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Increased activity of guanylate cyclase in the atherosclerotic rabbit aorta: role of non-endothelial nitric oxide synthases

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1 Experiments were performed to examine the effects of putative non-endothelial nitric oxide on the soluble guanylate cyclase activity of severe atherosclerotic aortae from hypercholesterolaemic rabbits fed a cholesterol rich diet for 45 weeks.

2 The guanosine 3':5'-cyclic monophosphate (cyclic GMP) content of aortae from rabbits fed either a control diet or a diet containing 0.3% cholesterol for 45 weeks was quantified in saline extracts or in trichloracetic acid/ether extracts by use of a competitive immunoenzymatic assay. Rabbit anti-cyclic GMP immunoglobulin G was covalently linked to the solid phase, in order to avoid false positive results due to high rabbit immunoglobulin G concentrations in the atherosclerotic saline extracts.

3 Saline extracts of atherosclerotic aortae which were harvested immediately after death (intact aortae) contained about 6 fold more cyclic GMP than control aortae when expressed in pmol cyclic GMP mg^{-1} protein. The cyclic GMP concentrations in trichloracetic acid/ether extracts of atherosclerotic and control aortae expressed in pmol mg^{-1} fresh tissue were not significantly different.

4 Neointimal-medial explants from atherosclerotic and control aortae were placed in a physiological saline solution and incubated at 37°C for six hours in an incubator gassed with 5% CO_2. Before the incubation, the cyclic GMP concentrations in saline extracts of atherosclerotic explants (0.74 ± 0.27 pmol mg^{-1}) were found to be 17 fold higher than those of control explants (0.043 ± 0.008 pmol mg^{-1}). The cyclic GMP content of control explants decreased significantly after 6 h of incubation, while that of atherosclerotic explants remained elevated.

5 Chronic administration of N^G-nitro-L-arginine methyl ester, a non selective inhibitor of nitric oxide synthases, at 12 mg kg^{-1} day^{-1} subcutaneously for one month did not reduce the cyclic GMP concentration of intact atherosclerotic aortae, while that of intact aortae from control rabbits decreased by $63.4 \pm 7.6\%$.

6 These data show that atherosclerotic aortae harvested immediately after death from hypercholesterolaemic rabbits contain higher concentrations of cyclic GMP than control aortae when measured in saline extracts. *In vitro*, the persistence of the cyclic GMP production in atherosclerotic neointimal medial explants suggests that the guanylate cyclase is activated by an endogenous mediator. This mediator could be NO, synthesized by non endothelial nitric oxide synthases. The results confirm our previous findings on atherosclerotic blood vessel reactivity, but further studies are needed to elucidate why treatment with N^G-nitro-L-arginine methyl ester did not decrease the cyclic GMP content of atherosclerotic rabbit aortae.

Keywords: Atherosclerosis; NO synthase; guanylate cyclase; guanosine 3':5'-cyclic monophosphate; N^G-nitro-L-arginine methyl ester

Introduction

It is now well accepted that both experimental and human atherosclerosis profoundly alters the contractile and relaxant properties of the arterial wall (Verbeuren, 1993; Harrisson, 1995). Thus, atherosclerosis causes a marked inhibition of the endothelium-dependent relaxations by a multifactorial process which implicates a decreased release of endothelium-derived relaxing factor (nitric oxide, NO) as well as destruction of NO by constituents of the atherosclerotic plaque (Verbeuren *et al.*, 1986; 1990; 1993) the latter can be explained by excess degradation of NO by superoxide anions generated in endothelial cells or in cells closely related to the endothelium from cholesterol-fed aortic segments (Minor *et al.*, 1990; Ohara *et al.*, 1993). At the smooth muscle level, NO activates the soluble guanylate cyclase, resulting in the generation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) and vascular relaxation by activation of cyclic GMP-dependent protein kinase (Gryglewski *et al.*, 1988; Furchgott & Vanhoutte, 1989; Moncada *et al.*, 1991; Wong & Garbers, 1992; Moncada & Higgs, 1993; Berdeaux, 1993; Robertson *et al.*, 1993). Hypercholesterolaemia and atherosclerosis also decrease the guanylate cyclase activity of aortae and thus participate in the inhibition of the endothelium-dependent relaxations found in these pathological vessels (Schmidt *et al.*, 1993). Recently, we described pharmacological evidence for the induction of non-endothelium NO synthase in atherosclerotic aortae of cholesterol-fed rabbits (Simonet *et al.*, 1993; Verbeuren *et al.*, 1993). This non-endothelial NO might be responsible for the decreased reactivity of these tissues to vasoconstriction. However, in those studies we were unable to demonstrate an increased concentration of cyclic GMP in the atherosclerotic blood vessels (Simonet *et al.*, 1993).

The goal of the present study was to re-examine this problem and to measure accurately under different experimental conditions the cyclic GMP content in atherosclerotic aortae from hypercholesterolaemic rabbits.

Methods

Cholesterol feeding

For the present study, two groups of male New-Zealand rabbits obtained from Charles River, France were used. One group of rabbits was fed a control diet (control rabbits) and the other

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Table 1 Body weight and plasma lipid concentrations of control and hypercholesterolaemic rabbits untreated or chronically treated with N^G-nitro-L-arginine methyl ester (L-NAME)

	Control rabbits		*Hypercholesterolaemic rabbits*	
	Untreated	*L-NAME-treated*	*Untreated*	*L-NAME-treated*
Weight[a] (kg)	4.65±0.13	4.52±0.22	4.11±0.27	3.80±0.28
Lipids ($mmol\,l^{-1}$)				
Total cholesterol[b]	0.31±0.04	0.41±0.06	31.1±3.6*	38.2±7.1*
HDL cholesterol[b]	0.12±0.02	0.17±0.03	0.45±0.25	0.58±0.38
Triglycerides[b]	1.17±0.20	1.13±0.27	2.01±0.59	1.78±0.66

[a]Body weights are given as determined just before and after the chronic treatment with L-NAME ($12\,mg\,kg^{-1}\,day^{-1}$ for 4 weeks, $n=5$). [b]Total, high density lipoproteins cholesterol and triglycerides were measured in blood obtained at death in the different groups of animals ($n=10$ without L-NAME (untreated) and $n=5$ with chronic L-NAME treatment). *Value significantly increased as compared to control rabbits ($P<0.05$; Student's t test for unpaired observations).

group was fed a diet containing cholesterol for 45 weeks (cholesterol-fed rabbits). All animals received 200 g of food daily. The diets were obtained from L. Pietrements Ets (Provins, France). The final cholesterol content of the atherogenic data averaged 0.30±0.02%. The protocols were identical to those described previously (Verbeuren *et al.*, 1986). The cholesterol diet markedly increased the plasma levels of total cholesterol from 0.31 ± 0.04 mmol l^{-1} to 31.1 ± 3.6 mmol l^{-1} but did not significantly modify those of high density lipoprotein cholesterol or those of triglycerides (Table 1).

Treatment of rabbits with L-NAME

To study the effect of chronic administration of N^G-nitro-L-arginine methyl ester (L-NAME), 5 control rabbits and 5 cholesterol-fed rabbits were treated with L-NAME for 4 weeks. Osmotic pumps (Alzet model 2ML4) were loaded with a sterile filtered solution of L-NAME (Sigma) that had been dissolved in a sterile saline solution (NaCl 0.9% Biosedra, Louviers, France) and made up to a concentration based on the initial weight of each rabbit to deliver $12\,mg\,kg^{-1}$ per day. The pumps were surgically implanted subcutaneously under sterile conditions in the scapular region as described previously (Cayatte *et al.*, 1994). All animals survived the intervention and the plasma lipid concentrations were not significantly modified by the L-NAME treatment (Table 1).

Tissue preparation and in vitro *procedures*

After cervical dislocation, rabbits were exsanguinated and a blood sample was taken for determination of cholesterol and triglyceride levels. Then, a 1 cm in length segment of the ascending thoracic aorta was immediately prepared, rinsed and frozen in liquid nitrogen and stored at −80°C (referred to as 'intact' aorta). The remainder of the thoracic aorta was then removed asceptically and immersed in a physiological saline solution (PSS) of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, Ca-EDTA 0.026 and glucose 11.1. Extraneous tissue was removed, the vessel opened and pinned luminal side up on a silicon sheet. The endothelial layer was removed by gentle scraping of the luminal surface with a scalpel and extensive washing with PSS. The exposed control medial or atherosclerotic neointimal-medial smooth muscle layer was then stripped from the adventitia with watchmakers' forceps and placed in PSS. By use of a scalpel blade, the tissues were then cut into 1 mm^2 explants and incubated individually for 6 h in the wells of a 96-well plate (Costar) with 150 µl of PSS in a humidified incubator gassed with 5% CO_2 at 37°C. After the incubation period, explants were removed and immediately frozen in liquid nitrogen and stored at −80°C. In the case of aortae from cholesterol-fed rabbits, only those parts of the aortae containing continuous atherosclerotic plaques were used to prepare explants.

Cyclic GMP extraction

Cyclic GMP from intact aortae was extracted from frozen tissues by the trichoracetic acid (TCA)/ether method (Pradelles & Grassi, 1989). Homogenization was performed in a glass/glass potter homogenizer in a phosphate buffer (50 mM, pH 7.4) containing 6% TCA. One millilitre of buffer per gram of aorta was added to homogenize the tissue. The homogenates were then centrifuged at 5000 *g* for 10 min and extracted three times with diethyl ether that had been saturated with water. Residual ether were removed by heating the samples to 70°C for 5 min and TCA/ether extracts frozen at −30°C to be assayed.

Saline extracts from intact tissue and explants of control and atherosclerotic aortae were also prepared. In this case, tissues were homogenized for 2 min with the glass potter homogenizer that contained 1 ml phosphate/theophylline buffer (phosphate buffer 50 mM, theophylline 1 mM, pH 7.4). This homogenization was followed by a 30 s sonication period. An aliquot of the final homogenate was used for protein determination by the DC protein assay (Biorad) based on the Lowry procedure (Lowry *et al.*, 1951). The remaining homogenate was centrifuged at 3,000 *g* for 15 min and the supernatants or saline extracts frozen at −30°C to be assayed for cyclic GMP.

Cyclic GMP assay

The quantification of cyclic GMP was performed with a commercially available cyclic GMP enzyme immunoassay kit (Cayman Chemical). A competitive assay (protocol I) was used where the sample, the tracer (a fixed concentration of purified cyclic GMP labelled with acetylcholinesterase) and the rabbit cyclic GMP antiserum were coincubated overnight at room temperature on the solid phase sensitized by a monoclonal anti-rabbit IgG antibody. Under these conditions, the cyclic GMP to be assayed competed with the cyclic GMP tracer which enables it to be quantified by use of a calibration curve performed with known concentrations of cyclic GMP. B and B° represented the bound enzyme activity measured in the presence or absence of competitor, respectively. The results are expressed in terms of $B/B° \times 100$ (B/B°) as a function of the logarithm of the dose. The B/B° value of 80% was arbitrary considered as the cut-off level in this assay (Pradelles & Grassi, 1989).

A second protocol was developed (protocol II) to assay cyclic GMP of saline extracts where the rabbit anti-cyclic GMP IgG was covalently linked to the goat anti-rabbit IgG to avoid the displacement of rabbit anti-cyclic GMP IgG by contaminant rabbit IgG of atherosclerotic saline extracts. To perform this protocol, the rabbit polyclonal anti-cyclic GMP was incubated alone overnight at 4°C in the wells of the coated microplate. After a washing step, a 0.25% glutaraldehyde solution in phosphate buffer (0.1 M) pH 7.4 was added for 10 min under constant agitation at 20°C. The glutaraldehyde was then neutralized by the addition in each

well of a solution of $NaBH_4$ 4 mg ml^{-1} in pure water for 10 min. Then the plate was washed and the cyclic GMP extract to be assayed plus the cyclic GMP tracer were incubated together for 18 h at 20°C. The cyclic GMP was quantified by performing an assay similar to protocol I by use of a calibration curve realized with increasing concentrations of purified cyclic GMP.

Rabbit IgG quantification

Rabbit IgG quantification was performed by use of an indirect enzyme linked immunosorbent assay. The solid phase sensitized by the monoclonal anti-rabbit IgG antibody (Cayman Chemical) was used to capture rabbit IgG of saline extracts for one hour at room temperature. After four washes with phosphate buffer saline at pH 7.4 (Sigma) containing 0.05% of Tween 20 (Sigma), a purified IgG from a swine polyclonal anti-rabbit IgG labelled with peroxidase (Dako, Glostrup, Denmark) was incubated, diluted at 1/10000, for one hour at room temperature. Four washings were then performed and orthophenylene diamine (1 mg ml^{-1}) in citrate buffer containing 0.1% (v/v) hydrogen peroxide was incubated at room temperature for 30 min. Absorbances were registered with an ieMS spectrophotometer at 492 nm (Labsystem, Finland). The simultaneous performance of a calibration curve with increasing concentrations of purified rabbit IgG (Sigma) allowed the quantification of rabbit IgG in saline extracts.

Statistical analysis

Results are shown as the mean ± s.e.mean of n experiments. Statistical analysis was performed by use of Student's t test for paired or unpaired observations with $P<0.05$ considered statistically significant.

Results

Influence of rabbit IgG on the assay of cyclic GMP

Rabbit IgG contained in saline extracts from control and atherosclerotic rabbit aortae was quantified with an indirect enzyme linked immunosorbent assay. Saline extracts from intact atherosclerotic aortae contained 160 fold more rabbit IgG than saline extracts from control aortae (312 ± 53 mg l^{-1} versus 1.95 ± 0.35 mg l^{-1}, $P<0.05$ unpaired t test, $n=5$). The influence of rabbit IgG on a classical cyclic GMP competitive enzyme immunoassay (protocol I) with a cyclic GMP antiserum prepared in the rabbit is shown in Figure 1a. Samples without cyclic GMP but containing purified rabbit IgG 3 mg l^{-1} gave B/B° values lower than 80% which decreased to 0% when the concentration of rabbit IgG was increased to 30 mg l^{-1}. Thus, samples without cyclic GMP but containing concentrations of rabbit IgG equal or higher than 3 mg ml^{-1} were falsely cyclic GMP positive when protocol I was used. To avoid this problem rabbit anti-cyclic GMP IgG was covalently linked on the monoclonal anti-rabbit IgG sensitized solid phase by the use of glutaraldehyde (protocol II). Under these methodological conditions, B/B° values obtained with samples without cyclic GMP but containing rabbit IgG concentrations up to 1000 mg l^{-1} remained higher than 80% and thus were negative for cyclic GMP. Calibration curves performed with increasing concentrations of purified cyclic GMP gave similar results whatever the protocol used (Figure 1b).

Cyclic GMP in intact aortae as a function of the protocol used

The concentration of cyclic GMP, expressed in pmol mg^{-1} of protein was first determined in saline extracts with protocol I. Protein concentrations of saline extracts from control and atherosclerotic aortae were, respectively, 6.4 ± 2.1 and 15.3 ± 0.9 mg ml^{-1} ($n=5$). With these methodological conditions, the cyclic GMP concentrations of saline extracts were found to be 50 fold higher in atherosclerotic aortae than in control aortae (42.2 ± 5.1 versus 0.84 ± 0.20 pmol mg^{-1}, $P<0.05$ unpaired t test, $n=5$). In a second step, the cyclic GMP contained in saline extracts of intact control and atherosclerotic aortae was determined with protocol II. In control aortae which contained low levels of rabbit IgG (1.95 ± 0.35 mg l^{-1}), similar concentrations of cyclic GMP were found with both protocols (1.05 ± 0.38 pmol mg^{-1} versus 0.84 ± 0.20 pmol mg^{-1}, NS, unpaired t test, $n=5$). In contrast, the cyclic GMP concentration of saline extracts from athero-

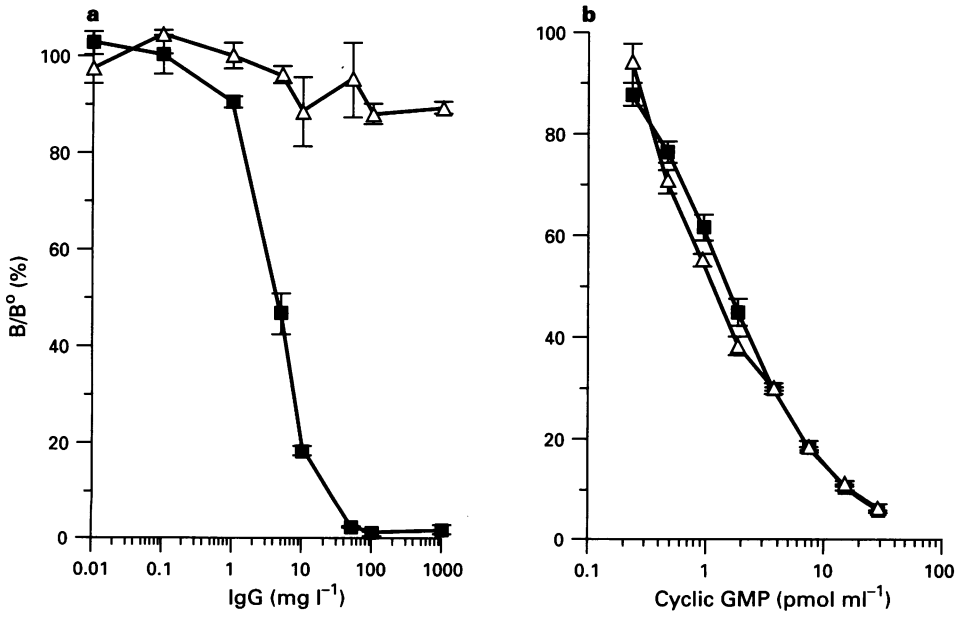

Figure 1 Effects of purified IgG (a) and purified cyclic GMP (b) on the ratio of bound enzyme activity measured in the presence or absence of competitor (B/B° expressed as percentage of the total activity in the absence of competitor). Measurements were performed by use of either a classical competitive protocol (■; protocol I) where cyclic GMP rabbit antiserum was incubated in the presence of labelled cyclic GMP and the cyclic GMP to be assayed, or a modified protocol (▲, protocol II) where the cyclic GMP rabbit antiserum was covalently linked to the anti-rabbit IgG coated solid phase.

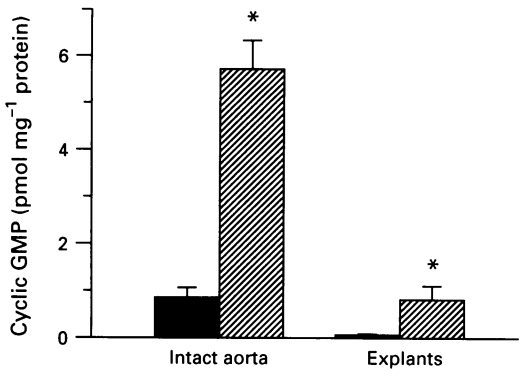

Figure 2 Cyclic GMP measured in saline extracts from intact aorta and neointimal-medial explants from control (solid columns) and atherosclerotic aortae (hatched columns). The immunoenzymatic protocol II was used to quantify cyclic GMP in atherosclerotic aortae. Cyclic GMP in atherosclerotic intact aortae and explants was significantly higher than that of intact aortae and explants from controls (*$P<0.05$, unpaired t test, $n=5$).

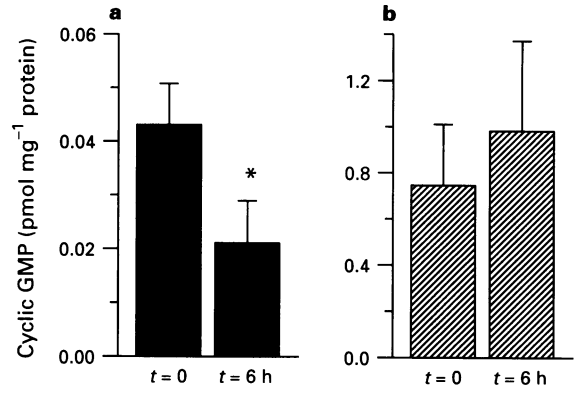

Figure 3 Cyclic GMP measured in saline extracts from medial explants of control aortae (a) and de-endothelialized neointimal-medial explants from atherosclerotic aortae (b) before and after a 6 h period of incubation in a physiological saline solution at 37°C in the presence of 5% CO_2. The cyclic GMP of medial control explants decreased significantly after 6 h of incubation (*$P<0.05$, unpaired t test, $n=5$). Note the difference in scale between control and atherosclerotic explants.

sclerotic aortae, which contained high levels of rabbit IgG (312 ± 53 mg l^{-1}) was found to be significantly lower with protocol II than with protocol I (5.71 ± 0.65 versus 42.4 ± 5.1 pmol mg^{-1}, $P<0.05$, unpaired t test, $n=5$) but remained 6 fold higher than that of control aortae (Figure 2).

The cyclic GMP content of deproteinized TCA/ether extracts of intact aortae from control and cholesterol-fed rabbits was also determined with protocol I. The masses of intact control and intact atherosclerotic aortae were, respectively, 52.5 ± 8.2 mg and 181.2 ± 35.2 mg ($n=4$). This assay revealed similar concentrations of cyclic GMP, expressed in pmol/mg^{-1}, in control and atherosclerotic aortae (3.62 ± 0.32 versus 4.46 ± 0.93 pmol mg^{-1}, NS, unpaired t test, $n=4$).

Cyclic GMP in de-endothelialized medial explants

De-endothelialized medial explants were dissected from intact control and atherosclerotic aortae. Half of them were directly frozen and the other half were placed in a physiological saline solution and incubated at 37°C for six hours in an incubator gassed with 5% CO_2 before being frozen. Cyclic GMP of saline extracts from incubated and freshly prepared explants from atherosclerotic aortae were determined with protocol II. Directly frozen explants from atherosclerotic aortae contained 8 fold less cyclic GMP than intact aortae (0.74 ± 0.27 pmol mg^{-1} versus $5.71+0.65$ pmol mg^{-1} $n=5$) and those from control aortae 20 fold less (0.043 ± 0.008 pmol mg^{-1} versus 0.84 ± 0.20 pmol mg^{-1}, $n=5$, Figure 2). Before their incubation, atherosclerotic explants contained 17 fold more cyclic GMP than control explants. After an incubation period of 6 h, the cyclic GMP concentration decreased significantly in control explants (0.021 ± 0.008 pmol mg^{-1} versus 0.043 ± 0.008 pmol mg^{-1}, $P<0.05$, paired t test, $n=5$) while it remained unchanged in atherosclerotic explants (0.98 ± 0.39 pmol mg^{-1} versus 0.74 ± 0.27 pmol mg^{-1}, $P<0.05$, paired t test, $n=5$) (Figure 3).

Effect of L-NAME on cyclic GMP concentrations

The subcutaneous administration of L-NAME by osmotic pumps for 4 weeks did not significantly modify the weight of control rabbits or that of hypercholesterolaemic rabbits (Table 1). Similarly, with both groups of rabbits, the L-NAME treatment did not significantly influence the total cholesterol, the high density lipoprotein cholesterol and the triglyceride levels of control and hypercholesterolaemic rabbits (Table 1).

Figure 4 illustrates the effect of chronic administration of L-NAME for 1 month on the cyclic GMP production by aortae

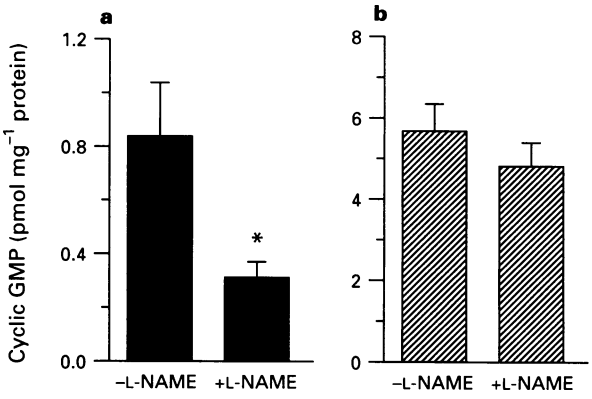

Figure 4 Cyclic GMP measured in saline extracts from intact aortae of control rabbits (a) and intact atherosclerotic aortae from hypercholesterolaemic rabbits (b) before (−L-NAME) or after (+L-NAME) chronic treatment (4 weeks) with N^G-nitro-L-arginine methyl ester 12 mg kg^{-1} day^{-1}, subcutaneously. The cyclic GMP of intact aortae from control rabbits decreased significantly after the L-NAME treatment (*$P<0.05$, unpaired t test, $n=5$). Note the difference in scale between control and atherosclerotic aortae.

of both groups of rabbits. The cyclic GMP concentration of intact aortae from control rabbits was decreased by $63.4\pm7.6\%$ (0.84 ± 0.20 pmol mg^{-1} before and 0.31 ± 0.06 pmol mg^{-1} after the treatment, $P<0.05$, unpaired t test, $n=5$, Figure 4a). In contrast, cyclic GMP from intact atherosclerotic aortae measured with protocol II was not significantly modified after the L-NAME treatment of hypercholesterolaemic rabbits (4.84 ± 0.57 pmol mg^{-1} before and 5.71 ± 0.65 pmol mg^{-1} after the treatment, NS, unpaired t test, $n=5$, Figure 4b).

Discussion

The major new finding of this investigation was that atherosclerotic rabbit aortae from long-term cholesterol-fed rabbits contain very high concentrations of cyclic GMP in comparison to control rabbit aortae. The production of cyclic GMP by de-endothelialized atherosclerotic vessels persisted *in vitro* suggesting that non endothelial NO-synthases of the atherosclerotic rabbit aorta activate guanylate cyclases in the vessel.

In the first part of the study, we demonstrated that advanced atherosclerotic lesions of thoracic aortae from rabbits fed a cholesterol diet for a long period and frozen immediately after isolation ('intact' aortae) contain 6 fold more cyclic GMP than thoracic aortae obtained from control rabbits. This result was obtained by use of a saline extraction of cyclic GMP from the tissue followed by an enzyme immunoassay protocol which is not influenced by the presence of rabbit IgG contained in atherosclerotic saline extracts. We could indeed demonstrate that saline extracts of atherosclerotic aortae contained high levels of rabbit IgG which interfered with the solid phase of the competitive enzyme immunoassay leading to overestimated cyclic GMP results. This problem was probably due to the displacement of rabbit anti-cyclic GMP IgG from the anti-rabbit IgG coated solid phase by contaminant polyclonal rabbit IgG, since the covalent link of the rabbit cyclic GMP antiserum to the solid phase eliminated false positive cyclic GMP responses even if very high concentrations of rabbit IgG contaminate a sample free of cyclic GMP. The presence of such high concentrations of rabbit IgG in atherosclerotic aortae has been described previously (Ylä-Herttuala *et al.*, 1994) and thus must be taken into account when a rabbit antiserum is used to perform the immunoenzymatic quantification of a rabbit biological substance in media or extracts contaminated by rabbit IgG.

Another possibility to avoid false positive results when immunoenzymatic assays are used to quantify a biological substance, is to deproteinize samples by the TCA/ether method. In our study we performed such a classical protocol (Pradelles & Grassi, 1989) where cyclic GMP is expressed as a function of the total weight of the tissue assayed. Under these conditions we found comparable concentrations of cyclic GMP in intact atherosclerotic aortae to those in control aortae. A disadvantage of this method in the context of our study, is that it takes into account all lipids incorporated in the atherosclerotic plaque which are not present in control tissue. This might have resulted in an underestimation of the cyclic GMP concentration of atherosclerotic aortae which is calculated as a function of the mass of the tissue. Moreover, the cyclic GMP extraction in atherosclerotic aortae by TCA/ether could be lower than in control tissues because of the high concentrations of lipids in this tissue. These two possibilities might explain the fact that similar cyclic GMP levels were detected in control and atherosclerotic aortae after TCA/ether extraction.

We and others have previously shown that the basal levels of cyclic GMP are lower in the atherosclerotic rabbit aorta than in control aorta (Bossaler *et al.*, 1987; Simonet *et al.*, 1993). Those studies were performed in blood vessel segments mounted in organ chambers and the levels of cyclic GMP were analysed in samples obtained several hours after collection of the tissues. Moreover, the rabbits used in our previous study (Simonet *et al.*, 1993) received the hypercholesterolaemic diet for only 20 weeks as compared to the 45 weeks in the present study.

In view of the fact in the present study that (1) the aortae were prepared immediately after surgery; (2) cyclic GMP was measured in saline extracts which does not underestimate its tissue content and (3) a modified cyclic GMP immunoassay was used which eliminates interference from tissue IgGs, our results, which demonstrate a marked increase of the cyclic GMP concentrations in advanced atherosclerosis in rabbit aortae, are validated.

The present study also demonstrated that de-endothelialized neointimal-medial explants from the atherosclerotic aortae contain higher concentrations of cyclic GMP than control de-endothelialized explants. Since this high level of cyclic GMP persisted in atherosclerotic explants after 6 h of *in vitro* incubation at a time when normally the cyclic GMP concentration in control explants decreases, we hypothesized that the production of a non-endothelial mediator might be responsible for this sustained and high cyclic GMP level in atherosclerotic explants. The cyclic GMP levels in control medial explants were about 20 fold lower than those obtained in intact control aortae. This most likely represents the destruction of the endothelium during the preparation of explants; indeed, in control aortae the endothelium is believed to be the principal source of guanylate cyclase activation through EDRF-NO production. Interestingly, the cyclic GMP levels of neointimal-media explants prepared from atherosclerotic aortae were also lower than those of intact aortae but they were only decreased by a factor of 8 indicating that in the atherosclerotic vessels, a part of the guanylate cyclase activation comes from the endothelium and/or from the subendothelial layer that was scraped off during the preparation of the explants. The findings obtained with the intimal-medial explants provide evidence for our previous work suggesting that a non-endothelial NO synthase activity could be operative in atherosclerotic aortae from hypercholesterolaemic rabbits (Verbeuren, 1993; Simonet *et al.*, 1993). In those studies, it was shown that the decreased contractile responses of atherosclerotic rabbit aortae to noradrenaline and 5-hydroxytryptamine were partially restored by the addition of nitro-L-arginine (L-NNA) and that this effect was reversed by the addition of an excess of L-arginine.

Another goal of the present study was to investigate the effect of chronic administration of an NO synthase inhibitor, L-NAME, on the cyclic GMP content of atherosclerotic aortae using the technique recently described by Cayatte *et al.* (1994). In control animals, we were able to confirm the data of the original paper and found that chronic administration of L-NAME with the minipumps significantly reduced the cyclic GMP content in intact aortae thereby illustrating that L-NAME was indeed inhibiting NO synthesis in the control animals. Surprisingly, treatment with L-NAME, using exactly the same procedure failed to inhibit the production of cyclic GMP in intact atherosclerotic aortae of the cholesterol-fed rabbits. If we accept that EDRF-NO is responsible for the enhanced cyclic GMP production in the atherosclerotic aorta, then one possible explanation of our data would be that L-NAME, administered *in vivo*, was unable to inhibit the inducible form of NO-synthase in the atherosclerotic aorta. Indeed, L-NAME which was described as a non selective inhibitor of NO-synthase is now known to be a rather poor inhibitor of the inducible form of the enzyme *in vitro* on stimulated macrophagic cell lines (Southan *et al.*, 1995). It is likely that the non-endothelial production of EDRF-NO comes from the numerous spumous macrophagic cells present in the advanced atherosclerotic lesions. This would be in agreement with a recent preliminary study illustrating that WHHL rabbit aortic lesions express the inducible form of NO-synthase which was confined to sites where the majority of cells were macrophages (Luoma *et al.*, 1994). Another possibility would be that L-NAME does not penetrate atherosclerotic blood vessels as readily as control vessels. However *in vitro* studies, performed on aortae of hypercholesterolaemic rabbits, have shown that L-arginine analogues are capable of inhibiting inducible NO-synthase both in tissues with and without endothelium suggesting that the compounds readily penetrate the aortic wall (Verbeuren *et al.*, 1993). Another possible explanation for the data would be that the high level of cyclic GMP found in the atherosclerotic aorta results from a NO-synthase independent pathway. Atrial natriuretic factor (ANF) or its analogues which activate the particulate guanylate cyclase (Hamet *et al.*, 1989; Wong & Gabers, 1992), guanilin or related peptides (Wong & Garbers, 1992) or hydroxyl radicals which are postulated to be activators of soluble guanylate cyclase (Waldman & Furad, 1987) could be implicated in this phenomenon. It has been shown that hydroxyl radicals are indeed generated from superoxide anion by the atherosclerotic endothelium via xanthine oxidase activation (Ohara *et al.*, 1993). Hypercholesterolaemia could also induce the production of an unknown factor leading to the activation of soluble guanylyl cyclase such as that described in the aorta of rats treated with endotoxin (Wu *et al.*, 1994).

Finally, our results demonstrate that the guanylyl cyclase of atherosclerotic vessels is sufficiently active to produce high concentrations of cyclic GMP in the aorta, and that the stimulating factor, most likely NO or another guanylate cyclase activator, is efficiently transported toward the enzyme. Even if macrophagic guanylyl cyclase has not been demonstrated in this context, it is a possibility that spumous macrophagic cells are responsible for the cyclic GMP production in atherosclerotic plaques.

In conclusion, the present data are the first to demonstrate the presence of a high concentration of cyclic GMP in the atherosclerotic plaque. This observation may explain the decreased responsiveness of atherosclerotic blood vessels to vasoconstrictor substances (Verbeuren *et al.*, 1986; 1990; 1993; Simonet *et al.*, 1993). This enhanced cyclic GMP production may also be responsible in part for the compensatory artery dilatation observed during progressive plaque development which is associated with arterial narrowing (Steinke *et al.*, 1994). Since cyclic GMP has been suggested to be an antiproliferative agent (Assender *et al.*, 1992), its intense production by the plaque may decrease smooth muscle cell proliferation and thus help to counteract the atherosclerotic process. This would be in agreement with the observation that chronic L-NAME treatment during hypercholesterlaemia enhances the atherosclerotic process (Cayatte *et al.*, 1994).

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