British Journal of Pharmacology (1996) 118, 1681-1688

Effect of verapamil on intimal thickening and vascular reactivity in the collared carotid artery of the rabbit

[1]Levent Üstünes, Mukadder Yasa, Zeliha Kerry, *Necmettin Özdemir, Tayfun Berkan, *Yildiz Erhan & Asli Özer

Department of Pharmacology, Faculty of Pharmacy and *Department of Pathology, Faculty of Medicine, Ege University, Izmir, Turkey

1 Intimal thickening is a common site for atherosclerosis. Therefore, we investigated whether the calcium entry blocker verapamil (10 mg kg^{-1} body weight day^{-1}, s.c.) can retard intimal thickening and changes in vascular reactivity induced by a non-occlusive, silicone collar positioned around the left carotid artery of rabbits. The contralateral carotid artery was sham-operated and served as a control.

2 Verapamil and placebo (saline 0.1 ml kg^{-1} day^{-1}, s.c.) treatments were initiated 7 days before placing the collar and lasted 3 weeks. Thereafter, segments were cut from collared and sham-treated arteries for histology and isometric tension recording.

3 The intima/media (I/M) ratio increased after 14 days of collar treatment, but intimal thickening was not inhibited by verapamil (I/M ratio placebo 0.31 ± 0.07, verapamil 0.32 ± 0.09).

4 The collar decreased the capacity to develop force, as indicated by the response to a supramaximal concentration of KCl, decreased the sensitivity (pD_2) to acetylcholine (ACh) and phenylephrine (Phe), but increased the sensitivity to 5-hydroxytryptamine (5-HT).

5 Although verapamil did not affect intimal thickening, it normalized the hypersensitivity to 5-HT in collared arteries.

6 The contraction to the supramaximal concentration of KCl was not affected by verapamil. Verapamil decreased the E_{max} of ACh, but this was only seen in collar-treated arteries. Verapamil also decreased the sensitivity to ACh and Phe, in both sham- and collar-treated arteries.

7 We conclude that verapamil, without preventing thickening of the intima, can modify collar-induced changes in vascular reactivity.

Keywords: Atherosclerosis; intima; collar; endothelium; 5-hydroxytryptamine; acetylcholine; phenylephrine; verapamil; vascular reactivity; calcium antagonist

Introduction

Atherosclerosis is an arterial disease characterized by localized accumulation of collagen, elastin, lipids and calcium at sites associated with macrophage infiltration and altered smooth muscle metabolism. Although there are some conflicting data, it is now suggested that verapamil (Rouleau *et al.*, 1983; Blumlein *et al.*, 1984; Sievers *et al.*, 1987) and calcium antagonists from chemically different groups (Weinstein & Heider, 1989; Henry, 1990; Catapano, 1992) inhibit the development of fatty streaks in the aorta of cholesterol-fed animals, a model of early atherosclerosis. Furthermore, patients treated with verapamil showed a slower development and a more pronounced regression of coronary artery disease (Kober *et al.*, 1989).

Thickening of the intima, due to smooth muscle cell migration and proliferation, is an essential step before atherosclerosis develops in man (Stary *et al.*, 1992). The effects of calcium antagonists on intimal thickening have rarely been studied. Intimal thickening can be evoked by placing a rigid polyethylene collar (Nomoto *et al.*, 1987) or a flexible silastic collar (Booth *et al.*, 1989) around the carotid artery of the rabbit. The first objective of the present *in vivo* study was therefore to determine whether verapamil could retard the thickening in the latter model.

Moreover, it has been shown that intimal thickening induced by the silastic collar is accompanied by marked alterations of the responsiveness of the artery to both vasoconstrictor and vasodilator agents (De Meyer *et al.*, 1990; 1991; 1994). These changes resemble those seen in models of experimental atherosclerosis (Henry & Yokoyama, 1980; Kalsner & Richards, 1984; Tesfamariam *et al.*, 1989). The possible effects of calcium antagonists on the altered vascular responsiveness due to atherosclerosis or intimal thickening have received little attention. Therefore, the second aim was to test whether verapamil could overcome the modifications in vascular reactivity associated with the collar.

[1]Author for correspondence at: Department of Pharmacology, Faculty of Pharmacy, Ege University, 35100 Bornova, Izmir, Turkey.

Methods

Treatments

White rabbits of either sex ($n = 20$) weighing 2.1 ± 0.07 kg were divided into 2 groups. The treatment group ($n = 10$) was injected once daily with 10 mg kg^{-1} verapamil subcutaneously. The placebo group of animals ($n = 10$) received saline (0.1 ml kg^{-1}). During the whole experiment rabbits were housed in individual cages and had *ad libitum* access to food and water.

Induction of intimal thickening

After 7 days of treatment with or without verapamil the rabbits were anaesthetized with sodium pentobarbitone, 30 mg kg^{-1} i.v. Subsequently, the left carotid artery was dissected free and surrounded by a non-occlusive, flexible silicone collar of 2.0 cm length (Booth *et al.*, 1989; De Meyer *et al.*, 1991). The right carotid artery was sham-operated, i.e. separated from the surrounding connective tissue and the vagus nerve, receiving a similar stretch as the contralateral collared artery. The carotid arteries were then returned to their original position and the wounds were sutured. After recovery from anaesthesia the animals were given their respective treatment for 2 weeks.

Morphometry

After anticoagulation with heparin (150 u kg^{-1}, i.v.) the rabbits were killed with an overdose of sodium pentobarbitone and two segments (4 mm) were cut from both the collared and sham-operated artery, one for morphometry, the other for isometric tension recording (cf. infra). The former was immediately placed in formalin fixative solution (0.4%) for 24 h, dehydrated in a graded series of isopropyl alcohol (60 to 100%) followed by toluol before being embedded in paraffin. Transverse sections were cut and stained with sirius red haematoxylin. Two transverse sections from the segment were randomly chosen and video images were recorded by a video-camera (JVC Colour Video Camera, Head Model No. TK-890E, Japan) connected to a light microscope (Olympus BH-2, Japan). The cross-sectional area of lumen, intima and media was measured by a computerized system. Briefly, the image of each segment from the video-player (Sony Video Cassette Recorder SL-C6E) was captured via a video-card (Video Blaster SE, Creative Labs, Creative Labs. Inc., U.S.A.). The cross-sectional area of lumen, intima, media and adventitia was traced by use of a software package (CorelDraw, Version 4.00.A5, Corel Corporation 1993, U.S.A.) and measured by Autocad (release 12-cl, 1993, Autodesk, Inc., U.S.A.) in both sections and the means were calculated. The intima/media ratio of each section was also calculated.

Vascular reactivity

The two remaining rings from the right (sham) and the left (collar-treated) carotid artery were used to study vascular reactivity. The rings were suspended in organ chambers filled with 25 ml physiological salt solution containing indomethacin (3×10^{-6} M), maintained at 37°C and continuously gassed with 95% oxygen-5% carbon dioxide (De Meyer *et al.*, 1991). Tension was measured isometrically with a Grass FT3 force transducer and recorded by IOSlab software package (IOSlab version 3.23 MS8, EMKA Technologies, Paris, France) using a 80486 based micro-computer (IBM PS/1, U.K.). After an equilibration period of 15 min the preparations were gradually stretched to a tension of 7 g, which was determined in preliminary experiments to bring both sham and collar segments to the optimal point of their length-tension relationship. The segments were then allowed to equilibrate for 45 min at their optimal length. During this period the bath solution was changed every 15 min.

At the end of the equilibration period, a cumulative concentration-response curve (CRC) to 5-HT was constructed in each preparation by increasing the concentration of 5-HT (10^{-9} to 3×10^{-5} M) by a half log unit once the preceding response had peaked or reached an equilibrium. Following attainment of a plateau contraction, a cumulative concentration-relaxation curve to acetylcholine (ACh, 10^{-9} to 10^{-4} M) was constructed in each segment. This CRC to ACh also confirmed the presence of functional endothelium in the rings. The rings that failed to relax to ACh less than 40% of the initial contraction were discarded from the experiment. Thereafter, segments were washed three times and then exposed to a cumulative (0.5 log unit) concentration range of phenylephrine (10^{-9} to 3×10^{-5} M). After washing three times, the maximum contractile force to 120 mM KCl (with equimolar replacement of NaCl; Tesfamariam *et al.*, 1989) was determined at the end of the experiment.

Materials

The physiological salt solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. Indomethacin sodium was included in all experiments to inhibit cyclo-oxygenase activity. Acetylcholine chloride, phenylephrine hydrochloride and 5-hydroxytryptamine creatinine sulphate were obtained from Sigma, St. Louis, MO, U.S.A. Indomethacin sodium was purchased from Merck, Sharp & Dohme, München, F.R.G.; sodium pentobarbitone solution from Psyphac, Brussels, Belgium and heparin solution from Roche, Istanbul, Turkey. Silicone (Silastic E, Dow Corning) was obtained from the Compagnie Commerciale de Matière Premières, Antwerp, Belgium. 5-Hydroxytryptamine creatinine sulphate monohydrate was dissolved in an aqueous solution of ascorbic acid (0.01%) and diluted in distilled water. The other drugs were made up in distilled

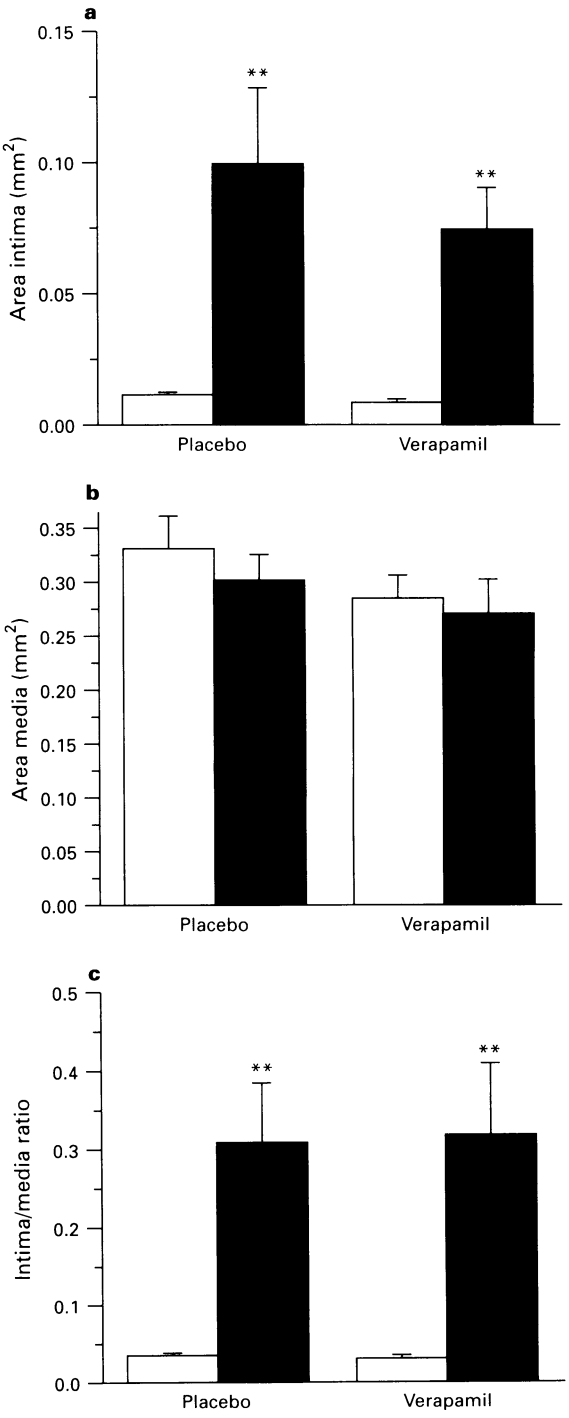

Figure 1 Effect of verapamil on intimal thickening in the rabbit carotid artery induced by 14 days collar treatment; (open columns) sham arteries and (solid columns) collared arteries. Cross-sectional areas of the intima (a), media (b) and intima/media ratio (c). Each segment measured in duplicate, distance between sections 250 μm. Data are expressed as mean ± s.e.mean; verapamil group ($n = 8$) and placebo group ($n = 10$); $^{**}P < 0.01$ sham vs. collar (Wilcoxon matched-pairs signed-ranks test).

water. Verapamil was kindly provided by Birlesik Alman Ilaç Fabrikalari T.A.S., Istanbul, Turkey.

Data analysis

All data are given as mean ± s.e.mean. The number of arteries reported (*n*) equals the number of rabbits used. For the statistical analysis the SPSS/PC$^+$ package (SPSS, Chicago, IL, U.S.A.) was applied. A 5% level of significance was selected. To compare the intima/media ratio, areas of intima and media between placebo and verapamil group the Mann-Whitney U test was used. The effect of the collar treatment (i.e. sham vs. collar) was evaluated in each group by the Wilcoxon matched-pairs signed-ranks test.

Relaxations were expressed as a percentage of the initial contraction. The negative logarithm of the molar concentration of the agonist that produced a half-maximal contraction or relaxation for that agonist ($-\log EC_{50}$ or pD_2) was determined for each segment. IOSlab software package was used for this purpose. For statistical analysis of these data a factorial analysis of variance was used with drug treatment (2 levels, verapamil or placebo) as between rabbit factor and collar (2 levels, present or not) as within rabbit factor. Only when the interaction between two factors in ANOVA was statistically significant was the ANOVA test supplemented by an additional statistical analysis (Student's *t* test).

Results

Survival and intimal thickening

All rabbits of the placebo group survived, but two rabbits of the verapamil group died. The eight remaining rabbits tolerated the verapamil treatment without visible side-effects, and weight gain was similar in placebo and verapamil-treated rabbits.

The area of the intima, and the intima/media ratio were significantly increased after the positioning of the silicone collar as compared to the sham operation (Figure 1a and c). Neither the intimal area nor the intima/media ratio were significantly lower in the verapamil group. Neither the collar implantation, the verapamil treatment nor the combination significantly altered the area of the media (Figure 1b).

Vascular reactivity

KCl Contractions to a supramaximal concentration of KCl (120 mM) were significantly diminished in collar-treated rings. Verapamil treatment did not affect the force-development to KCl in either sham- or collar-treated rings (Table 1).

Table 1 Effects of verapamil (10 mg kg^{-1} day^{-1}) and collar on contractile responses (E_{max}) to KCl in rings from rabbit carotid arteries

	E_{max} (g)	
	Placebo	*Verapamil*
Treatment	*(n=10)*	*(n=7)*
Sham	6.1±0.8	5.5±0.3
Collar	1.2±0.4	0.3±0.0
Significance of factors in analysis of variance		
- Collar:	$P<0.001$	
- Verapamil:	NS ($P=0.284$)	
- Interaction: verapamil by collar:	NS ($P=0.749$)	

Values are shown as mean±s.e.mean, *n* is the number of rabbits in each group. NS: not significant.

5-HT 5-HT induced concentration-dependent contractions in sham- and collar-treated segments. In collar-treated rings the maximum contractile response (E_{max}) was significantly diminished (Figure 2, $P<0.05$, collared versus sham-operated arteries, paired Student's *t* test). In spite of the reduced E_{max}, low concentrations of 5-HT evoked larger contractions in collared arteries (Figure 2). This sensitization was clearly reflected by increased pD_2 values (Table 2). Verapamil treatment attenuated 5-HT-induced contractions of collared arteries, but not of sham-operated arteries (Figure 2). Moreover, verapamil normalized the pD_2 values of collared segments, leading to highly significant interaction in the ANOVA (Table 2).

Phenylephrine Phenylephrine induced concentration-dependent contractions. The E_{max} and the pD_2 to phenylephrine were significantly diminished in collar-treated rings when compared to sham-treated rings (Figure 3, Table 3). Verapamil did not significantly influence the E_{max}, but reduced the pD_2 of phenylephrine to the same extent in collared and sham-treated segments (Figure 3, Table 3).

Acetylcholine Acetylcholine induced concentration-dependent relaxations in sham- and collar-treated segments constricted with 5-HT (3×10^{-5} M). Collar did not significantly influence the maximum relaxations, but the pD_2 was significantly decreased in collared segments (Figure 4, Table 4). Verapamil decreased the pD_2 to the same extent in sham and collared arteries (Table 4). Moreover, the treatment with verapamil significantly depressed amplitude of relaxations in

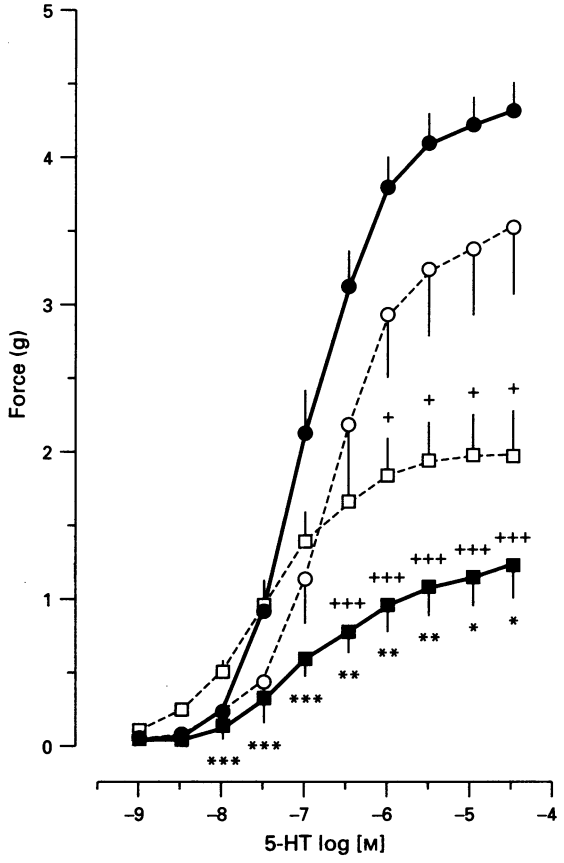

Figure 2 The effect of collar and verapamil treatment on the vascular reactivity to 5-HT. Cumulative dose-response curves were made both in placebo-treated sham (○) and collar (□) rings and verapamil-treated sham (●) and collar (■) rings. Data are expressed as gram contraction. Each point represents the mean±s.e.mean (placebo group $n=8$ and verapamil group $n=8$). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Student's *t* test for unpaired data (verapamil vs. placebo). $^+P<0.05$, $^{+++}P<0.001$, Student's *t* test for paired data (sham vs. collar).

Table 2 Effects of verapamil (10 mg kg^{-1} day^{-1}) and collar on contractile responses (EC_{50}) to 5-hydroxytryptamine in rings from rabbit carotid arteries

Treatment	EC_{50} (−log M) Placebo (n=8)	Verapamil (n=8)
Sham	6.63±0.14	6.89±0.08
Collar	7.23±0.09*	6.76±0.11

Significance of factors in analysis of variance
- Collar: $P=0.046$
- Verapamil: NS ($P=0.367$)
- Interaction: verapamil by collar: $P=0.004$

Values are shown as mean±s.e.mean, n is the number of rabbits in each group. NS: not significant. EC_{50}: concentration producing half maximum contraction of a segment. Because of the interaction between collar and verapamil treatment two-tailed paired Student's t tests were performed: *Collar vs. sham in the placebo group, $P=0.012$; collar vs. sham in the verapamil group $P=0.23$.

collared arteries, but was without effect in sham-treated segments (Figure 4).

Discussion

Verapamil and intimal thickening

In contrast to atherosclerotic lesions, the collar-induced intimal thickening consists predominantly of smooth muscle cells, which have first migrated to the intima, where a high rate of proliferation is maintained for two weeks (Kockx *et al.*, 1992; 1993). Verapamil, the prototype of the phenylalkylamine calcium antagonists, did not decrease this intimal thickening in our study. This result is consistent with the failure of verapamil to suppress intimal thickening induced by a rigid collar, a model in which nilvadipine, another calcium entry blocker was effective (Nomoto *et al.*, 1987). Interestingly, nilvadipine was also more potent than verapamil as inhibitor of *in vitro* smooth muscle cell migration (Nomoto *et al.*, 1987). Local perivascular application of diltiazem in a silastic collar has also been shown to inhibit neo-intima formation after balloon denudation of the rat carotid artery (Hadeishi *et al.*, 1994), but in that study the local dose was extremely high and led to plasma drug levels that exceeded the therapeutic range.

The failure of verapamil to prevent intimal thickening in the collar model could be due to insufficient plasma concentrations. Indeed, plasma levels of verapamil were undetectable in a previous study on rabbits after oral administration due to poor resorption and/or considerable first-pass metabolism (Stender *et al.*, 1986). Parenteral administration of verapamil is required to obtain detectable plasma levels exerting anti-atherosclerotic effects in cholesterol-fed rabbits (Blumlein *et al.*, 1984). Considering this, in our study, verapamil was given subcutaneously, in a near lethal dose (Blumlein *et al.*, 1984; Nomoto *et al.*,1987), which indeed resulted in the death of two rabbits in the treatment group, presumably as a result of acute myocardial depression (Nomoto *et al.*, 1987). This parenteral dose has also been shown to obtain plasma levels that exceed the human therapeutic range (Stender *et al.*, 1986). Hence, it is unlikely that plasma levels of verapamil were insufficient in our study. Moreover, verapamil did alter the sensitivity of the smooth muscle cells to several vasoactive agents (cf. infra).

The failure of verapamil to have an effect due to inadequate timing of drug administration seems to be unlikely as well. When lesions have begun to form, calcium antagonists usually have little or no effect (Jackson *et al.*, 1988; Catapano, 1992).

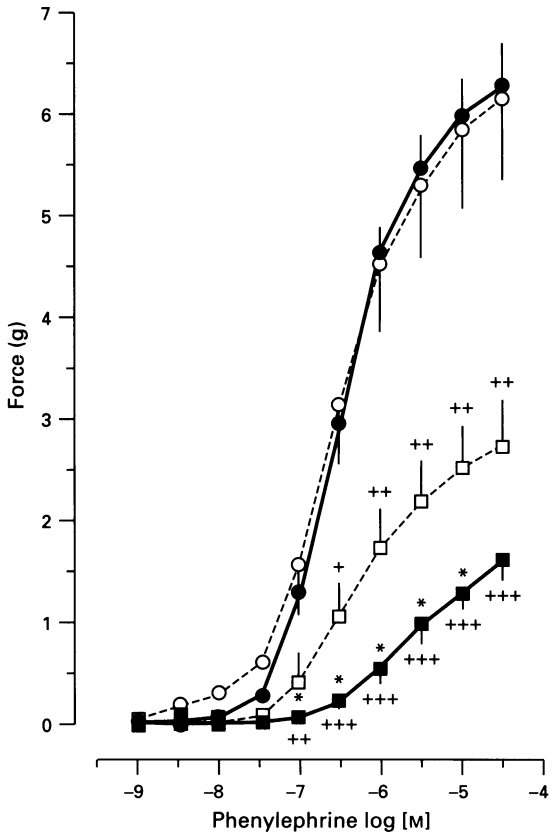

Figure 3 The effect of collar and verapamil treatment on the vascular reactivity to phenylephrine. Cumulative dose-response curves were made both in placebo-treated sham (○) and collar (□) rings and verapamil-treated sham (●) and collar (■) rings. Data are expressed as gram contraction. Each point represents the mean± s.e.mean (placebo group $n=8$ and verapamil group $n=6$). *$P<0.05$, Student's t test for unpaired data (verapamil vs. placebo). $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$, Student's t test for paired data (sham vs. collar).

Table 3 Effects of verapamil (10 mg kg^{-1} day^{-1}) and collar on contractile responses (EC_{50}) to phenylephrine in rings from rabbit carotid arteries

Treatment	EC_{50} (−log M) Placebo (n=8)	Verapamil (n=6)
Sham	6.44±0.14	6.25±0.16
Collar	6.17±0.12	5.68±0.10

Significance of factors in analysis of variance
- Collar: $P=0.009$
- Verapamil: $P=0.019$
- Interaction: verapamil by collar: NS ($P=0.296$)

Values are shown as mean±s.e.mean, n is the number of rabbits in each group. EC_{50}: concentration producing half maximum contraction of a segment. NS: not significant.

However in our experiments verapamil administration was started 7 days before collar implantation and was continued thereafter. It has also been pointed out that in some studies the fact that the lesions were small precluded detection of protective effects of calcium antagonists (Catapano, 1992). However, in our study this is unlikely because the collar led to a statistically significant intimal thickening, as found previously (Booth *et al.*, 1989; Kockx *et al.*, 1992; 1993; De Meyer *et al.*, 1994), which can be suppressed by drugs such as a cysteine-containing donor of nitric oxide (De Meyer *et al.*, 1995) or a

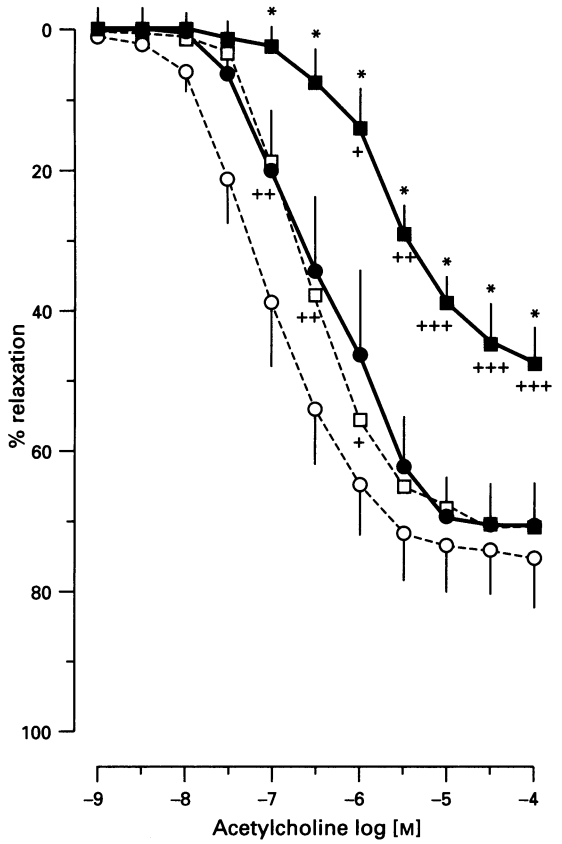

Figure 4 The effect of collar and verapamil treatment on the vascular relaxations to acetylcholine. Cumulative dose-response curves for acetylcholine (10^{-9} to 10^{-4} M) in rabbit carotid artery rings precontracted with 5-HT (3×10^{-5} M) were made both in placebo-treated sham (○) and collar (□) rings and verapamil-treated sham (●) and collar (■) rings. The responses are shown as mean ± s.e.mean (placebo group $n=7$ and verapamil group $n=6$) and expressed as % of initial contraction to 5-HT (3×10^{-5} M) (shown in Figure 2). $^{*}P<0.05$, Student's t test for unpaired data (verapamil vs. placebo). $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$, Student's t test for paired data (sham vs. collar).

Table 4 Effects of verapamil (10 mg kg^{-1} day^{-1}) and collar on EC_{50} values to acetylcholine in rings from rabbit carotid arteries

	EC_{50} (−log M)	
Treatment	*Placebo* (n=7)	*Verapamil* (n=6)
Sham	7.10±0.15	6.45±0.24
Collar	6.56±0.12	5.74±0.18

Significance of factors in analysis of variance
- Collar: $P<0.001$
- Verapamil: $P=0.006$
- Interaction: verapamil by collar: NS ($P=0.481$)

Values are shown as mean ± s.e.mean, n is the number of rabbits in each group. EC_{50}: concentration producing half maximum relaxation of a segment. NS: not significant.

glucocorticoid (Van Put *et al.*, 1996). Hence we conclude that the very high dose of verapamil used in the present study was not able to suppress smooth muscle cell migration and proliferation. This suggests that the possible beneficial effects of verapamil against cholesterol-induced atherosclerotic lesions should be explained by another mechanism, such as prevention of progressive mural calcium incorporation in these models (Fleckenstein-Grün *et al.*, 1994).

Verapamil and vascular reactivity

Although most investigators stress the potential influence of calcium antagonists on the atherosclerotic process itself, the beneficial effects of calcium antagonists on the behaviour of atherosclerotic blood vessels, which have an unusual inclination to develop spasms (Henry & Yokoyama, 1980; Yokoyama *et al.*, 1983; Heistad *et al.*, 1984; Kalsner & Richards, 1984; Verbeuren *et al.*, 1986; Tesfamariam *et al.*, 1989) has received little attention. Therefore, our second goal was to determine whether verapamil interfered with the modifications in vascular reactivity associated with intimal thickening (De Meyer *et al.*, 1990; 1991; 1994). The major finding was that verapamil, although inactive as an inhibitor of intimal thickening, normalized the hypersensitivity to 5-HT.

KCl In accordance with previous experiments (De Meyer *et al.*, 1994), the force development to KCl was reduced in collar-treated segments. Several explanations have been proposed for the diminished force development (De Meyer *et al.*, 1994). First, the smooth muscle cells in the thickened intima are oriented longitudinally (De Meyer *et al.*, 1991; Kockx *et al.*, 1992) and may oppose force developed by media. Second, there may be mechanical damage of vascular smooth muscle cells in the media induced by the collar, although this seems unlikely since histological examination did not show damage or necrosis in the media of collared arteries. Moreover, the area of the media did not change after collar application in either the placebo or verapamil treated groups. Third, a change of smooth muscle phenotype from a contractile to a synthetic stage occurs in the media of collared arteries (Beesley *et al.*, 1992) and this might contribute to the decreased force development in response to KCl. Fourth, localised production of discrete amounts of nitric oxide by vascular smooth muscle could occur at sites of collar-induced injury (Schini *et al.*, 1994). However, this possibility has been eliminated previously in functional experiments, which failed to demonstrate an inducible nitric oxide synthase (De Meyer *et al.*, 1994). Similar explanations must be considered for the diminished force development to receptor stimulation (see below).

KCl-induced contractions depend exclusively upon an influx of extracellular Ca^{2+} (Yamashita *et al.*, 1977; Heaslip & Rahwan, 1982), which is normally blocked by verapamil (Marriot, 1988). However, in the present study verapamil treatment did not affect the *ex vivo* development of force to KCl in sham-treated or collared arteries. This is not surprising, since a supramaximal concentration of KCl was selected to calibrate force development in each segment. Verapamil concentrations required to inhibit contractions induced by 120 mM KCl exceed 1 μM (unpublished results), which is orders of magnitude above the maximum plasma levels of verapamil (Blumlein *et al.*, 1984; Stender *et al.*, 1986).

5-HT In view of the reduced responsiveness to KCl, the reduced E_{max} of 5-HT came as no surprise. Furthermore, the present study confirmed that collared arteries show an increased sensitivity to 5-HT even when the animals are not fed a cholesterol-rich diet (De Meyer *et al.*, 1990; 1994).

Different mechanisms have been suggested to elucidate this hypersensitivity, namely, modification of the 5-HT receptors on the smooth muscle cells (De Meyer *et al.*, 1990), dysfunction of endothelial 5-HT receptors (Dusting *et al.*, 1990) and removal of 5-HT by the endothelium (Verbeuren *et al.*, 1988).

The main new findings of the present study were that the collar-induced hypersensitivity to 5-HT can be effectively blunted by therapy, and secondly that this occurred independently of intimal thickening. Indeed, verapamil normalized the increased sensitivity of the artery to 5-HT and depressed the E_{max} values in collared segments, but not in sham-treated segments. The finding that verapamil was mainly effective in collared segments, not in sham-operated segments, is in accordance with the observation that another calcium antagonist, isradipine antagonized vasoconstrictor effects more

effectively in atherosclerotic animals than in normal rabbits (Hof & Hof, 1988). Moreover, it has been shown that compounds containing a verapamil moiety interact with 5-HT receptors in rabbit vascular smooth muscles (Ohashi *et al.*, 1985). In addition, it has recently been shown that verapamil partially inhibits 5-HT contractions in human coronary and internal mammary arteries and that this inhibition may be related to blockade of L-calcium channels (Godfraind *et al.*, 1992).

The selective anti-vasoconstrictor effect of verapamil to 5-HT in collared arteries can be explained by 'use-dependency'. Use-dependence, by definition, suggested that the effect of a drug depends on the state of activity of its target tissue (Hof & Rüegg, 1991). This property has been characterized for verapamil in cardiac tissues (McDonald *et al.*, 1980; Kanaya *et al.*, 1983; Ellenbogen *et al.*, 1985). The selective anti-vasoconstrictor effect of verapamil on collared arteries may also be use-dependent, since atherosclerotic vessels are, in general, hyperresponsive and may therefore be preferential targets for use-dependence of verapamil. In addition, the anti-vasoconstrictor effects of calcium antagonists are more widespread than their vasodilator effects (Hof & Rüegg, 1989).

Phenylephrine In contrast to 5-HT, the sensitivity to the α_1-adrenoceptor agonist phenylephrine was decreased in collared arteries. Again, the E_{max} was significantly diminished, as expected from the KCl data. The decreased sensitivity in collared arteries points to a diminished responsiveness of α_1-adrenoceptors. As stated above, this is presumably not due to the induction of nitric oxide synthase activity (De Meyer *et al.*, 1994).

Verapamil shifted the dose-response curves for phenylephrine to the right in both sham- and collar-operated carotid arteries without affecting E_{max}. This result is consistent with the observation that verapamil may antagonize noradrenaline competitively at α_1-adrenoceptors in the rabbit thoracic aorta (Koike *et al.*, 1988), and many other tissues of different species (for review see Godfraind *et al.*, 1986). Moreover, it has been shown that verapamil produces a more selective reduction of responses mediated by Ca^{2+} entry through receptor-operated channels than either diltiazem or flunarizine (Marriot, 1988). The effect of verapamil tended to be larger in collared arteries, again suggesting that the effects of verapamil may be more pronouced in vessels with contractile abnormalities.

Acetylcholine Acetylcholine was used to induce relaxations mediated by the biosynthesis of nitric oxide. The collar decreased the sensitivity of acetylcholine, a finding consistent with previous observations (De Meyer *et al.*, 1991; 1992). This effect appears to be due to a defect at the level of the endothelial muscarinic receptors (De Meyer *et al.*, 1991; 1992; Arthur & Dusting 1992). In addition, verapamil appeared to alter the nitric oxide formation in response to muscarinic receptor stimulation as well, as pD_2 was suppressed in sham- and collar-treated arteries. This effect of verapamil was more pronounced in collared arteries since the amplitude of relaxations was also reduced.

The initial contractions elicited by 5-HT were reduced by verapamil and the collar. These decreased initial contractions could have enhanced the sensitivity to vasodilators (Karliner *et al.*, 1982). However, in the present study the opposite occurred: arteries treated with collar and/or verapamil became less sensitive to acetylcholine. In fact it has already been shown that a smaller initial contraction does not contribute to the decreased endothelium-dependent relaxations (De Meyer *et al.*, 1991). Hence, the right-ward shift in sham and collared arteries and the reduced amplitude of the relaxations in collared arteries of the verapamil-treated rabbits are presumably not explained by the decreased initial contraction to 5-HT. Both alterations could be due to interference with muscarinic receptors, as Karliner *et al.* (1982) found that verapamil is a competitive antagonist of muscarinic receptors, and Singer & Peach (1982) showed that verapamil inhibited maximum methacholine-induced relaxations of the rabbit aorta by 39%, a result consistent with the present study. The precise mode of action of verapamil at the level of calcium channels or at the level of muscarinic receptors is not clear.

Nitric oxide release is strictly dependent on a rise of cytosolic Ca^{2+} concentrations $[Ca^{2+}]_i$, which is often biphasic (reviewed in Adams *et al.*, 1989). It has been shown that verapamil has no effect on resting $[Ca^{2+}]_i$ in endothelial cells, but attenuates agonist-induced transient peak responses, which may contribute to the inhibition of endothelium-dependent relaxations (Morgan-Boyd *et al.*, 1987; Peach *et al.*, 1987). However, recently it has been found that verapamil does not alter bradykinin-stimulated release of nitric oxide from cultured bovine aortic endothelial cells (Mügge *et al.*, 1991). This suggests that the inhibitory effects of verapamil on both pD_2 and maximum relaxation of acetylcholine in the rabbit carotid artery are due to antagonism at the level of muscarinic receptors on endothelial cells.

In conclusion, the main new finding of our study is that although verapamil did not prevent collar-induced intimal thickening, it effectively blunted the enhanced vascular reactivity of these collared arteries in response to 5-HT. This may be of clinical importance, since atherosclerotic vessels often demonstrate an increased responsiveness to vasoconstrictors, especially 5-HT and calcium antagonists are often used in patients with atherosclerotic disease.

This study was supported by grant 93.ECZ.002 of the Ege University Fund for Scientific Research. The authors wish to thank research assistants Pharm.M.S. Bahar Yilmaz, Pharm. Riyad Akpinar, Pharm. Gülnur Karaoglu, Pharm. Buket Özgür and Pharm. Günay Yetik for their excellent technical assistance. The scientific support and contributions of Prof. Dr Arnold G. Herman, Prof. Dr Hidde Bult and Pharm. Dr Guido De Meyer (The University of Antwerp (UIA), Division of Pharmacology, Antwerp, Belgium) are gratefully acknowledged.

References

ADAMS, D.J., BARAKEH, J., LASKEY, R. & VAN BREEMEN, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.*, **3**, 2389–2400.

ARTHUR, J.F. & DUSTING, G.J. (1992). Selective endothelial dysfunction in early atheroma-like lesions in the rabbit. *Coronary Artery Dis.*, **3**, 623–629.

BEESLEY, J.E. HONEY, A.C. & MARTIN, J.F. (1992). Ultrastructural assessment of lesion development in the collared rabbit carotid artery model. *Cell Materials*, **2**, 201–203.

BLUMLEIN, S.L., SIEVERS, R., KIDD, P. & PARMLEY, W.W. (1984). Mechanism of protection from atherosclerosis by verapamil in the cholesterol-fed rabbit. *Am. J. Cardiol.*, **54**, 884–889.

BOOTH, R.F.G., MARTIN, J.F., HONEY, A.C., HASSAL, J.E., BEESLEY, J.E. & MONCADA, S. (1989). Rapid development of atherosclerotic lesions in the rabbit carotid artery induced by perivascular manipulation. *Atherosclerosis*, **76**, 257–268.

CATAPANO, A.L. (1992). The antiatherogenic potential of calcium antagonists: experimental evidence. *J. Cardiovasc. Pharmacol.*, **20, (Suppl.A)** S44–S50.

DE MEYER, G.R.Y., BULT, H., MARTIN, J.F., HOYDONCK, A.V. & HERMAN, A.G. (1990). The effect of a developing neointima on serotonergic and adrenergic contractions. *Eur. J. Pharmacol.*, **187**, 519–524.

DE MEYER, G.R.Y., BULT, H., VAN HOYDONCK, A.-E., JORDAENS, F.H., BUYSSENS, N. & HERMAN, A. (1991). Neointima formation impairs endothelial muscarinic receptors while enhancing prostacyclin-mediated responses in the rabbit carotid artery. *Circ. Res.*, **68,** 1669–1680.

DE MEYER, G.R.Y., BULT, H. & HERMAN, A. (1992). Selective muscarinic alterations of nitric oxide-mediated relaxations by neointima. *J. Cardivasc. Pharmacol.*, **20 (Suppl.12),** S205–S207.

DE MEYER, G.R., BULT, H., ÜSTÜNES, L., KOCKX, M., JORDAENS, F.H., ZONNEKEYN, L.L. & HERMAN, A.G. (1994). Vasoconstrictor responses after neointima formation and endothelial removal in the rabbit carotid artery. *Br. J. Pharmacol.*, **112,** 471–476.

DE MEYER, G.R.Y., BULT, H., ÜSTÜNES, L., KOCKX, M.M., FEELISCH, M. & HERMAN, A.G. (1995). Effect of nitric oxide donors on neointima formation and vascular reactivity in the collared carotid artery of rabbits. *J. Cardiovasc. Pharmacol.*, **26,** 272–279.

DUSTING, G.J., CURCIO, A., HARRIS, P.J., LIMA, B., ZAMBETIS, M & MARTIN, J.F. (1990). Supersensitivity to vasoconstrictor action of serotonin precedes the development of atheroma-like lesions in the rabbit. *J. Cardiovasc. Pharmacol.*, **16,** 667–670.

ELLENBOGEN, K.A., GERMAN, L.D. & O'CALLAGHAN, W.G. (1985). Frequency dependent effects of verapamil on atrioventricular nodal conduction in man. *Circulation*, **72,** 344–352.

FLECKENSTEIN-GRÜN, G., THIMM, F., CZIRFUZS, A., MATYAS, S. & FREY, M. (1994). Experimental vasoprotection by calcium antagonists against calcium-mediated arteriosclerotic alterations. *J. Cardiovasc. Pharmacol.*, **24 (Suppl.2)** S75–S84.

GODFRAIND, T., DESSY, C. & SALOMONE, S. (1992). A comparison of the potency of selective L-calcium channel blockers in human coronary and internal mammary arteries exposed to serotonin. *Pharmacol. Exp. Ther.*, **263,** 112–122.

GODFRAIND, T., MILLER, R. & WIBO, M. (1986). Calcium antagonism and calcium entry blockade. *Pharmacol. Rev.*, **38,** 321–416.

HADEISHI, H., MAYBERG, M.R., SETO, M., DACEY, R.G. & SOLOMON, R.A. (1994). Local application of calcium antagonists inhibits intimal hyperplasia after arterial injury. *Neurosurgery*, **34,** 114–121.

HEASLIP, R.J. & RAHWAN, R.G. (1982). Evidence for the existence of two distinct pools of intracellular calcium in the rat aorta accessible to mobilization by norepinephrine. *Pharmacol. Exp. Ther.*, **221,** 7–13.

HEISTAD, D.D., ARMSTRONG, M.L., PIEGORS, D.J. & MARK, A. (1984). Augmented responses to vasoconstrictor stimuli in hypercholesterolemic and atherosclerotic monkeys. *Circ. Res.*, **54711,** 711–718.

HENRY, P.D. (1990). Atherogenesis, calcium and calcium antagonists. *Am. J. Cardiol.*, **66,** 31–61.

HENRY, P.D. & YOKOYAMA, M. (1980). Supersensitivity of atherosclerotic rabbit aorta to ergonovine. *J. Clin. Invest.*, **66,** 306–313.

HOF, R.P. & HOF, A. (1988). Vasoconstrictor and vasodilator effects in normal and atherosclerotic conscious rabbits. *Br. J. Pharmacol.*, **95,** 1075–1080.

HOF, R.P. & RÜEGG, T.U. (1989). Antivasoconstrictor effects of isradipine. A quantitative approach in anaesthetised rats and conscious rabbits. *Am. J. Med.*, **86 (Suppl 4A),** 50–56.

HOF, R.P. & RÜEGG, T.U. (1991). Calcium antagonists in experimental atherosclerosis use-dependence of isradipine: a potential explanation for enhanced action in atherosclerotic animals and tissue selectivity. *Am. J. Hypertens*, **4,** 107–113.

JACKSON, C.L., BUSH, R.C. & BOWYER, D.E. (1988). Inhibitory effect of calcium antagonists on balloon catheter-induced arterial smooth muscle cell proliferation and lesion size. *Atherosclerosis*, **69,** 115–122.

KALSNER, S. & RICHARDS, R. (1984). Coronary arteries of cardiac patients are hyperreactive and contain stores of amines. A mechanism for coronary spasm. *Science*, **223,** 1435–1437.

KANAYA, S., ARLOCK, P., KATZUNG, B.G. & HONDEGHEM, L.M. (1983). Diltiazem and verapamil preferentially block inactivated cardiac calcium channels. *J. Mol. Cell. Cardiol.*, **15,** 145–148.

KARLINER, J.S., MOTULSKY, H.J., DUNLAP, J., BROWN, J.H. & INSEL, P.A. (1982). Verapamil competitively inhibits a_1-adrenergic and muscarinic but not beta-adrenergic receptors in rat myocardium. *J. Cardiovasc. Pharmacol.*, **4,** 515–520.

KOBER, G., SCHNEIDER, W. & KALTENBACH, M. (1989). Can the progression of coronary sclerosis be influenced by calcium antagonists? *J. Cardiovasc. Pharmacol.*, **4,** S2–S6.

KOCKX, M.M., DE MEYER, G.R.Y., JACOB, W.A., BULT, H. & HERMAN, A.G. (1992). Triphasic sequence of neointimal formation in the cuffed carotid artery of the rabbit. *Arterioscler. Thromb.*, **12,** 1447–1457.

KOCKX, M.M., DE MEYER, G.R.Y., ANDRIES, L.J., BULT, H., JACOB, W.A. & HERMAN, A.G. (1993). The endothelium during cuff-induced neo-intima formation in the rabbit carotid artery. *Arterioscler. Thromb.*, **13,** 1874–1884.

KOIKE, K., TAKAYANAGI, I., OHASHI, M., NAKAZAWA, T., KUMAGAI, N. & KISHII, K. (1988). Ca-entry blockers, verapamil and diltiazem, on a_1-adrenoceptors in thoracic aorta, renal artery and portal vein from rabbit. *Gen. Pharmacol.*, **19,** 541–545.

MARRIOT, J.F. (1988). A comparison of the effects of the calcium entry blockers, verapamil, diltiazem and flunarizine against contractions of the rat isolated aorta and portal vein. *Br. J. Pharmacol.*, **95,** 145–154.

MCDONALD, T.F., PELZER, D. & TRAUTWEIN, W. (1980). On the mechanism of slow calcium channel block in heart. *Pflugers Arch.*, **385,** 175–179.

MORGAN-BOYD, R., STEWART, J.M., VAVREK, R.J. & HASSID, A. (1987). Effects of bradykinin and angiotensin II on intracellular Ca^{2+} dynamics in endothelial cells. *Am. J. Physiol.*, **253,** C588–C598.

MÜGGE, A., PETERSON, T. & HARRISON, D.G. (1991). Release of nitrogen oxides from cultured bovine aortic endothelial cells is not impaired by calcium channel antagonists. *Circulation*, **83,** 1404–1409.

NOMOTO, A., HIROSUMI, J., SEKIGUCHI, C., MUTOH, S., YAMAGUCHI, I. & AOKI, H. (1987). Antiatherogenic activity of FR34235 (Nilvadipine), a new potent calcium antagonist. *Atherosclerosis*, **64,** 255–261.

OHASHI, M., KANAI, R. & TAKAYANAGI, I. (1985). Do D 600 and diltiazem interact with serotonin receptors in rabbit vascular tissues? *J. Pharmacol. Exp. Ther.*, **233,** 830–835.

PEACH, M.J., SINGER, H.A., IZZO, N.J. & LOEB, A.L. (1987). Role of calcium in endothelium-dependent relaxation of arterial smooth muscle. *Am. J. Cardiol.*, **59,** 35A–43A.

ROULEAU, J.L., PARMLEY, W.W., STEVENS, J., WIKMAN-COFFELT, J., SIEVERS, R., MAHLEY, R.W. & HAVEL, R.J. (1983). Verapamil suppresses atherosclerosis in cholesterol-fed rabbits. *J. Am. Coll. Cardiol.*, **1,** 1453–1460.

SCHINI, V.B., BUSSE, R. & VANHOUTTE, P.M. (1994). Inducible nitric oxide synthase in vascular smooth muscle. *Arzneim. Forsch./Drug Res.*, **44,** 432–435.

SIEVERS, R.E., RASHID, T., GARRETT, J., BLUMLEIN, S. & PARMLEY, W.W. (1987). Verapamil and diet halt progression of atherosclerosis in cholesterol fed rabbits. *Card. Drugs Ther.*, **1,** 65–69.

SINGER, H.A. & PEACH, M.J. (1982). Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension*, **4 (Suppl II),** II-19–II-25.

STARY, H.C., BLANKENHORN, D.H., CHANDLER, A.B., GLAGOV, S., INSULL, W., JR., RICHARDSON, M., ROSENFELD, M.E., SCHAFFER, S.A., SCHWARTZ, C.J., WAGNER, W.D. & WISSLER, R.W. (1992). A definition of the intima of human arteries and of its atherosclerosis-prone regions. *Arterioscler. Thromb.*, **12,** 120–134.

STENDER, S., RAVN, H., HAUGEGAARD, M. & KJELDSEN, K. (1986). Effect of verapamil on accumulation of free and esterified cholesterol in the thoracic aorta of cholesterol fed rabbits. *Atherosclerosis*, **61,** 15–23.

TESFAMARIAM, B., WEISBROD, R.M. & COHEN, R.A. (1989). Augmented adrenergic contractions of carotid arteries from cholesterol-fed rabbits due to endothelial cell dysfunction. *J. Cardiovasc. Pharmacol.*, **13,** 820–825.

VAN PUT, D.J.M., VAN HOVE, C.E., DE MEYER, G.R.Y., WUYTS, F., HERMAN, A.G. & BULT, H. (1996). Dexamethasone influences intimal thickening and vascular reactivity in the rabbit collared carotid artery. *Eur. J. Pharmacol.*, (in press).

VERBEUREN, T.J., JORDAENS, F.H., ZONNEKEYN, L., VAN HOVE, C., COENE, M.-C. & HERMAN, A. (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ. Res.*, **58,** 552–564.

VERBEUREN, T.J., JORDAENS, F.H., BULT, H. & HERMAN, A. (1988). The endothelium inhibits the penetration of serotonin and norephinephrine in the isolated canine saphaneous vein. *J. Pharmacol. Exp. Ther.*, **244,** 276–282.

WEINSTEIN, D.B. & HEIDER, J.G. (1989). Antiatherogenic properties of calcium antagonists, State of the art. *Am. J. Med.*, **86**, 27–32.

YAMASHITA, K., TAKAGI, T. & HOTTA, K. (1977). Mobilization of cellular calcium and contraction-relaxation of vascular smooth muscle. *Jpn. J. Physiol.*, **27**, 551–564.

YOKOYAMA, M., AKITA, H., MIZUTANI, T., FUKUZAKI, H. & WATANABE, Y. (1983). Hyperreactivity of coronary arterial smooth muscle in response to ergonovine from rabbits with hereditary hyperlipidemia. *Circ. Res.*, **53**, 63–71.

(Received August 3, 1995
Revised April 2, 1996
Accepted April 15, 1996)