ELSEVIER

European Journal of Pharmacology 388 (2000) 275–279

ejp

www.elsevier.nl/locate/ejphar

Short communication

Urinary nitrate excretion in cholesterol-fed rabbits: effect of a chronic treatment by *N*-iminoethyl-L-lysine, a selective inhibitor of inducible nitric oxide synthase

Delphine Behr-Roussel [a], Alain Rupin [b], Serge Simonet [b], Jean-Noël Fabiani [a], Tony J. Verbeuren [b,*]

[a] *Department of Cardiovascular Surgery and Laboratoire d'Etude des Greffes et Prothèses Cardiaques, Hôpital Broussais, 16 Rue Didot, 75014 Paris, France*

[b] *Division of Angiology, Servier Research Institute, 11 Rue des Moulineaux, 92150 Suresnes, France*

Received 17 November 1999; accepted 23 November 1999

Abstract

To evaluate the influence of atherosclerosis on the global production of NO, we studied the effect of a 0.3% cholesterol-enriched diet on urinary nitrate excretion in rabbits during 69 weeks. To examine whether the inducible nitric oxide synthase (iNOS) present in atherosclerotic lesions could participate in NO excretion, hypercholesterolemic rabbits were treated chronically with the selective iNOS inhibitor, *N*-iminoethyl-L-lysine (L-NIL; 5 mg/kg/day). Urinary nitrate excretion was higher in hypercholesterolemic than in control rabbits throughout the study period and decreased progressively with time in both groups; L-NIL had no significant effect on urinary nitrate excretion. These data illustrate that systemic NO production is enhanced in hypercholesterolemia and that iNOS, present within the plaque, might not participate in this enhanced NO production.

Keywords: (Rabbit); Nitrate; Atherosclerosis; Nitric oxide (NO) synthase; Nitric oxide (NO); L-NIL (*N*-iminoethyl-L-lysine)

1. Introduction

Experimental and human atherosclerosis has been shown to profoundly alter the contractile and relaxant properties of the arterial wall (Verbeuren et al., 1986, 1990). Indeed, endothelium-dependent vasodilation in atherosclerotic arteries is abnormal due to a reduced ability of the endothelium to produce and/or release biologically active nitric oxide (Ohara et al., 1993; Böger et al., 1997). In addition, we recently demonstrated the presence of a functional inducible nitric oxide synthase (iNOS) in rabbit atherosclerotic lesions (Verbeuren et al., 1993; Rupin et al., 1996), which has been recently confirmed by different immuno-histochemical studies (Esaki et al., 1997; Behr et al., 1999). In vitro, a high production of nitrogen oxides was observed by chemiluminescence in effluents from aortic segments of hypercholesterolemic rabbits (Minor et al., 1990) suggesting that NO produced by atherosclerotic plaques could be released in the bloodstream.

The in vivo activity of NO must be monitored indirectly because the unstable NO rapidly oxidizes to nitrite and predominantly nitrate (collectively referred to as NO_x) (Ignarro et al., 1993). Measurement of plasma NO_x reflects the level of systemic NO production, but also depends on the level of glomerular filtration and proximal tubule NO generation since there is substantial tubular reabsorption of NO_x (Süto et al., 1995). So, it has been recently reported that a more straightforward and useful measurement of the total NO production can be obtained by quantifying the 24-h urinary nitrate excretion in animals on a controlled diet after a suitable period of fasting (Böger et al., 1997).

The aim of the present study was first to assess the kinetics of total NO formation in rabbits fed a cholesterol-enriched diet during 69 weeks by quantifying the nitrate levels in urine samples and second, to examine whether the presence of a functional iNOS in atherosclerotic lesions had an impact on nitrate excretion levels of hypercholesterolemic rabbits.

* Corresponding author. Tel.: +33-1-55-722518; fax: +33-1-55-722430.

E-mail address: tover@netgrs.com (T.J. Verbeuren).

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00836-5

2. Material and methods

2.1. Animals

Eight-week-old New Zealand white rabbits (Charles River, France) were fed a diet (Aliment pour lapin extralbo C15, L. Pietrements Ets, France) containing $0.30 \pm 0.02\%$ of cholesterol (hypercholesterolemic rabbits) or not (control rabbits), as previously described (Verbeuren et al., 1986). Eight 8-week-old rabbits were included in the study to represent Week 0 of the hypercholesterolemic and the control diet. Then, eight hypercholesterolemic and eight control rabbits were randomly selected at 8, 17, 24, 28, 32, 36, 45, 53, and 69 weeks of diet, fasted overnight and placed in metabolic cages to collect 24-h urine samples. A blood sample was drawn from the central ear artery for plasma total cholesterol, triglyceride, high-density lipoprotein cholesterol and creatinine level determination. Creatinine was also assayed in urine samples to assess its clearance and limit variability due to changes in renal excretory function. A total of 38 control rabbits and 63 hypercholesterolemic rabbits were included in this study; some rabbits were used for more than one time point. All experiments were conducted in accordance with the institutional guidelines for animal studies.

2.2. Determination of urinary and plasma nitrate / nitrite levels

The concentration of nitrate and nitrite in urine and plasma were determined using a commercially available assay kit (Cayman Chemical, USA). Urine and plasma samples were filtered through a 10-kDa-molecular weight cut-off filter (Pall Filtron, USA) and appropriately diluted. Conversion of nitrates into nitrites was performed using nitrate reductase and the resulting products (NO_x) were assayed either with the Griess reagent for the determination in urine or with a fluorimetric method relying on the use of diamino-naphtalene to increase the sensitivity of the assay for the determination in plasma. Nitrite levels were subtracted from NO_x levels assayed to obtain urinary nitrate concentrations. Urinary levels were corrected by the clearance of creatinine.

2.3. Studies with N-iminoethyl-L-lysine (L-NIL)

After 24 weeks of hypercholesterolemic diet, eight rabbits were treated chronically by an inhibitor of iNOS, L-NIL, at 5 mg/kg/day via osmotic pump delivery into the jugular vein (2ML4 mini-osmotic pumps, Charles River) during 12 weeks (L-NIL), while eight rabbits received a physiological saline solution via the same route for the same period of time (SALINE). All rabbits continued their cholesterol-enriched diet during the treatment period. Urinary and plasma samples were obtained before initiation of the treatment and after 12 weeks of treatment. To verify the selectivity of L-NIL, mean arterial pressures were recorded on a pressure monitor (Gould) prior to and at the end of the treatment period in each group of rabbits by central ear catheterization (Unicath 20G, Abbott). Aortic segments from control rabbits ($n = 3$) were studied in isolated organ chambers to measure the effect of L-NIL (10 μM) on endothelium-dependent relaxations to acetylcholine (10^{-7} M) using the technique described by Verbeuren et al. (1986).

2.4. Statistical analysis

Assay results are expressed as the mean $\pm$ SEM of n rabbits. Statistical analysis was performed by use of a two-way ANOVA analysis with replications. Complementary analysis for group's effect at fixed number of weeks on diet and vice versa was based on Newman–Keuls test with $p < 0.05$ considered statistically significant. For L-NIL studies, two-way ANOVA with repeated measures analysis was performed with $p < 0.05$ considered statistically significant. For endothelium-dependent relaxations, results are expressed as a percentage of initial contraction to phenylephrine (1.5^{-7} M) and the effect of L-NIL was analyzed with the Student's t-test for unpaired observation with $p < 0.05$ considered statistically significant.

3. Results

Plasma total cholesterol concentrations decreased in the control group within the first 8 weeks of the study (2.11 ± 0.13 mmol/l versus 0.42 ± 0.13 mmol/l, $p < 0.05$), while they increased in the hypercholesterolemic group to stabilize around 20 mmol/l beginning at 17 weeks of diet (Table 1). Levels of high-density lipoprotein-cholesterol and triglycerides were not altered significantly by the cholesterol diet (data not shown).

Plasma nitrite concentrations were below the threshold limit of detection of the assay. Plasma nitrate levels from control and hypercholesterolemic rabbits were not significantly different throughout the study and varied between 40 and 100 μmol/l (two-way ANOVA, $n = 8$) (Table 1).

Nitrate urinary excretion in control animals decreased during the first 8 weeks of the study period (85.0 ± 14.7 μmol/l versus $28.5.0 \pm 5.9$ μmol/l, $p < 0.05$, Week 0 versus Week 8) and showed a slight, although not statistically significant decrease until the end of the study period to reach 13.9 ± 2.2 μmol/l (Week 8 versus Week 69) (Fig. 1). Nitrate urinary excretion in hypercholesterolemic rabbits was always higher than that in control rabbits of the same age, although subject to greater variability ($p < 0.05$, two-way ANOVA with replications) (Fig. 1). During the first 8 weeks, nitrate urinary excretion did not differ significantly (85.0 ± 14.7 μmol/l versus 110.24 ± 21.2 μmol/l, Week 0 versus Week 8), but then decreased

Table 1
Plasma total cholesterol and NO_x levels and values for the clearance of creatinine
Values are mean ± SEM.

Duration of diet (weeks)	Control rabbits			Hypercholesterolemic rabbits		
	Total cholesterol levels (mmol/l)	Plasma NO_x (μmol/l)	Clearance of creatinine (μmol/l)	Total cholesterol levels (mmol/l)	Plasma NO_x (μmol/l)[§]	Clearance of creatinine (μmol/l)[§]
0	2.11 ± 0.13	86.9 ± 15.43	2.42 ± 0.10	–	–	–
8	0.42 ± 0.13	74.0 ± 24.5	4.27 ± 0.17	11.64 ± 1.46*	53.4 ± 11.2	4.51 ± 0.62
17	0.53 ± 0.07	100.8 ± 17.2	5.26 ± 0.40	19.29 ± 3.19*	81.3 ± 10.2	7.59 ± 1.05
24	0.47 ± 0.09	63.4 ± 10.2	4.72 ± 0.31	17.69 ± 0.88*	64.1 ± 8.7	5.69 ± 1.28
28	0.42 ± 0.05	92.2 ± 15.8	4.45 ± 0.32	25.48 ± 4.56*	40.3 ± 7.7	5.77 ± 1.79
32	0.33 ± 0.06	74.7 ± 10.9	5.46 ± 0.88	23.36 ± 5.25*	45.5 ± 10.7	5.53 ± 1.10
36	0.46 ± 0.07	78.6 ± 7.9	4.44 ± 0.56	18.89 ± 3.07*	68.7 ± 15.3	5.03 ± 0.88
45	0.33 ± 0.02	62.9 ± 20.63	7.99 ± 1.31	23.61 ± 2.86*	42.7 ± 10.9	5.34 ± 1.14
53	0.33 ± 0.05	101.8 ± 29.8	7.12 ± 1.35	21.49 ± 1.55*	87.0 ± 26.1	6.51 ± 1.48
69	0.34 ± 0.05	84.7 ± 12.3	4.97 ± 0.87	13.38 ± 2.06*	60.2 ± 12.7	5.32 ± 0.43

[§] Not significant versus control, two-way ANOVA with replications.
* $p < 0.05$ versus control, Newman–Keuls complementary analysis of two-way ANOVA with replications.

throughout the study period to reach 30.6 ± 9.0 μmol/l at 69 weeks ($p < 0.05$, Week 0 versus Week 69) (Fig. 1).

In a second study, we looked at urinary nitrate excretion levels of rabbits fed a 0.3% enriched-cholesterol diet for 24 weeks and then, treated by L-NIL (5 mg/kg/day) or its vehicle for 12 more weeks. This treatment had no effect on the lipid profile, the clearance of creatinine or mean arterial blood pressure (data not shown). Before treatment, the urinary nitrate excretion levels were not significantly different in both groups (24.93 ± 6.04 μmol/l SALINE versus 73.08 ± 22.61 μmol/l L-NIL, paired two-way ANOVA with repeated measures). These levels were not significantly modified by a chronic administration of L-NIL (50.33 ± 21.48 μmol/l SALINE versus 93.47 ± 17.94 μmol/l L-NIL, paired two-way ANOVA with repeated measures) although the presence of iNOS within these lesions had been verified in both treated and untreated rabbits, as previously reported (data not shown) (Behr et al., 1999). In isolated aortas with endothelium of control rabbits ($n = 3$), acetylcholine (10^{-7} M) caused relaxations (66.7 ± 7.7%) that were not significantly altered by L-NIL (10 μM) (63.1 ± 9.1%) (Student's unpaired *t*-test).

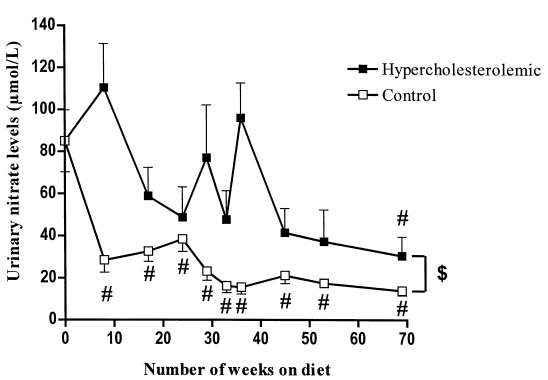

Fig. 1. Urinary nitrate excretion levels in 24-h urines collected at different time intervals corresponding to the number of weeks on regular (Control, open squares) or hypercholesterolemic (full squares) diet. Data are mean ± SEM of eight rabbits for each time point. [§] $p < 0.05$, Two-way (Group versus number of weeks on diet) ANOVA with replications; [#] $p < 0.05$, Newman–Keuls complementary comparison of each level versus week 0 in each group.

4. Discussion

The principal finding of this study is that 24-h urinary nitrate excretion is increased in hypercholesterolemic rabbits fed a 0.3% cholesterol-enriched diet compared to control rabbits throughout a 69-week study period. Furthermore, in both groups, the excretion of urinary nitrate decreases with time. Finally, the selective iNOS inhibitor, L-NIL (Moore et al., 1994; Hallinan et al., 1998), administered between 24 and 36 weeks of hypercholesterolemic diet, did not modify urinary nitrate excretion levels suggesting that the NO production by iNOS may not participate to the enhanced urinary excretion of nitrate in hypercholesterolemic rabbits.

In this study, measurement of 24-h urinary nitrate excretion was used to evaluate the global NO production by hypercholesterolemic rabbits released in the bloodstream. In fact, this parameter was chosen over plasma NO_x since the latter is a complex parameter that reflects the level of renal function and plasma volume and also is an indirect index of systemic NO production. Moreover, NO generated in the proximal tubule may also contribute to plasma NO_x levels, because there is substantial tubular reabsorption of NO_x. Thus, we chose to study the 24-h urinary nitrate excretion from rabbits in a steady state to give a qualitative and reliable measure of total NO production. This parameter has been previously used in rats and rabbits (Granger et al., 1991; Böger et al., 1995). Nonetheless, it should be noted that this parameter also takes into account the renal metabolism of nitrate (Süto et al., 1995; Baylis

and Vallance, 1998). In this context, the only parameter that could influence our results, apart from the NO production, is the tubular reabsorption of nitrate.

Between 0 and 8 weeks of normal diet, we found a strong decrease in the control rabbit urinary excretion of nitrate. During the same period, the cholesterol levels of these young male control animals decreased significantly suggesting a possible link between the levels of cholesterol and nitrate tubular reabsorption. Both parameters have never been monitored in the same study previously; however, transient high levels of cholesterol have already been reported in normal 8-week-old rabbits (Roberts et al., 1974).

We also found in our study that urinary nitrate excretion levels were always higher in hypercholesterolemic rabbits than in control rabbits. This observation appears to be in contradiction with the reduced endothelium-dependent relaxation observed in atherosclerotic arteries, which may be due, at least in part, to a reduced release of EDRF or NO (Verbeuren et al., 1990; Böger et al., 1997). However, Kanazawa et al. (1996) has suggested that endothelial NOS expression could be increased in WHHL rabbits in spite of altered endothelium-dependent relaxations. This paradox could be explained by an inactivation of endothelial NO by superoxide radicals resulting in a decreased bioavailability (Minor et al., 1990; Ohara et al., 1993). Since NO and superoxide radicals can combine to be excreted as nitrates (Baylis and Vallance, 1998), the increased urinary nitrate excretion we found in hypercholesterolemic rabbits could reflect an increased production of these entities. Another possibility to explain the enhanced excretion of NO in atherosclerosis would be an increased NO synthesis by the iNOS. In fact, we have demonstrated the presence and functional activity of iNOS in atherosclerotic arteries from rabbits fed a 0.3% cholesterol-enriched diet for more than 30 weeks (Verbeuren et al., 1993; Rupin et al., 1996; Behr et al., 1999) and others have described the activity of calcium-independent NO synthase in other organs such as the lungs as soon as 8 weeks after a hypercholesterolemic diet (Lang et al., 1993). Selective inhibitors of iNOS have only very recently become available and successful in vivo inhibition with L-NIL has been reported in models of chronic inflammation in rats and dogs (Faraci et al., 1996; Garvey et al., 1997). In our present study, we used a dose of L-NIL that resulted in a plasma concentration of about 10 μM; this concentration markedly decreases iNOS activity in cholesterol-fed rabbits without interfering with eNOS activity, as evidenced by the lack of influence on endothelium-dependent relaxations and mean blood pressure. Urinary nitrate excretion levels from rabbits treated with such a selective dose of L-NIL were not modified as compared to rabbits receiving a saline solution, which seems to rule out the hypothesis of iNOS implication. However, this inhibition of iNOS may not be as effective depending on which organ the isoform is located and thus be undetectable when considering global NO production. Furthermore, we cannot exclude that the apparent lack of effect of L-NIL may be linked to the large individual variability in response to cholesterol feeding in rabbits. It is, unfortunately, a concern with this kind of model (Verbeuren et al., 1986, 1990) and the reason why we chose to include relatively large groups of rabbits in this study. Nonetheless, it may also be that the presence of iNOS within atherosclerotic plaque may not be sufficient to elevate NO levels so as to influence systemic NO production.

In this study, we also show that urinary nitrate excretion decreases progressively with time between 8 and 69 weeks of diet both in control and hypercholesterolemic rabbits. An initial decrease in nitrate excretion has been described between 0 and 16 weeks in control rabbits (Böger et al., 1997) as well as in aging rats (Reckelhoff et al., 1997) and it has also been reported that urinary nitrate excretion was higher in younger humans (Lyons et al., 1997). Thus, our results are in accordance with other studies showing a tendency to a decrease in global NO production with aging, independently of the patho-physiological state of the vessel.

In conclusion, this study shows an increase in systemic NO production via an elevation in urinary nitrate levels in hypercholesterolemic rabbits compared to control animals. Furthermore, systemic NO production decreases with time in both control and hypercholesterolemic rabbits, which does not reflect the presence of iNOS described in atherosclerotic vessels.

References

Baylis, C., Vallance, P., 1998. Measurements of nitrite and nitrate levels in plasma and urine — what does this measure tell us about the activity of the endogenous nitric oxide system. Curr. Opin. Nephrol. Hypertens. 7, 59–62.

Behr, D., Rupin, A., Fabiani, J.-N., Verbeuren, T.J., 1999. Distribution and prevalence of inducible nitric oxide synthase in atherosclerotic vessels from long-term cholesterol-fed rabbits. Atherosclerosis 142, 335–344.

Böger, R., Bode-Böger, S., Brandes, R., Phivthong-ngam, L., Böhme, M., Nafe, R., Mügge, A., Frölich, J., 1997. Dietary L-Arginine reduces the progression of atherosclerosis in cholesterol-fed rabbits. Comparison with Lovastatin. Circulation 96, 1282–1290.

Böger, R., Bode-Böger, S., Mügge, A., 1995. Supplementation of hypercholesterolaemic rabbits with L-Arginine reduces the vascular release of superoxide anions and restores NO production. Atherosclerosis 117, 273–284.

Esaki, T., Hayashi, T., Muto, E., Yamada, K., Kuzya, M., Iguchi, A., 1997. Expression of inducible nitric oxide synthase in T lymphocytes and macrophages of cholesterol-fed rabbits. Atherosclerosis 128, 39–46.

Faraci, W., Nagel, A., Verdries, K., Vincent, L., Xu, H., Nichols, L., Labasi, J., Salter, E., Pettipher, E., 1996. 2-Amino-4-methylpyridine as a potent inhibitor of inducible NO synthase activity in vitro and in vivo. Br. J. Pharmacol. 119, 1101–1108.

Garvey, E., Oplinger, J., Furfine, E., Kiff, R., Laszlo, F., Whittle, B., Knowles, R., 1997. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J. Biol. Chem. 272, 4959–4963.

Granger, D.L., Hibbs, J.B., Broadnax, L.M., 1991. Urinary nitrate excretion in relation to murine macrophage activation. Influence of dietary L-arginine and oral N^G-monomethyl-L-arginine. J. Immunol. 146, 1294–1302.

Hallinan, E.A., Tsymbalov, S., Finnegan, P.M., Moore, W.M., Jerome, G.M., Currie, M.G., Pitsele, B.S., 1998. Acetamidine lysine derivative, *N*-(5(*S*)-amino-6,7-dihydroxyheptyl)-ethanimidamide dihydrochloride: a highly selective inhibitor of human inducible nitric oxide synthase. J. Med. Chem. 41, 775–777.

Ignarro, L., Fukuto, J., Griscavage, J., Rogers, N., Byrns, R., 1993. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. Proc. Natl. Acad. Sci. U. S. A. 90, 8103–8107.

Kanazawa, K., Kawashima, S., Mikami, S., Miwa, Y., Hirata, K.-I., Suematsu, M., Hayashi, Y., Itoh, H., Yokoyama, M., 1996. Endothelial constitutive nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired endothelium-dependent vascular relaxation. Am. J. Pathol. 148, 1949–1956.

Lang, D., Smith, J., Lewis, M., 1993. Induction of a calcium-independent NO synthase by hypercholesterolaemia in the rabbit. Br. J. Pharmacol. 108, 290–292.

Lyons, D., Roy, S., Patel, M., Benjamin, N., Swift, C., 1997. Impaired nitric oxide-mediated vasodilatation and total body nitric oxide production in healthy old age. Clin. Science 93, 519–525.

Minor, R.L., Myers, P.R., Guerra, R., Bates, J.N., Harrison, D.G., 1990. Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. J. Clin. Invest. 86, 2109–2116.

Moore, W., Webber, R., Jerome, G., Tjoeng, F., Misko, T., Currie, M., 1994. L-*N*6-(1-Iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. J. Med. Chem. 37, 3886–3888.

Ohara, Y., Peterson, T.E., Harrison, D.G., 1993. Hypercholesterolemia increases endothelial superoxide anion production. J. Clin. Invest. 91, 2546–2551.

Reckelhoff, J., Kellum, J.J., Racusen, L., Hildebrandt, D., 1997. Long-term dietary supplementation with L-arginine prevents age-related reduction in renal function. Am. J. Physiol. 272, R1768–R1774.

Roberts, D., West, C., Redgrave, T., Smith, J., 1974. Plasma cholesterol concentration in normal and cholesterol-fed rabbits. Atherosclerosis 19, 369–380.

Rupin, A., Behr, D., Verbeuren, T.J., 1996. Increased activity of guanylate cyclase in the atherosclerotic rabbit aorta: role of non-endothelial nitric oxide synthases. Br. J. Pharmacol. 119, 1233–1238.

Süto, T., Losonczy, G., Qiu, C., Hill, C., Samsell, L., Ruby, J., Charon, N., Venuto, R., Baylis, C., 1995. Acute changes in urinary excretion of nitrite + nitrate do not necessarily predict renal vascular NO production. Kidney Int. 48, 1272–1277.

Verbeuren, T.J., Bonhomme, E., Laubie, M., Simonet, S., 1993. Evidence for induction of non-endothelial NO synthase in aortas of cholesterol-fed rabbits. J. Cardiovasc. Pharmacol. 21, 841–845.

Verbeuren, T.J., Jordaens, F.H., van Hove, C.E., van Hoydonck, A.E., Herman, A.G., 1990. Release and vascular activity of endothelium derived relaxing factor in atherosclerotic rabbit aorta. Eur. J. Pharmacol. 191, 173–184.

Verbeuren, T.J., Jordaens, F.H., Zonnekeyn, L.L., van Hove, C.E., Coene, M.-C., Herman, A.G., 1986. Effect of hypercholesterolemia and vascular reactivity in the rabbit: endothelium-dependent and endothelium-independant contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. Circ. Res. 58, 552–564.