis.* Differences between parameters with a Gaussian distribution were sought by Student's *t* test. For parameters with a non-Gaussian distribution the Mann–Whitney *U* test was used.

## RESULTS

### *Plasma Lipids and Lipoproteins*

As we have previously reported [22], plasma lipids were significantly higher in the transgenic animals due to the greater than 3-fold increase in HDL (Table 1), although there was no significant difference in non HDL-cholesterol.

**TABLE 1**

Plasma Lipids and Lipoproteins in Control and Human Apo A-I Transgenic Rabbits

| | Controls | Transgenics |
|---|---|---|
| Total cholesterol | 1.50 ± 0.72 | 2.87 ± 0.70* |
| Free cholesterol | 0.31 ± 0.18 | 0.83 ± 0.18*** |
| Triglyceride | 0.66 ± 0.20 | 1.70 ± 1.26** |
| Phospholipid | 1.07 ± 0.21 | 2.40 ± 0.40*** |
| HDL-cholesterol | 0.57 ± 0.21 | 1.84 ± 0.54*** |
| Non-HDL-cholesterol | 0.93 ± 0.54 | 1.01 ± 0.21 |
| Non-HDL:HDL cholesterol ratio | 1.64 | 0.55 |

*Note.* All values mean ± SD and are in mmol/L. Significantly different from control: *$P = 0.0013$; **$P = 0.0007$; ***$P = 0.0001$.

Feeding the high cholesterol diet for 14 weeks to both control and transgenic rabbits caused a 35-fold increase in plasma total cholesterol concentration (Fig. 1a) which was entirely due to increases in non-HDL-cholesterol (Fig. 1b). HDL in the control and transgenic animals fell by 70% ($P = 0.008$ at 14 weeks compared to pre-diet) (Fig. 1c). The plasma human apo A-I concentration also fell by 70% on the high fat diet from 153 ± 21 mg/dl pre-diet to 46 ± 17 mg/dl at week 14.

### *Paraoxonase and Lesion Development*

Serum PON1 activity was higher in the transgenic rabbits at 4006.1 ± 716.7 nmol/min/ml serum (mean ± SD) compared to controls 3078.5 ± 623.3 nmol/min/ml serum ($P < 0.01$). There was no difference between male and female rabbits. Serum PON1 concentration was significantly higher in transgenic rabbits (0.785 ± 0.387 μg/ml) (mean ± SD) than in controls (0.547 ± 0.245 μg/ml) ($P < 0.001$).

After 14 weeks on the high cholesterol diet serum PON1 activity had fallen to 1632.72 ± 454.3 nmol/min/ml serum in controls and 1987.8 ± 504.1 nmol/min/ml serum in the transgenics (both $P < 0.001$ compared to pre-diet). In the subset of rabbits from which samples were obtained at 0, 2, 6, 10, and 14 weeks, serum PON1 activity progressively fell in both control and transgenic rabbits (Fig. 2) on the high cholesterol diet until after 14 weeks activity had fallen by 43% in controls and 50% in the transgenic rabbits (both $P < 0.001$ compared to pre-diet). In the subset of rabbits which continued on the diet for a further 16 weeks serum PON1 activity fell even further in both control and transgenic animals (Fig. 2), while in the subset which were returned to a normal chow diet, PON1 activity increased in both control and transgenic animals (Fig. 2), but failed to recover to pre-diet levels. Serum PON1 concentration did not change during the experimental period in control rabbits (0.54 ± 0.26 μg/ml pre-diet, 0.571 ± 0.364 μg/ml post-diet), but decreased significantly in transgenic rabbits from

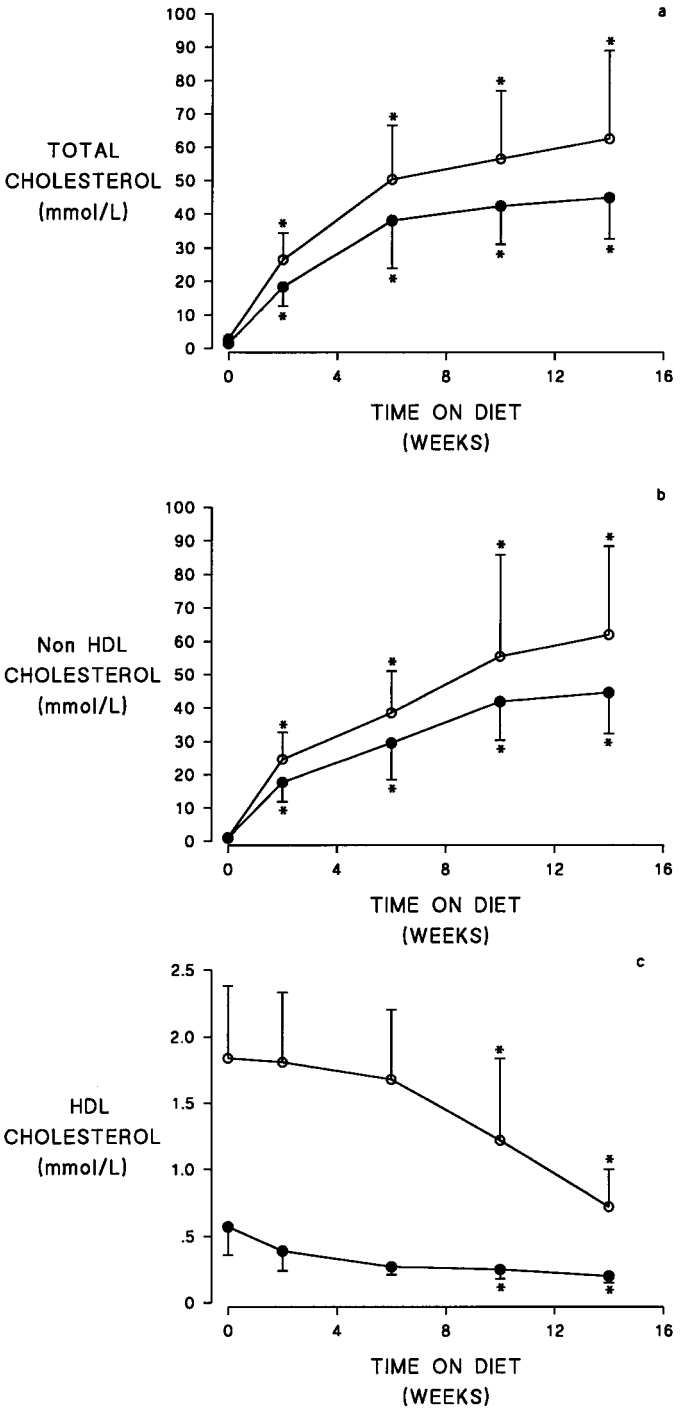


**FIG. 1.** Plasma total cholesterol (a), non-HDL cholesterol (b), and HDL cholesterol (c) in control (●) and transgenic (○) rabbits fed a 0.5% cholesterol diet. Details of diet and analysis are given under Methods. *Significantly different from week 0. $P < 0.001$.

0.775 ± 0.375 μg/ml pre-diet to 0.562 ± 0.387 μg/ml post-diet.

Analysis of the profiles produced by the FPLC System for PON1 indicated that the enzyme was entirely associated with HDL in control rabbits. However, in the transgenic animals, 10% of the PON1 activity was associated with VLDL and 90% with HDL whether analysed by activity or immunologically (results not shown). The reasons for this difference are unclear, but could be due to an association between HDL components and VLDL in the transgenic animals due to overproduction of apo A-I.

After 14 weeks on the pro-atherogenic diet, the control group had 29 ± 16% of their thoracic aorta surface covered by lesions which was not statistically different from the transgenic group (26 ± 15%). Similar results were obtained for the abdominal aortas. There was no correlation between serum PON1 activity, concentration or dietary change in PON1 activity and the quantity of atherosclerosis measured by computed tomography.

## DISCUSSION

The effect of expressing the human apo A-I gene in rabbits in this investigation was to increase both HDL-C and apo A-I as previously described [21, 22]. Both HDL-associated PON1 activity and concentration were also increased compared to nontransgenic littermates. However, the increases were not to a comparable extent to those in HDL-C. This would indicate that there is a relatively smaller effect of increasing the HDL carrier by threefold on PON1 mass and activity in rabbits which is consistent with PON1 being physically associated with a subspecies of HDL.

Feeding a 0.5% cholesterol diet caused a 70% fall in HDL-C and human apo A-I concentrations in the transgenic animals. PON1 activity declined by approximately 50% in both transgenic and nontransgenic animals, while PON1 concentration was not significantly different before and after diet. The decrease in serum PON1 activity was therefore due to a reduction in

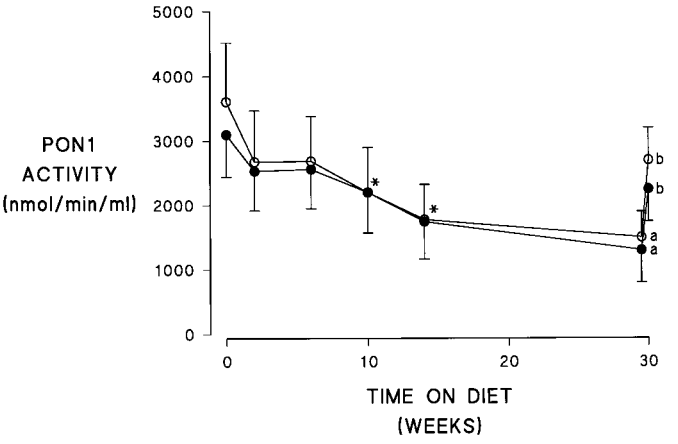


**FIG. 2.** Effect of high cholesterol diet on PON1 activity in control (●) and transgenic (○) rabbits. Experimental details are described under Methods. *Significantly different from pre-diet $P < 0.01$. (a) Continued on pro-atherogenic diet. (b) Discontinued pro-atherogenic diet and returned to normal chow.

specific activity. The reason(s) for the reduction in PON1 specific activity are unclear. Low PON1 activity may be a general consequence of hypercholesterolaemia and the consequential changes to HDL composition. PON1 activity is reduced in subjects with familial hypercholesterolaemia, diabetes mellitus, and renal disease, [24, 25] all of which are associated with hypercholesterolaemia and excess CHD compared to matched controls. PON1 activity is also reduced in apo E gene knock-out mice which exhibit gross hypercholesterolaemia [26]. Atherosclerosis-prone strains of mice also exhibit reduced PON1 activity when fed a pro-atherogenic diet [27]. However, this is due to reduced hepatic PON mRNA synthesis. This was clearly not the case in our experiments with rabbits as PON1 mass was largely unaffected indicating that synthesis and secretion may be unaffected by the high cholesterol diet. Feeding a high cholesterol diet or inducing hypercholesterolaemia by ablation of the apo E gene has been shown to increase the plasma and hepatic concentrations of lipid peroxides [26, 27]. Lipid peroxides have been shown to inhibit PON1 activity [28]. It is therefore entirely feasible that feeding high cholesterol diets to rabbits in this study caused an increase in lipid peroxide concentration resulting in inhibition of PON1 activity which would be reflected as a decrease in specific activity as found in this study.

The reduction of PON1 activity by a pro-atherogenic diet is indeed intriguing and could by itself be pro-atherogenic. PON1 retards the oxidation of LDL, reducing its pro-inflammatory/pro-atherosclerotic properties [13–17]. Studies on atherosclerosis susceptible strains of mice have shown that feeding a pro-atherogenic diet reduces HDL-PON1 and HDL isolated from these mice loses its ability to protect LDL from oxidation [27]. Although we did not investigate the ability of HDL to protect LDL in this study, it seems likely that the reduction in PON1 found in our investigation would have resulted in reduced ability of HDL to protect LDL from oxidation. HDL from PON1 gene knock-out mice is also unable to retard LDL-oxidation and such mice are more susceptible to the development of atherosclerosis than non-transgenic littermates [19]. HDL from subjects who have normal HDL concentrations but low PON1 activity is also unable to retard LDL-oxidation [29]. Both PON1 activity and mass have been reported to be reduced by 60% within hours of the onset of myocardial infarction, a finding which strongly suggests PON1 was low prior to the event [30]. It is therefore possible that nutrition may influence serum PON1 activity in man influencing its ability to retard the oxidation of LDL. It is possible that under certain conditions the anti-atherogenic effects are negated by a "bad" diet.

In this investigation we found no difference in lesion development between transgenic and non-transgenic littermates. This is different from results previously reported for another strain of human apo A-I transgenic rabbits which were resistant to the development of atherosclerosis [22]. However, the strain of transgenic rabbit used in this study contains only two copies of the human apo A-I transgene while the animals used in the previous study express five copies of the transgene [21]. In the previous study resistance to atherosclerosis in the transgenic rabbits was attributed to an increase in reverse cholesterol transport, which was not measured in the present study. There were also differences in the way the diet was applied in the two studies: in the present study animals were fed a higher concentration of cholesterol and the amount of cholesterol was not altered to keep non-HDL cholesterol concentrations the same in both groups as was the case in the previous study [22]. PON1 was not measured previously. In the present study PON1 declined to the same extent in both transgenic and nontransgenic littermates when they were fed a pro-atherogenic diet. There was no correlation between serum PON1 activity, specific activity or percentage change in activity and the quantity of atherosclerosis present. However, PON1 activity was generally lower in animals which developed most atherosclerosis, although this did not reach statistical significance (result not shown).

In conclusion, we have shown that PON1 activity is reduced by feeding a pro-atherogenic diet to both transgenic and nontransgenic rabbits. This activity reduction may be a generalised property of hypercholesterolaemia that could have mechanistic consequences for the development of atherosclerosis through an inhibition of the ability of PON1 to retard LDL oxidation.

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## REFERENCES

1. Gordon, D. J., Probstfield, J. L., Garrison, R. J. (1989) *Circulation* **79,** 8–15.
2. Miller, N. E. (1987) *Am. Heart J.* **113,** 589–597.
3. Badimon, J. J., Badimon, L., and Faster, V. (1990) *J. Clin. Invest.* **85,** 1234–1241.
4. Rubin, E. M., Krauss, R. M., Spungier, E. A., Verstuyft, J. G., and Gift, S. M. (1991) *Nature* **353,** 265–267.
5. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *New Engl. J. Med.* **320,** 915–924.
6. Witztum, J. L., and Steinberg, D. (1991) *J. Clin. Invest.* **88,** 1785–1792.
7. Parthasarathy, S., Barnett, J., and Fong, L. G. (1990) *Biochim. Biophys Acta* **1044,** 275–283.
8. Ohta, T., Takata, S., Morino, Y., and Matsuda, I. (1989) *FEBS Lett.* **257,** 435–438.
9. Navab, M., Imes, S. S., Hama, S. Y., Hough, G. P., Ross, L. A.,

Bork, R. W., Valente, A. J., Berliner, J. A., Drinkwater, D. C., Laks, H., and Fogelman, A. M. (1991) *J. Clin. Invest.* **88,** 2039–2046.

10. Mackness, M. I., Abbott, C. A., Arrol, S., and Durrington, P. N. (1993) *Biochem. J.* **294,** 829–835.
11. Klimov, A. N., Gurevich, V. S., Nikiforova, A. A., Shatilina, L. V., Kuzmin, A. A., Plavinsky, S. L., and Teryukova, N. P. (1993) *Atherosclerosis* **100,** 13–19.
12. Mackness, M. I., and Durrington, P. N. (1995) *Atherosclerosis* **115,** 243–253.
13. Mackness, M. I., Arrol, S., and Durrington, P. N. (1991) *FEBS Lett.* **286,** 152–154.
14. Mackness, M. I., Arrol, S., Abbott, C. A., and Durrington, P. N. (1993) *Atheroscl.* **104,** 129–135.
15. Mackness, M. I., Arrol, S., Mackness, B., and Durrington, P. N. (1997) *Lancet* **349,** 851–852.
16. Watson, A. D., Berliner, J. A., Hama, S. Y., La Du B. N., Fault, K. F., Fogelman, A. M., and Navab, M. (1995) *J. Clin. Invest.* **96,** 2882–2891.
17. Aviram, M., Billecke, S., Sorenson, R., Bisgaier, C., Newton, R., Rosenblat, M., Erogul, J., Hsu, C., Dunlop, C., and La Du, B. N. (1998) *Arterioscl. Thromb. Vasc. Biol.* **10,** 1617–1624.
18. Mackness, B., Durrington, P. N., and Mackness, M. I. (1998) *Biochem. Biophys. Res. Commun.* **247,** 443–446.
19. Shih, D. M., Gu, L., Xia, Y-R., Navab, M., Li, W-F., Hama, S., Castellani, L. W., Furlong, C. E., Costa, L. G., Fogelman, A. M., and Lusis, A. J. (1998) *Nature* **394,** 284–287.
20. Mackness, M. I., Mackness, B., Durrington, P. N., Fogelman, A. M., Berliner, J., Lusis, A. J., Navab, M., Shih, D., and Fonarow, G. C. (1998) *Curr. Opin. Lipidol.* **9,** 319–324.
21. Duverger, N., Viglietta, C., Berthou, L., Emmanuel, F., Tailleux, A., Parmentier-Nihoul, L., Laine, B., Fievet, C., Castro, G., Fruchart, J-C., Houdebine, L. M., and Denèfle, P. (1996) *Arterioscl. Thromb. Vasc. Biol.* **16,** 1424–1429.
22. Duverger, N., Kruth, H., Emmanuel, F., Caillaud, J-M., Viglietta, C., Castro, G., Tailleux, A., Fiever, C., Fruchart, J-C., Houdebine, L. M., and Denèfle, P. (1996) *Circulation* **94,** 713–717.
23. Abbott, C. A., Mackness, M. I., Kumar, S., Boulton, A. J. M., and Durrington, P. N. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 1812–1818.
24. Mackness, M. I., Harty, D., Bhatnagar, D., Winocour, P. H., Arrol, S., Ishola, M., and Durrington, P. N. (1991) *Atherosclerosis* **86,** 193–199.
25. Hasselwander, O., McMaster, D., Fogarty, D. G., Maxwell, A. P., Nichols, D. P., and Young, I. S. (1998) *Clin. Chem.* **44,** 179–181.
26. Hayek, T., Fuhrman, B., Vaya, J., Rosenblat, M., Belinky, P., Coleman, R., Elis, A., and Aviram, M. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 2744–2752.
27. Shih, D. M., Gu, L., Hama, S., Xia, Y-R., Navab, M., Fogelman, A. M., and Lusis, A. J. (1996) *J. Clin. Invest.* **97,** 1630–1639.
28. Aviram, M., Rosenblat, M., Billecke, S., Erogul, J., Sorenson, R., Bisgaier, C. L., Newton, R. S., and La Du B. N. (1999) *Free Radical Biol. Med.* **26,** 892–904.
29. Navab, M., Hama-Levy, S., Van Lenten, B. J., Fonarow, G. C., Carelinez, C. J., Castellanl, L. W., Brennan, M-L., La Du B. N., Lusis, A. J., and Fogelman, A. M. (1997) *J. Clin. Invest.* **99,** 2005–2019.
30. Ayub, A., Mackness, M. I., Arrol, S., Mackness, B., Patel, J., and Durrington, P. N. (1999) *Arterioscl. Thromb. Vasc. Biol.* **19,** 330–335.