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The role of endothelin receptor antagonism in collar-induced intimal thickening and vascular reactivity changes in rabbits

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Abstract

Intimal thickening, due to smooth muscle cell migration and proliferation, is considered to be one of the major components of vascular proliferative disorders such as atherosclerosis and restenosis. One experimental model, resulting in intimal thickening in the rabbit, involves placing a silicon collar around the carotid artery, and is used in this study. Endothelin is known to act as a strong mitogen and to stimulate smooth muscle cell proliferation and migration. We investigated the contribution of endothelin to the development of collar-induced intimal thickening and the effects of TAK-044, (5mg kg^{-1} daily, s.c.), a non-selective ET_A/ET_B receptor antagonist, on intimal thickening and vascular reactivity changes in the collared rabbit carotid artery. Endothelin levels and the intimal cross-sectional area, as well as the ratio of intimal area to media (index), increased significantly in collared arteries as compared with those in sham-operated arteries. TAK-044 significantly inhibited intimal thickening and also decreased the index without affecting increased endothelin levels in collared arteries. Vascular reactivity changes in response to collaring produced predictable effects, such as decreased contractile responses to vasoconstrictor agents and increased sensitivity to serotonin (5-hydroxytryptamine, 5-HT). In terms of contractile responses in this model, TAK-044, in particular, did not affect collar-induced vascular reactivity changes. These results suggest that endothelin may be involved in the pathogenesis of collar-induced intimal thickening. As an endothelin receptor antagonist, TAK-044 may potentially be beneficial in the treatment of atherosclerosis.

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Introduction

Intimal thickening, due to smooth muscle cell (SMC) migration and proliferation, is known to be a critical factor in the development of vascular proliferative disorders such as atherosclerosis and restenosis (Schwartz et al 1998). Experimental models in which intimal thickening is induced by different methods have been widely used in a variety of studies (Kikuchi et al 1998; Walsh 2000). The advantages and disadvantages of these experimental models have been discussed in detail (De Meyer & Bult 1997; Lafont & Faxon 1998; Bayes-Genis et al 2000). One of these experimental models, in which intimal thickening was induced by placing a collar around the rabbit carotid artery, was used in this study (Booth et al 1989). In this model, independently of formation of intimal thickening, it has been reported that changes in the functional characteristics of collared arteries to vasoconstrictor agents have occurred (Soma et al 1994; Van Put et al 1995; Kerry et al 1999). Among these, hypersensitivity to serotonin (5-hydroxytryptamine, 5-HT) is the most pronounced (De Meyer et al 1990, 1994).

On the other hand, endothelins consist of a family of peptides with potent biological properties (Webb & Meek 1997). Endothelin-1 (ET-1), a 21-amino-acid peptide, is primarily produced by endothelial (Yanagisawa et al 1988) and vascular SMC (Resink et al 1990). ET-1 acts on two receptor subtypes — $endothelin_A$ (ET_A) and $endothelin_B$ (ET_B) (Lüscher & Barton 2000). ET_A receptor predominates on vascular SMC and is responsible for causing vasoconstriction, cell growth, cell adhesion, thrombosis and cell proliferation. In contrast, ET_B receptor exists on endothelial and vascular SMC

and mediates the release of relaxing factors such as nitric oxide and prostacyclin. However, ET_B receptors, present on vascular SMC, can mediate vasoconstriction (Masaki 1998; Lüscher & Barton 2000). It has been shown that ET-1 acts as a strong mitogen and chemoattractant and also stimulates proliferation and migration of SMC. Increased ET-1 levels have been found in atherosclerosis, myocardial infarction, pulmonary hypertension, heart failure and renal failure (reviewed in Lüscher & Barton 2000). Moreover, ET-1, ET-converting enzyme, ET_A and ET_B receptor expression have been found to increase in rat carotid artery after balloon angioplasty (Wang et al 1996). Furthermore, a number of studies showed that endothelin receptor antagonists could inhibit the development of intimal thickening (reviewed in Kirchengast & Münther 1998). Additionally, ET-1 acts as a stimulus for autocrine growth mechanisms. The fact that it also acts as a growth factor (Berk 2001) has brought up the question of the role (either direct or indirect) of endothelin or endothelin antagonism in the formation of intimal thickening in the collar model (Marano et al 1998; Rectenwald et al 2000). In this respect, the possible effect of TAK-044 (cylo [D-α-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl] disodium), a new nonselective ET_A/ET_B receptor antagonist, in the development of intimal thickening in the collar model has not been studied. Therefore, in this study, we had two main aims: firstly, to investigate the effects of TAK-044 ($5\,mg\,kg^{-1}$ daily, s.c.) (Tsujino et al 1995) and, secondly, by measuring plasma and tissue ET-1 levels to elucidate the role of ET-1 in the development of intimal thickening and vascular reactivity changes in the collared rabbit carotid artery.

Materials and Methods

Materials

Acetylcholine chloride, phenylephrine hydrochloride, 5-hydroxytryptamine creatinine sulphate, potassium chloride and indometacin sodium were purchased from Merck (Darmstadt, Germany), sodium pentobarbital solution from Psyphac (Brussels, Belgium), *p*-aminophenyl methansulfonyl fluoride, pepstatin A and leupeptin from Sigma-Aldrich (Steinheim, Germany) and silicone (MED-401) from Nusil Silicone Technology (Anglet, France). TAK-044 was kindly provided by Takeda Chemical Industries, Osaka, Japan.

Animals

The animal experiments were carried out in accordance with guidelines described by the Ethics Committee of the Faculty of Pharmacy, Ege University.

A total of 34 rabbits were used in this study. White rabbits of either sex (4 female, 6 male in each group) were divided into two groups. The first group (n = 17) received a single subcutaneous injection of TAK-044 ($5mg\,kg^{-1}$ daily, throughout the 3-week treatment period). The second group (n = 17) received only the vehicle (0.9% NaCl $0.8\,mL\,kg^{-1}$ daily, throughout the same treatment period). Throughout the 3-week treatment period each rabbit was kept in a separate cage and allowed free access to regular food and tap water. Ten rabbits from each group were used in morphometric analyses, blood pressure measurements and vascular reactivity experiments, while the remaining 7 rabbits from each group were used in the determination of ET-1 levels.

Model

After the 7th day of treatment with TAK-044 and placebo, the rabbits were anaesthetized with intravenous sodium pentobarbital ($30\,mg\,kg^{-1}$). Subsequently, the left common carotid artery was surgically accessed and surrounded by a non-occlusive, flexible silicone collar (2 cm long). The right common carotid artery was sham-operated (i.e. it was separated from surrounding connective tissue and vagus nerve and received a similar stretch to that of the contralateral carotid artery) (Booth et al 1989). The carotid arteries were then returned to their original positions and the incisions were closed. After recovery from the anaesthesia, the rabbits were kept in their individual cages for a further 2 weeks before tissue isolation. At the end of the treatment period (placebo and TAK-044) the rabbits (n = 20) were killed by means of an overdose of sodium pentobarbital. Isolated vessel segments from sham-operated (right) and collared (left) arteries were cut into three pairs of rings, each one 4 mm long, one pair of rings for morphometry and two pairs for organ-chamber experiments.

To obtain arterial tissue samples to measure ET-1 levels, the rabbits from both groups (n = 14) were killed by sodium pentobarbital overdose. Vessel segments taken from both the right (sham-operated) and left (collared) carotid arteries were carefully cleared from loose connective tissue, frozen under liquid nitrogen and stored at −80°C until the analysis was performed.

Immunohistochemistry

The first pair of rings was immediately fixed in 4% buffered formalin solution and embedded in paraffin blocks after routine tissue processing. Four-micrometre sections were cut and mounted on poly-L-lysine-coated slides. To assess cell proliferation, the avidin–biotin–peroxidase method was performed using primary monoclonal antibody against Ki-67 protein (pre-dilute; Neomarkers, Fremont, USA) (Hilker et al 2002). In brief, the sections were de-paraffinized and endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxidase in physiological buffer solution (PBS) at room temperature for 10 min. After microwave treatment, primary antibody was applied for 30 min at room temperature and washed in PBS. Linking antibody and streptavidin–peroxidase complex (Neomarkers, Fremont, USA) were applied for 5 min. Appropriate positive (tonsil) and negative (cerebellum) controls were also labelled with the primary antibody. Ki-67 proliferative index was determined as the

percentage of the cells showing positive nuclear staining in 10 randomly selected fields of the intima at ×400 magnification (Hilker et al 2002).

Morphometry

Areas of lumen, intima and media were measured at three cross-sectional levels by two morphometrists (E.O. and S.O.) with no prior knowledge of the experimental data, using a computer-assisted image analyser system consisting of a microscope (Labophot-2; Nikon, Japan) equipped with a high-resolution video camera (VKC22OE; Hitachi, Tokyo, Japan). The images were processed using an IBM-compatible personal computer, high-resolution video monitor and image analysis software (BS 200Docu Version 2.0; BAB Imaging Systems, Ankara, Turkey). The images were obtained with the video camera at ×4 magnification and the areas were viewed on the monitor and outlined by drawing.

Organ chamber experiments

The two remaining rings from both the right (sham-operated) and left (collared) carotid arteries were used in organ chamber experiments to study vascular reactivity. After careful removal of loose connective tissue the rings were suspended in organ chambers filled with physiological salt solution (Krebs) at 37°C, continuously oxygenated with 95%O_2–5%CO_2. In addition, special care was taken not to cause damage to endothelium. Indometacin was added to Krebs solution (3 μM) to inhibit endogenous prostanoid synthesis. Isometric contractile force development was measured by means of a Grass FT3 force transducer and recorded (Polywin 95 1.0; Commat, Ankara, Turkey) by means of a microcomputer (IBM PS/1). After a 15-min equilibration, tissues were gradually stretched to a tension of 7 g, a previously determined optimum resting tension based on the length–tension relationship (Üstünes et al 1996), and left to equilibrate for a further 45 min. At the end of the equilibration period the following concentration–response curves were constructed: acetylcholine (10^{-9} to 10^{-4} M) pre-contracted by 10^{-6} M phenylephrine; phenylephrine (10^{-9} to 10^{-4} M); 5-hydroxytryptamine (10^{-9} M to 3×10^{-5} M); potassium chloride (KCl) (120 mM, in depolarizing Krebs). Each agonist was washed out by changing the bath solution three times in a 30-min time period before addition of the next agonist. Krebs solution contained (in 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.

Body weight and blood pressure measurements

Body weight, arterial pressure (systolic and diastolic) and heart rate were measured before the collar application (on day 8) and at the end of the treatment period (on day 22). Arterial pressure and heart rate were recorded by cannulation of the central ear artery in conscious rabbits. After insertion of the catheter, systolic and diastolic blood pressure (mmHg) and heart rate (heart beats/min) were measured by means of a blood pressure transducer (Baxter Uniflow; Baxter Healthcare Group, CA) and a transducer interface (May 9601; Commat Ltd., Ankara, Turkey) for 15 min in a computerized system. In this system, a direct blood pressure measurement program (Diasys; Commat Ltd, Ankara, Turkey) was used.

Sampling of plasma

Blood samples were collected from the marginal ear artery before the collar application (on day 8) and at the end of the treatment period (on day 22). For this purpose, the skin of the ear of the rabbits was sprayed with Xylocaine (AstraZeneca, Södertalje, Sweden), a 24-gauge cannula was inserted into the central ear artery and approximately 6 mL of blood was taken. Samples were centrifuged (1000 g, 4°C) (Minifuge RF; Heraeus Sepatech, Germany) for 10 min within an hour. After centrifugation the serum samples were stored in polypropylene tubes at −80°C before analysis.

Measurement of ET-1 (ET 1–21) levels in arterial tissue and plasma samples

On the day of analysis, pre-weighed arterial tissue samples were pulverized under liquid nitrogen. Thereafter, tissues were homogenized by previously reported methods (Mitani et al 2000). All samples were then cut into small pieces and 10% (w/v) tissue homogenates were prepared in ice-cold homogenization buffer, which consisted of 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 20 μM pepstatin A (acid protease inhibitor), 20 μM leupeptin (serine and cysteine protease inhibitor) and 50 μM p-aminophenyl methanesulfonyl fluoride (serine protease inhibitor), for 30 s using an ultrasonic homogenizator. After the homogenates were centrifuged (1000 g, 4°C) for 10 min supernatant was used for the assay of tissue ET-1 level. Arterial tissue and plasma ET-1 levels were measured using a commercial enzyme immunoassay kit (Biomedica ELISA kit; Vienna, Austria). Tissue and plasma ET-1 levels were expressed as fmol (mg protein)$^{-1}$ or fmol (mL plasma)$^{-1}$, respectively. Total protein amount was measured by using a commercially available kit (Bichinconinic acid, BCA-1; Sigma), using bovine serum albumin (BSA) as standard.

Statistical methods

Statistical analyses of data from morphometric measurements and vascular reactivity studies were evaluated with repeated measures analysis of variance with between-subjects factors. Analysis was designed for drug treatments (two levels, TAK-044 or placebo) as between rabbit factor and collar (two levels, present or not) as within rabbit factor. If there were interactions between the factors in repeated measures analysis of variance, the Student's t-test was used. In the other analyses, the Wilcoxon signed ranks test and Mann–Whitney U-test were used for paired and unpaired comparisons. All data are expressed as means ± s.e.m.; n indicates the number of rabbits. Significance was accepted at $P = 0.05$. Values of maximum effect

(E_{max}) and 50% effective concentration (EC50) were derived for each cumulative concentration–response curve by non-linear curve fitting (Polywin 95 1.0; Commat, Ankara, Turkey). Means of the negative logarithm of EC50 values (pD_2) were compared. Acetylcholine-induced relaxations were normalized to the initial phenylephrine contraction. Intima/media ratio (index) was calculated for statistical analyses. Means of the areas (μm^2) and index were compared. To assess interobserver variation, the morphometric and immunohistochemical measurements were performed under code by two pathologists (E.O. and S.O.) who had no prior knowledge of the experimental data. The results of each measurement were assessed by the other observer. The results of the two assessments were compared and there was no significant difference by Student's *t*-test for paired samples.

Results

Survival and body weight

Only one rabbit from each group died during the treatment period. TAK-044 did not appear to cause any visible side effects. The body weight of the rabbits in the placebo group was not changed by collar placing (2.24 ± 0.05 kg on day 8, 2.22 ± 0.05 kg on day 22; $n = 9$, repeated measures analysis of variance). Treatment with TAK-044 did not affect the body weight of the rabbits (2.15 ± 0.04 kg on day 8, 2.13 ± 0.04 kg on day 22; $n = 9$, repeated measures analysis of variance).

Arterial tissue and plasma ET-1 (1–21) levels

ET-1 (1–21) levels were measured in sham-operated and collared arteries from both groups. The collar significantly increased the endothelin levels in collared arteries from both groups (Table 1). TAK-044 did not significantly affect these increased levels of endothelin in collared arteries (Table 1).

ET-1 (1–21) levels were measured in blood samples taken on day 8 (before collar application) and on day 22 (after collar application) of the treatment period from both placebo and TAK-044 groups. Neither collaring nor TAK-044 treatment affected plasma endothelin levels (ET-1 levels as fmol mL^{-1}: 0.51 ± 0.08, 0.57 ± 0.04 in placebo group and 0.37 ± 0.11, 0.37 ± 0.08 in TAK-044 group, before and after collar application, respectively, repeated measures analysis of variance, $n = 7$).

Table 1 Effects of collar and TAK-044 ($5\,mg\,kg^{-1}$ daily, s.c.) on ET-1 levels in rabbit carotid artery tissues

	Placebo (n=7)	TAK-044 (n=7)
Endothelin (ET-1) (fmol (mg protein)$^{-1}$)		
Sham	34.08 ± 9.53	23.01 ± 3.36
Collared	58.67 ± 14.18	51.33 ± 9.42
Significance of factors in analysis of variance		
Collar	$P < 0.05$	
TAK-044	$P = 0.396$	
Interaction	TAK-044 by collar	$P = 0.845$

Shown are means ± s.e.m. n, no. of rabbits in each group.

Morphometry and Ki-67 immunoreactivity in intimal area

The intimal cross-sectional area was significantly increased in collared arteries as compared with those in sham-operated arteries in the placebo group (Figure 1, Table 2). TAK-044 treatment significantly inhibited the intimal thickening in collared arteries (Figure 1, Table 2). Collaring did not alter the medial cross-sectional area but increased the index in the placebo group. TAK-044 treatment did not affect the medial cross-sectional area but significantly decreased the index (Table 2).

Positive immunostaining for Ki-67 was clearly seen in collared (Figure 2a) but not in sham-operated arteries (Figure 2b) from the placebo group. Moreover, proliferative index increased significantly in collared arteries from the placebo group (Table 2). Treatment with TAK-044 significantly decreased the proliferative index in collared arteries (Table 2).

Effects of the treatment with TAK-044 in functional characteristics of the vessels

Contractile responses

The maximum contractile force development (E_{max}) in response to potassium chloride (KCl) was significantly decreased in collared arteries (Table 3). Treatment with TAK-044 did not affect this collar-induced decrease in response to KCl (Table 3).

As indicated by increased pD_2 values in collared arteries, the collar increased sensitivity to 5-HT (Table 3). TAK-044 altered the hypersensitivity to 5-HT in neither sham nor collared arteries as indicated by the absence of interaction (Table 3). With regard to E_{max} values, the collar placement did not affect the maximum contractile force development to 5-HT in carotid arteries (Table 3). TAK-044 increased E_{max} values in both sham and collared arteries (Table 3).

The sensitivity to phenylephrine was not influenced by collar placement in carotid arteries (pD_2 values: 6.14 ± 0.16, 5.94 ± 0.20 in placebo group, sham vs collared, repeated measures analysis of variance, $n = 6$). TAK-044 altered pD_2 values neither in sham nor collared arteries (pD_2 values; 6.31 ± 0.17, 6.31 ± 0.14 in TAK-044 group, sham vs collared, repeated measures analysis of variance, $n = 7$). In terms of maximum contractile force development to phenylephrine, as indicated by the presence of an interaction between the collar and TAK-044, neither collar nor TAK-044 treatment significantly affected E_{max} values of phenylephrine (E_{max}: 3.93 ± 0.48 g, 3.03 ± 0.55 g for placebo group, $n = 6$; 5.16 ± 0.66 g, 3.91 ± 0.71 g for TAK-

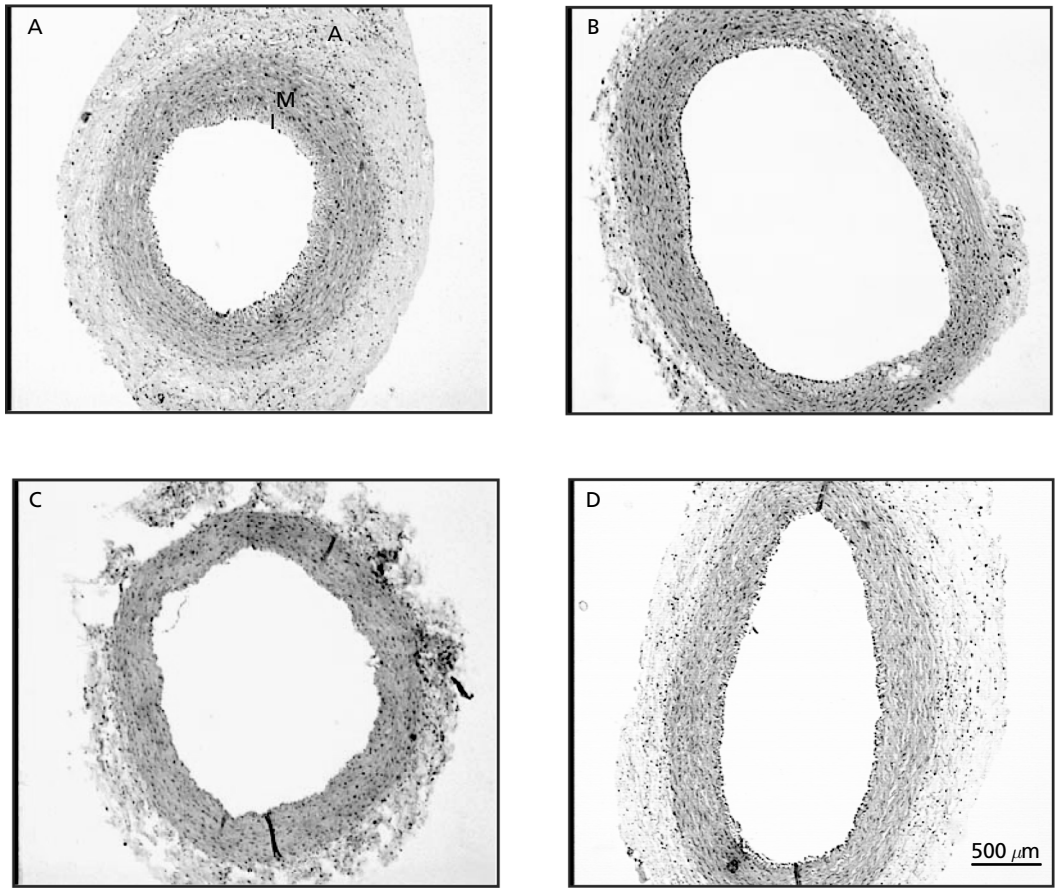

Figure 1. Photomicrographs of paraffin transverse sections after haematoxylin-eosin staining of rabbit carotid arteries, showing collared artery from placebo (A), collared artery from TAK-044 (B), sham-operated artery from placebo (C) and sham-operated artery from TAK-044 group (D) (original magnification × 4). Letters I, M and A represent tunica intima, tunica media and tunica adventitia, respectively.

044 group, sham and collared arteries, respectively, repeated measures analysis of variance, $n = 7$).

Relaxant responses

Acetylcholine-induced concentration-dependent relaxations in both sham-operated and collared arteries pre-contracted with 10^{-6} M phenylephrine. The maximal acetylcholine relaxation was not significantly altered in response to either collaring or to treatment with TAK-044 (Table 4). Sensitivity to acetylcholine decreased in response to collaring. TAK-044 treatment did not significantly alter the decreased pD_2 values in either sham or collared arteries (Table 4).

Blood pressure

There was no significant difference in blood pressure measurements before and after collaring (data not shown). Besides, treatment with TAK-044 did not alter mean arterial blood pressure (54 ± 1 mmHg, 54 ± 3 mmHg before collaring, $n = 6$; 57 ± 4 mmHg, 61 ± 5 mmHg after collaring, $n = 8$, for placebo and TAK-044-treated groups, respectively, repeated measures analysis of variance)

Discussion

In this study, arterial tissue endothelin levels were found to be statistically increased in collared arteries as compared with those in sham-operated arteries from both groups. Although the importance of endothelin in the development of intimal hyperplasia has already been mentioned (Marano et al 1998), our study demonstrates that, as a strong mitogen in smooth muscle (Tamirisa et al 1995), ET-1 is involved in the formation of intimal thickening in the collar model as indicated by augmented amounts of ET-1 in collared arteries. In their detailed study to elucidate the possible mechanisms in collar-induced intimal thickening, De Meyer et al (1997) suggested that obstruction of transmural fluid transport by the collar application might lead to retention of toxic metabolites or cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF-α), within the arterial wall. Consequently, in accordance with our findings, it is possible that toxic metabolites or cytokines within the segments enclosed by the collar may stimulate endothelin synthesis, an assumption that strongly correlates with

Table 2 Effects of collar and TAK-044 (5 mg kg^{-1} daily, s.c.) on intimal area, medial area, index and Ki-67 proliferative index of rabbit carotid arteries

	Placebo (n=9)	TAK-044 (n=9)
Intimal area (μm^2)		
Sham	64.627 ± 22.547	34.381 ± 10.829
Collared	183.217 ± 48.755*	33.870 ± 12.823#
Significance of factors in analysis of variance		
Collar	$P < 0.05$	
TAK-044	$P < 0.05$	
Interaction	TAK-044 by collar	$P < 0.05$
Medial area (μm^2)		
Sham	917.318 ± 49.567	940.262 ± 46.589
Collared	883.478 ± 41.154	902.339 ± 68.146
Significance of factors in analysis of variance		
Collar	$P = 0.373$	
TAK-044	$P = 0.744$	
Interaction	TAK-044 by collar	$P = 0.959$
Index		
Sham	0.069 ± 0.022	0.035 ± 0.010
Collared	0.212 ± 0.060*	0.041 ± 0.015†
Significance of factors in analysis of variance		
Collar	$P < 0.05$	$P < 0.05$
TAK-044	$P < 0.05$	
Interaction	TAK-044 by collar	$P < 0.05$
Ki-67 proliferative index		
Sham	0.100 ± 0.0999	0.200 ± 0.1330
Collared	1.300 ± 0.5170	0.100 ± 0.0999
Collar	†$P < 0.05$	
TAK-044	§$P < 0.05$	

Shown are means ± s.e.m. n, no. of rabbits in each group. *$P < 0.05$ Student's *t*-test for paired data (collar vs sham), #$P < 0.05$ Student's *t*-test for unpaired data (placebo vs TAK-044). †$P < 0.05$ Wilcoxon signed ranks test for paired data (collar vs sham), §$P < 0.05$ Mann–Whitney *U*-test for unpaired data (placebo vs TAK-044).

the finding that cytokines (IL-1 and TNF-α) stimulated ET-1 production (Nakano et al 1994; Woods et al 1999).

In contrast to arterial tissue endothelin levels, plasma endothelin levels did not change in response to collaring. This is hardly surprising, since one feature of the collar model is that accumulation of toxic metabolites or cytokines leading to the formation of intimal thickening is limited to the segment enclosed by the collar, suggesting that collar application does not produce a systemic effect (De Meyer et al 1997). Moreover, this local effect of the collar application enables some drugs to be administered by osmotic mini pumps to investigate the formation of intimal thickening in this model (Matthys et al 1997; De Meyer et al 2000; Bruijns & Bult 2001).

On the other hand, in our study, it was found that the endothelin receptor antagonist TAK-044 did not affect either plasma or tissue endothelin levels in the collar model. This finding demonstrates that TAK-044 does not interfere with endothelin synthesis or expression. Furthermore, treatment with TAK-044 significantly inhibited intimal thickening in the collar model, indicating that TAK-044 has an antagonistic effect on endothelin receptors in the rabbit carotid artery. In accordance with our findings, Tsujino et al (1995) showed that TAK-044, at the same dose and with the same method of administration, inhibited neointima formation in carotid artery balloon angioplasty in rat studies. Similarly, it has been reported that bosentan, a non-selective ET_A/ET_B receptor antagonist, reduced intimal hyperplasia in balloon-injured and collared arteries in rabbits, being more pronounced in the latter (Marano et al 1998). In our study, the fact that TAK-044 inhibits intimal thickening without affecting arterial tissue and plasma endothelin levels may suggest that the drug could possibly prevent migration or proliferation via blockage of ET_A and ET_B receptors, with intimal thickening not occurring as a result. In our study, TAK-044 reduced the proliferative index of Ki-67, which might possibly suggest that proliferation may have a dominant role in the formation of intimal thickening in this model. However, the effects of TAK-044

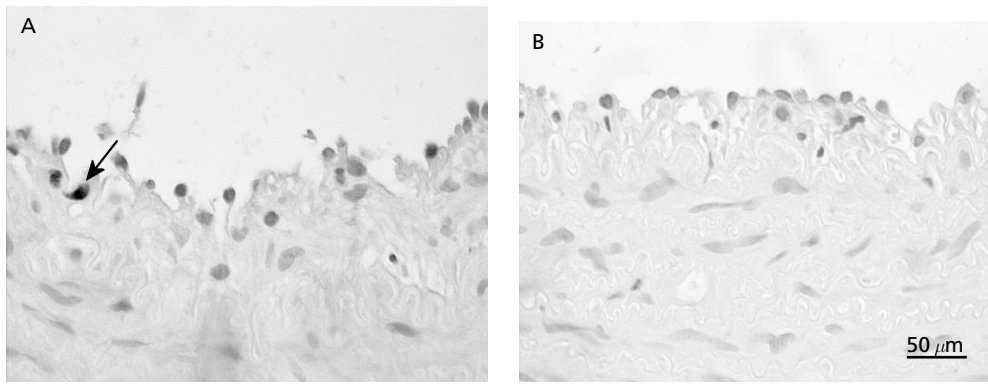

Figure 2. Photomicrographs of paraffin transverse sections of collared arteries from placebo (A) or TAK-044-treated (B) rabbits after immunohistochemical staining with Ki-67 antibody. In collared arteries (A), the arrow shows brown-stained Ki-67-positive smooth muscle cells (original magnification × 400)

Table 3 Effects of collar and TAK-044 (5 mg kg^{-1} daily, s.c.) on maximum contractions, E_{max} and pD_2 values for KCl- and 5-HT-induced contractions in rabbit carotid arteries

	Placebo (n=6)	TAK-044 (n=8)
KCl, maximum contraction, E_{max} (g)		
Sham	2.11 ± 0.75	2.40 ± 0.47
Collared	0.61 ± 0.10	0.89 ± 0.22
Significance of factors in analysis of variance		
Collar	$P < 0.05$	
TAK-044	$P = 0.536$	
Interaction	TAK-044 by collar	$P = 0.988$
5-HT, pD_2	(n = 7)	(n = 7)
Sham	6.60 ± 0.17	6.67 ± 0.10
Collared	6.83 ± 0.09	6.98 ± 0.09
Significance of factors in analysis of variance		
Collar	$P < 0.05$	
TAK-044	$P = 0.444$	
Interaction	TAK-044 by collar	$P = 0.652$
5-HT, maximum contraction, E_{max} (g)		
Sham	2.67 ± 0.52	3.62 ± 0.38
Collared	1.68 ± 0.41	3.40 ± 0.60
Significance of factors in analysis of variance		
Collar	$P = 0.171$	
TAK-044	$P < 0.05$	
Interaction	TAK-044 by collar	$P = 0.371$

Shown are means ± s.e.m. n, no. of rabbits in each group.

on the migration of smooth muscle cells was assessed neither in-vivo nor in-vitro in our study.

Considering that the role of the endothelin receptor subtypes in the formation of intimal thickening still requires further clarification (Azuma et al 1994), a selective ET_A receptor antagonist, ABT 147627, significantly reduced neointimal hyperplasia forming over porcine coronary-stented injuries (McKenna et al 1998). Similarly, SB 217242, a nonpeptide ET_A/ET_B receptor antagonist, has been shown to attenuate neointima formation in the rat carotid artery balloon angioplasty model (Chandra et al 1998). Although TAK-044 has been accepted as a non-selective ET_A/ET_B receptor antagonist, receptor-binding experiments revealed that TAK-044 has the characteristics of an ET_A-preferring antagonist (Masuda et al 1996). Regarding the effects of ET_A receptor antagonists, Maguire et al (2002) reported the ability of PDI156707, an ET_A receptor antagonist, to inhibit intimal proliferation in human saphenous veins maintained in organ culture. Moreover, LU 135252, another ET_A receptor antagonist, has been shown to reduce neointima formation following percutaneous transluminal coronary angioplasty (PTCA) (Dashwood et al 1999). However, Douglas et al (1995) reported that the ET_A-selective antagonist BQ-123 was found to be ineffective in angioplasty-induced neointima formation in the rat, while the ET_A/ET_B non-selective antagonist SB 20 9670 was effective, suggesting that the ET_B receptor subtype is implicated in the pathogenesis of intima formation in their study. Therefore, considering the results of these studies as a whole would lead to the implication that blockage of both endothelin receptors is superior to ET_A receptor antagonism by itself in inhibiting neointima formation in different models.

Alteration in functional characteristics of the vessels to vasoactive agents

Contraction

In the collar model, independently of development of intimal thickening, the alterations in vascular reactivity in response to collaring have been well documented, among them increased sensitivity to 5-HT and decreased sensitivity to phenylephrine being the most pronounced ones (Van Put et al 1995; Üstünes et al 1996). In 1999, in this model, Geerts et al demonstrated that the serotonergic receptor involved in the hypersensitivity to 5-HT of rabbit collared carotid artery is a $5HT_{1B}$ receptor subtype (Geerts et al 1999). Morover, Geerts et al (2000) further reported that collar placement elevates mRNA expression and activity of the $5HT_{1B}$ receptor in the rabbit carotid artery.

Table 4 Effects of collar and TAK-044 (5 mg kg^{-1} daily, s.c.) on maximum contractions, E_{max} and pD_2 values for KCl and 5-HT-induced contractions in rabbit carotid arteries

	Placebo (n=7)	TAK-044 (n=9)
Acetylcholine, pD_2		
Sham	6.55 ± 0.19	6.93 ± 0.07
Collared	6.36 ± 0.16	6.49 ± 0.13
Significance of factors in analysis of variance		
Collar	$P < 0.05$	
TAK-044	$P = 0.075$	
Interaction	TAK-044 by collar	$P = 0.411$
Acetylcholine, maximum relaxation, E_{max} (%)		
Sham	84.1 ± 8.2	72.0 ± 8.2
Collared	73.6 ± 8.0	85.9 ± 11.5
Significance of factors in analysis of variance		
Collar	$P = 0.856$	
TAK-044	$P = 0.993$	
Interaction	TAK-044 by collar	$P = 0.194$

Shown are means ± s.e.m. n represents the no. of rabbits in each group.

In our study, consistent with previous results (De Meyer et al 1994; Üstünes et al 1996; Kerry et al 1999; Yasa et al 1999), the collar placement suppressed 120 mM KCl-induced contractions in collared rabbit carotid arteries. In the collar model, attenuation of KCl-induced contractions is well known and has been discussed in detail (Kockx et al 1992; De Meyer et al 1994). Treatment with TAK-044 did not affect contractile responses to KCl in either sham-operated or collared arteries, suggesting that endothelin receptor antagonism does not interfere with non-receptor mediated contractions in the collar model.

In accordance with the findings of previous studies (Üstünes et al 1996; Kerry et al 1999), collaring increased the pD_2 values of 5-HT in arteries, a characteristic feature of the collar model (De Meyer et al 1990, 1994; Hickey et al 1996). As stated before, the 5-HT_{1B} receptor subtype has been found to be responsible for the development of hypersensitivity to 5-HT in the collar model (Geerts et al 1999, 2000). With regard to the effect of TAK-044 on 5-HT-induced contractions, chronic treatment with TAK-044 (21 days) seems to enhance the E_{max} values of 5-HT without affecting pD_2 values in both collared and sham arteries.

In contrast to previous experiments (Van Put et al 1995; Üstünes et al 1996; Matthys et al 1998; Yasa et al 1999; Kerry et al 2005), in this study the collar affected neither the sensitivity nor the E_{max} values of phenylephrine. At present, we are unable to suggest an explanation for the discrepancy in responses to phenylephrine in the collar model.

Treatment with TAK-044 did not affect pD_2 and E_{max} values of phenylephrine, suggesting that endothelin receptor antagonism does not interfere with the α-adrenergic receptor-mediated responses in the rabbit carotid artery.

Relaxation

Consistent with previous experiments (De Meyer et al 1995; Yasa et al 1999), collaring decreased sensitivity to acetylcholine in both the placebo and the TAK-044 group, suggesting that endothelin receptor antagonism does not interfere with muscarinic receptor-mediating responses. However, it has been shown that a non-selective ET_A/ET_B receptor antagonist PD 145 (in the incubation media) increased the relaxant responses to acetylcholine in aortic rings from rabbits with mixed dyslipidaemia (De Las Heras et al 2003). In our study, neither collar placement nor TAK-044 treatment affected E_{max} values of acetylcholine, a predictable effect of collaring (Üstünes et al 1996).

In conclusion, with regard to the vascular reactivity changes, TAK-044 did not particularly affect vascular reactivity changes occurring in this model.

Blood pressure

In this study, as was expected from results of previous experiments (De Meyer et al 1994; Hickey et al 1996; Marano et al 1999), positioning the collar around the carotid artery did not affect arterial blood pressure. As an endothelin receptor antagonist, TAK-044 has been shown to lower blood pressure in various experimental models of hypertension (Kohno et al 1997). However, in these studies, it has been stressed that TAK-044 did not affect resting arterial pressure (Bergstrom et al 2001). In our study, treatment with TAK-044 had no effect on arterial blood pressure, suggesting that endothelin receptor antagonism may only lower high blood pressure. On the other hand, in view of increased ET-1 levels in collared arteries in this study, it has been previously well documented that alterations in the amounts of molecules in response to collaring were found within the segment enclosed by the collar, resulting in a non-systemic effect of the collar (De Meyer et al 1997). Besides, in our study, plasma endothelin levels did not change before or after collaring.

Conclusion

In this study, we have shown that systemic ET_A/ET_B receptor blockade with the peptide TAK-044 causes inhibition of intimal thickening without affecting vascular reactivity in the rabbit collared artery. This result, along with the finding that collaring increases endothelin levels, may suggest the involvement of endothelin in the formation of intimal thickening in this model. The results of this and previous studies addressing the inhibitory effects of endothelin receptor antagonists may, therefore, indicate the potential effectiveness of these drugs in the treatment of atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty.

References

Azuma, H., Hamasaki, H., Niimi, Y., Terada, T., Matsubara, O. (1994) Role of endothelin-1 in neointima formation after endothelial removal in rabbit carotid arteries. *Am. J. Physiol.* **267**: 2259–2267

Bayes-Genis, A., Kantor B., Keelan, P. C., Altman, J. D., Lubbe, D. F., Kang, J., Schwartz, R. S. (2000) Restenosis and hyperplasia: animal models. *Curr. Intervent. Cardiol. Rep.* **2**: 303–308

Bergstrom, G., Nystrom, H., Jia, J., Evans, R. G. (2001) Effects of the ET_A/ET_B antagonist, TAK-044, on blood pressure and renal excretory function after unclipping of conscious one-kidney-one-clip hypertensive rats. *J. Hypertens.* **19**: 659–665

Berk, B. (2001) Vascular smooth muscle growth: autocrine growth mechanisms. *Physiol. Rev.* **81**: 999–1030

Booth, R. F. G, Martin, J. F, Honey, A. C, Hassal, D. G, Beesley, J. E., Moncada, S. (1989) Rapid development of atherosclerotic lesions in the rabbit carotid artery induced by perivascular manipulation. *Atherosclerosis* **76**: 257–268

Bruijns, R. H. J., Bult, H. (2001) Effects of local cytochalasin D delivery on smooth muscle cell migration and on collar-induced intimal hyperplasia in the rabbit carotid artery. *Br. J. Pharmacol.* **134**: 473–483

Chandra, S., Clark, L. V., Coatney, Y. R. W., Phan, L., Sarkar, S. K., Ohlstein, E. H. (1998) Application of serial in vivo magnetic resonance imaging to evaluate the efficacy of endothelin receptor antagonist SB 217242 in the rat carotid artery model of neointima formation. *Circulation* **97**: 2252–2258

Dashwood, M. R., Noertersheuser, P., Kirchengast, K., Münter, K. (1999) Altered endothelin-1 binding following balloon angioplasty of pig coronary arteries: effect of the ET_A receptor antagonist, LU 135252. *Cardiovasc. Res.* **43**: 445–456

De Las Heras, N., Cediel, E., Oubina, P. M., Aragoncillo, P., Sanz-Rosa, D., Lahera, V., Cachofeiro, V. (2003) Comparison between the effects of mixed dyslipidaemia and hypercholesterolaemia on endothelial function, atherosclerotic lesions and fibrinolysis in rabbits. *Clin. Sci.* **104**: 357–365

De Meyer, G. R. Y., Bult, H. (1997) Mechanisms of neointima formations – lessons from experimental models. *Vasc. Med.* **2**: 179–189

De Meyer, G. R. Y., Bult, H., Martin, J. F., Van Hoydunck, A. E., 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., Üstünes, L., Kockx, M. 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., Feelish, 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

De Meyer, G. R. Y., Vanput , D. J. M., Kockx, M. M., Van Schil, P., Bosmans, R., Bult, H., Buyssens, N., Vanmaele, R., Herman, A. G. (1997) Possible mechanisms of collar-induced intimal thickening. *Arterioscler. Thromb. Vasc. Biol.* **17**: 1924–1930

De Meyer, G. R. Y., Kockx, M. M., Cromheeke, K. M., Seye, C. I., Herman, A. G., Bult, H. (2000) Periadventitial inducible nitric oxide synthase expression and intimal thickening. *Arterioscler. Thromb. Vasc. Biol.* **20**: 1896–1902

Douglas, S. A., Vichery-Clark, L. M., Louden, C., Ohlstein, E. H. (1995) Selective ETA receptor antagonism with BQ-123 is insufficient to inhibit angioplasty induced neointima formation in the rat. *Cardiovasc. Res.* **29**: 641–646

Geerts, I. S., Matthys, K. E., Herman, A. G., Bult, H. (1999) Involvement of 5-HT_{1B} receptors in collar-induced hypersensitivity to 5-hydroxytryptamine of the rabbit carotid artery. *Br. J. Pharmacol.* **127**: 1327–1336

Geerts, I. S., De Meyer, G. R., Bult, H. (2000) Collar-induced elevation of mRNA and functional activity of 5-HT_{1B} receptor in the rabbit carotid artery. *Br. J. Pharmacol.* **131**: 1723–1731

Hickey, H., Makdissi, M., Hyland, R., Wilks, D., Dusting, G. J. (1996) Perindopril treatment prevents the loss of endothelial nitric oxide function and development of neo-intima formation in rabbits. *J. Mol. Cell Cardiol.* **28**: 1985–1994

Hilker, M., Tellmann, G., Buerke, M., Gloger, K., Moersig, W., Oerket, H., Hake, U. Lehr, H. (2002) Proliferative activity in stenotic human aortocoronary bypass grafts. *Cardiovasc. Pathol.* **11**: 284–290

Kerry, Z., Yasa, M., Akpinar, R., Sevin, G., Yetik, G., Tosun, M., Özdemir, N., Erhan, Y., Üstünes, L., Özer, A. (1999) Effects of nicardipine on collar-induced intimal thickening and vascular reactivity in the rabbit. *J. Pharm. Pharmacol.* **51**: 1–8

Kerry, Z., Yasa, M., Sevin, G., Reel, B., Yetik Anacak, G., Özer A. (2005) Diverse effect of calcium channel blockers in collar model. *Acta Cardiol.* In press

Kikuchi, S., Umemura, K., Kondo, K., Saniabadi, A. R., Nakashima, M. (1998) Photochemically induced endothelial injury in the mouse as a screening model for inhibitors of vascular intimal thickening. *Arterioscler. Thromb. Vasc. Biol.* **18**: 1069–1078

Kirchengast, M., Münther, K. (1998) Endothelin and restenosis. *Cardiovasc. Res.* **39**: 550–555

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

Kohno, M., Yokokawa, K., Yasunari, K., Kano, H., Minami, M., Ueda, M., Tatsumi, Y., Yoshikawa, J. (1997) Renoprotective effect of a combined endothelin type A/type B receptor antagonist in experimental malignant hypertension. *Metabolism* **46**: 1032–1038

Lafont, A., Faxon, D. (1998) Why do animal models of post-angioplasty restenosis sometimes poorly predict the outcome of clinical trials? *Cardiovasc. Res.* **39**: 50–59

Lüscher, T. F., Barton, M. (2000) Endothelins and endothelin receptor antagonists. Therapeutic considerations for a novel class of cardiovascular drugs. (2000) *Circulation* **102**: 2434–2440

Maguire, J. J., Yu, J. C., Davenport, A. P. (2002) ET_A receptor antagonist inhibits intimal smooth muscle cell proliferation in human vessels. *Clin. Sci.* **103**: 184–188

Marano, G., Palazzesi, S., Bernucci, P., Grigioni, M., Formigari, R., Ballerini L. (1998) ET_A/ET_B receptor antagonist bosentan inhibits neointimal development in collared carotid arteries of rabbits. *Life Sci.* **63**: 259–266

Marano, G., Palazzesi, S., Vergari, A., Ferrari, A. U. (1999) Protection by shear stress from collar-induced intimal thickening, role of nitric oxide. *Arterioscler. Thromb. Vasc. Biol.* **19**: 2609–2614

Masaki , T. (1998) The discovery of endothelins. *Cardiovasc. Res.* **39**: 530–533

Masuda, Y., Sugo, T., Kikuchi, T., Kawata, A., Satoh, M., Fujisawa, Y., Itoh, Y., Wakimasu, M., Ohtaki, T. (1996) Receptor binding and antagonist properties of a novel endothelin receptor antagonist, TAK-044 {Cylo [D-α-aspartyl-3- [(4-phenylpiperazin-1-yl) carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl] disodium salt}, in human endothelin A and endothelin B receptors. *J. Pharmacol. Exp. Ther.* **279**: 675–685

Matthys, K. E., Van Hove, C. E., Kockx, M. M., Andries, L. J., Van Osselaer, N., Herman, A. G., Bult, H. (1997) Local application of LDL promotes intimal thickening in the collared carotid artery of the rabbit. *Arterioscler. Thromb. Vasc. Biol.* **17**: 2423–2429

Matthys, K. E., Van Hove, C. E., Kockx, M. M., Andries, L. J., Van Osselaer, N., Herman, A. G., Bult, H. (1998) Exposure to oxidized low-density lipoprotein in vivo enhances intimal thickening and selectively impairs endothelium-dependent dilation in the rabbit. *Cardiovasc. Res.* **37**: 239–246

McKenna, C. J., Burke, S. E., Opgenorth, T. J., Padley, R. J., Camrud, L. J., Camrud, A. R., Johnson, J., Carlson, P. J., Lerman, A., Holmes, D. R., Schwartz, R. S. (1998) Selective ET_A receptor antagonism reduces neointimal hyperplasia in a porcine coronary stent model. *Circulation* **97**: 2551–2556

Mitani, M., Takimoto, M., Bandoh, T., Kimura, M. (2000) Increases of vascular endothelin-converting enzyme activity and endothelin-1 level on atherosclerotic lesions in hyperlipidemic rabbits. *Eur. J. Pharmacol.* **387**: 313–319

Nakano, J., Takizawa, H., Ohtoshi, T., Shoji, S., Yamaguchi, M., Ishil, A., Yanagisawa, M., Ito, K. (1994) Endotoxin and pro-inflammatory cytokines stimulate endothelin-1 expression and release by airway epithelial cells. *Clin. Exp. Allergy* **24**: 330–336

Rectenwald, J. E., Moldawer, L. L., Huber, T. S., Seeger, J. M., Ozaki, K. (2000) Direct evidence for cytokine involvement in neointimal hyperplasia. *Circulation* **102**: 1697–1702

Resink, T. J., Hahn, A. W., Scott-Burden, T., Powell, J., Weber, E., Buhler, F. R. (1990) Inducible endothelin mRNA expression and peptide secretion in cultured human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **168**: 1303–1310

Schwartz, S. R., Topol, E. J., Serruys, P. W., Sangiorgi, G., Holmes, D. R. (1998) Artery size, neointima, and remodeling. Time for some standards. *J. Am. Coll. Cardiol.* **32**: 2087–2094

Soma, M. R, Donetti, E., Parolini, C., Barberi, L., Paoletti, R., Fumagalli, R., Catapano, A. L. (1994) Effect of lacidipine on the carotid intimal hyperplasia induced by cuff injury. *J. Cardiovasc. Pharmacol.* **23**: 71–74

Tamirisa, P., Frishman, W., Kumar, A. (1995) Endothelin and endothelin antagonism: Roles in cardiovascular health and disease. *Am. Heart J.* **130**: 601–610

Tsujino, M., Hirata, Y., Egushi, S., Watanabe, T., Chatani, F., Marumo, F. (1995) Nonselective ETA/ETB receptor antagonist blocks proliferation of rat vascular smooth muscle cells after balloon angioplasty. *Life Sci.* **56**: 449–454

Üstünes, L., Yasa, M., Kerry, Z., Ozdemir, N., Erhan, Y., Berkan, T., Ozer, A. (1996) Effect of verapamil on intimal thickening and vascular reactivity in the collared carotid artery of the rabbit. *Br. J. Pharmacol.* **118**: 1681–1688

Van Put, D. J. M., Van Hove, C. E., De Meyer, G. R. Y, Wuyts, F., Herman, A. G., Bult, H. (1995) Dexamethasone influences intimal thickening and vascular reactivity in the rabbit collared carotid artery. *Eur. J. Pharmacol.* **294**: 753–761

Walsh, K. (2000) Building a better mouse model. *J. Mol. Cell Cardiol.* **32**: 1921–1922

Wang, X., Douglas, S. A., Louden, C., Vickery-Clark, L. M., Feuerstein, G. Z., Ohlstein, E. H. (1996) Expression of endothelin-converting enzyme-1, and endothelin-A and endothelin-B receptor mRNA after angioplasty-induced neointimal formation in the rat. *Circ. Res.* **78**: 322–328

Webb, M. L., Meek, T. D. (1997) Inhibitors of endothelin. *Med. Res. Rev.* **17**: 17–67

Woods, M., Mitchell, J. A., Wood, E. G., Barker, S., Walcot, N., Rees, G. M., Warner, T. D. (1999) Endothelin-1 is induced by cytokines in human vascular smooth muscle cells: Evidence for intracellular endothelin-converting enzyme. *Mol. Pharmacol.* **55**: 902–909

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**: 411–415

Yasa, M., Kerry, Z., Yetik, G., Sevin, G., Reel, B., Ozdemir, N., Erhan, Y., Üstünes, L., Berkan, T., Ozer, A. (1999) Effects of treatment with FK409, a nitric oxide donor, on collar-induced intimal thickening and vascular reactivity. *Eur. J. Pharmacol.* **374**: 33–39