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Effects of treatment with FK409, a nitric oxide donor, on collar-induced intimal thickening and vascular reactivity

Mukadder Yasa [a,*], Zeliha Kerry [a], Günay Yetik [a], Gülnur Sevin [a], Buket Reel [a], Necmettin Özdemir [b], Yildiz Erhan [b], Levent Üstünes [a], Tayfun Berkan [a], Asli Özer [a]

[a] *Department of Pharmacology, Faculty of Pharmacy, Ege University, 35100, Bornova, Izmir, Turkey*
[b] *Department of Pathology, Faculty of Medicine, Ege University, Izmir, Turkey*

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Abstract

Intimal thickening in arteries is considered a site of predilection for atherosclerosis. In a rabbit model of early atherosclerosis, a silastic collar was placed around the carotid artery, which resulted in the formation of intimal thickening. We investigated whether the oral application of FK409 ((±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide, 10 mg kg^{-1} day^{-1}, p.o.), a nitric oxide donor, inhibited the collar-induced intimal thickening as well as accompanying reactivity changes in rabbit carotid artery. The intimal thickening was significantly inhibited by FK409. The collar treatment increased the pD_2 value of 5-hydroxytryptamine (5-HT) whereas it decreased those of phenylephrine and acetylcholine and did not significantly alter that of nitroglycerine. Maximal contractile force development in response to potassium chloride (KCl), 5-HT and phenylephrine was decreased in collared arteries. The collar did not alter the maximal relaxant effects of acetylcholine and nitroglycerine. Despite the significant reduction of intimal thickening, FK409 treatment did not affect these collar-induced modifications in vascular reactivity.

Keywords: FK409; Intimal thickening; Vascular reactivity; Nitric oxide (NO); Carotid artery; (Rabbit)

1. Introduction

Intimal thickening, either adaptive or stimulated, represents the locations at which atherosclerotic lesions develop early. Smooth muscle cell migration and/or proliferation is the principal cause of intimal thickening (Basha and Sowers, 1995). Intimal thickening caused by different stimuli in experimental models, such as transmural electrical stimulation (Kling et al., 1993) or perivascular manipulation (Booth et al., 1989) in rabbit carotid arteries, is similar to that of human early atheroma (Arthur et al., 1997). The positioning of a soft silicone collar around the rabbit carotid artery results in thickening of the intima (Booth et al., 1989), a model which was also used in the present study. In this model, intima-bearing arterial segments functionally exhibit vascular reactivity changes such as increased sensitivity to 5-hydroxytryptamine (5-HT) and decreased sensitivity to phenylephrine (Üstünes et al., 1996). The possible mechanism(s) of intimal thickening in the collar model is not clear. Several proposals have been offered, such as a response to inflammation (Hirosumi et al., 1987), loss of the perivascular innervation (Scott et al., 1992), and hypoxia resulting from interrupting the vasa vasorum (Booth et al., 1989). Recently, De Meyer et al. (1997) reported that, in the collar model, the obstruction of transmural fluid transport may lead to retention of toxic metabolites, and/or cytokines within the segment.

Recent functional, biochemical and histological evidence from experimental animals demonstrates that atherosclerosis (Lang et al., 1993; Verbeuren et al., 1993) and intimal thickening (Hansson et al., 1994; Schini et al., 1994) lead to synthesis of inducible nitric oxide synthase in smooth muscle cells or macrophages within the arterial wall. On the other hand, nitric oxide (NO) and chemically different NO-generating drugs have been reported to inhibit DNA synthesis and proliferation of rabbit (De Meyer et al., 1995) and rat aortic smooth muscle cells (Nakaki et al., 1990). Furthermore, NO was suggested to be a modulator of vascular smooth muscle cell mitogenesis and proliferation through a cyclic GMP-mediated mechanism(s) (Sarkar et al., 1997).

* Corresponding author. Tel.: +90-232-388-5266; Fax: +90-232-388-4687; E-mail: yasam@eczacilik.ege.edu.tr

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FK409, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide, a novel NO donor was first isolated from the fermentation broth of *Streptomyces griseosporeus* (Hino et al., 1989a,b). NO is spontaneously released from the FK409 molecule without requiring enzymic activation (Isono et al., 1993; Kita et al., 1994).

Based on these reports, the present study investigated the effects of three-week FK409 treatment on collar-induced intimal thickening and on accompanying vascular reactivity changes caused by the collar.

2. Materials and methods

2.1. Treatment of animals

White rabbits (2–2.5 kg) of either sex ($n = 20$) were divided into 2 groups. The first group ($n = 10$) received a single p.o. dose of FK409 (10 mg kg^{-1} day^{-1}) via gastric gavage. The second group (placebo, $n = 10$) received only the vehicle (0.5% methylcellulose, 2.5 ml kg^{-1} day^{-1}). Throughout the 3-week treatment period, the rabbits were kept in separate cages and allowed access to their regular diet (standard rabbit chow and tap water ad libitum).

2.2. Induction of intimal thickening

After the 7th day of FK409 or placebo treatment, the rabbits were anesthetized with sodium pentobarbital (30 mg kg^{-1}, i.v.). Subsequently, the left carotid artery was surgically accessed and surrounded by a non-occlusive, flexible silicone collar 2 cm in length (Booth et al., 1989). The right carotid artery was sham-operated (i.e., separated from surrounding connective tissue and vagus nerve and received stretch similar to that of the contralateral collared artery). The carotid arteries were then replaced in their original positions and the incisions were sutured. After recovery from the anesthesia, the animals received their respective treatment for 2 weeks.

2.3. Morphometry

After anticoagulation with heparin (150 U kg^{-1}, i.v.), the rabbits were killed with an overdose of sodium pentobarbital. Artery segments from both sham-operated and collared arteries were isolated. Then, the segments were cut into two 4-mm long rings, one for morphometry and the other for organ bath experiments. The former ring was immediately placed in formalin fixative solution (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 each artery ring were randomly chosen and their video images were recorded with a video-camera (JVC Color Video Camera, Head Model No. TK-890E, Japan) connected to a light microscope (Olympus BH-2, Japan). Intimal and medial cross-sectional areas were measured with a computerized system. In brief, video (Sony VCR SL-C6E) images of each segment were captured via a video-card (Video Blaster SE, Creative Labs, USA). Intimal and medial cross-sectional areas were marked (CorelDraw, Version 4.00.A5, Corel 1993, USA) and measured (AutoCAD, release 12-cl, 1993, Autodesk, USA). The ratios of intimal to medial cross-sectional areas were also calculated and referred to as index.

2.4. Vascular reactivity

The two remaining rings from both the right (sham) and the left (collared) carotid artery were used in organ chamber experiments to study vascular reactivity. Following careful removal of loose connective tissue, the rings were suspended in organ chambers filled with 25 ml 37°C physiological salt solution (PSS) continuously gassed with 95% O_2–5% CO_2 (Üstünes et al., 1996). PSS 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. Isometric contractile force development was measured via a Grass FT3 force transducer and recorded (Polywin95 1.0, Commat, Ankara, Turkey) using a microcomputer (IBM PS/1). Following a 15-min equilibration period, the tissues were gradually stretched to a tension of 7 g, previously determined to be the optimal resting tension, based on the length–tension relationship, and allowed to equilibrate for an additional 45 min. During the equilibration period, the bath solution was changed every 15 min.

Acetylcholine-induced, endothelium-dependent, vasorelaxant responses resulting from the release of nitric oxide were tested at the end of the equilibration period. For this purpose, arterial rings were contracted with phenylephrine (10^{-6} M) and during plateau contraction, acetylcholine was added in a cumulative manner (10^{-9}–10^{-4} M). Tissues which relaxed by more than 40% of the initial contraction (indicative of functional presence of endothelium) were washed out three times with PSS, recontracted with phenylephrine (10^{-6} M), and then exposed to cumulative concentrations of nitroglycerine (10^{-9}–3×10^{-6} M). Later, the tissues were washed out three times and treated with cumulatively increased concentrations of phenylephrine (10^{-9}–10^{-4} M), 5-HT (10^{-9}–3×10^{-5} M). Each agonist was washed out by changing the bath solution three times within 30 min before addition of the next agonist. Concentration-response relationships of 5-HT-, phenylephrine-, acetylcholine- and nitroglycerine-induced responses were constructed for each preparation. At the end of the experiment, the tissues were washed out three times and contracted with 120 mM KCl (with equimolar replacement of NaCl) to determine the contractility.

2.5. Drugs

Reagent sources were as follows: Sigma (St. Louis, MO, USA): acetylcholine hydrochloride, phenylephrine

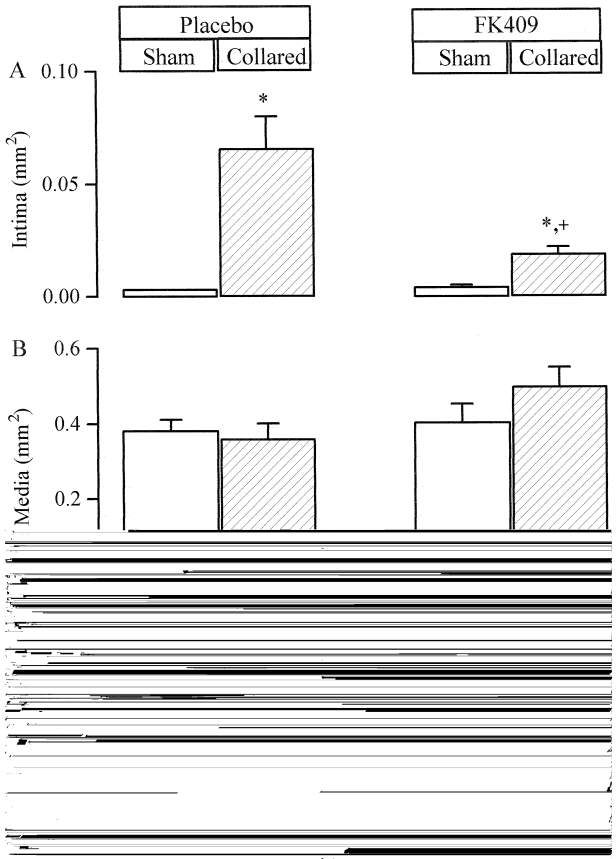

Fig. 1. Effects of FK409 treatment on collar-induced intimal thickening in rabbit carotid artery. Cross-sectional areas of intima (A), media (B), and intima/media ratio (C) are shown. Sham-operated arteries (open columns) and collared arteries (hatched columns) were isolated from rabbits receiving only the vehicle (placebo) or 3-week treatment with FK409 (10 mg kg^{-1} day^{-1}, p.o.). Shown are means ± S.E.M. (FK409-treated group, $n = 8$; placebo group, $n = 8$, each measurement is the average of two transverse sections from the same tissue). $^{*}P < 0.01$, sham vs. collared in placebo- or FK409-treated group (Student's *t*-test for paired data). $+P < 0.01$, placebo- vs. FK409-treated group (Student's *t*-test for unpaired data).

hydrochloride, 5-hydroxytryptamine creatinine sulphate; Merck, Sharp and Dohme (München, F.R.G.): nitroglycerine solution; Psyphac (Brussels, Belgium): sodium pentobarbital; Roche (Istanbul, Turkey): heparin solution; Nusil Silicone Technology (Anglet, France): silicone (MED-4011); Fujisawa Pharmaceutical (Osaka, Japan): FK409 (gift). 5-Hydroxytryptamine creatinine sulphate monohydrate was dissolved in an aqueous solution of ascorbic acid (0.01%) and diluted with distilled water. FK409 was suspended in 0.5% methylcellulose. The other drug solutions were prepared in distilled water.

2.6. Statistical methods

Statistical analyses were performed for drug treatments (2 levels, FK409 or placebo) and collar (2 levels, with or without collar) with a factorial analysis of variance (ANOVA) (SPSS/PC+, Chicago, IL, USA). If there were interactions between the factors, a paired or unpaired Student's *t*-test was applied.

Shown are means ± S.E.M. *n* represents the number of animals. Significance was accepted at $P = 0.05$. Values for maximal effect (E_{max}) and 50% effective concentration (EC_{50}) were derived for each cumulative concentration–response curve with iterative non-linear curve fitting (KaleidaGraph™ 3.06 by Synergy Software). Means of the negative logarithm of EC_{50} values (pD_2) were compared. Acetylcholine- and nitroglycerine-induced relaxations were normalized to the initial phenylephrine contraction.

3. Results

3.1. Survival and body weight

Only one rabbit from each group died during the treatment period. FK409 did not appear to cause any side-effects. The body weight of animals in the two groups was not changed by the treatment protocol.

3.2. Intimal thickening

The intimal cross-sectional area and the ratio of intimal area to medial area (index) were significantly increased in collared arteries as compared to those in sham-operated arteries (Fig. 1A and C). FK409 treatment significantly inhibited the intimal thickening (Fig. 1A). FK409 also significantly decreased the index (Fig. 1C). Collar or FK409 treatment did not alter the medial cross-sectional area (Fig. 1B).

3.3. Vascular reactivity

3.3.1. Contractions

The 120 mM KCl-induced contraction was significantly diminished in collared arteries (sham 3.4 ± 0.49 g, collared

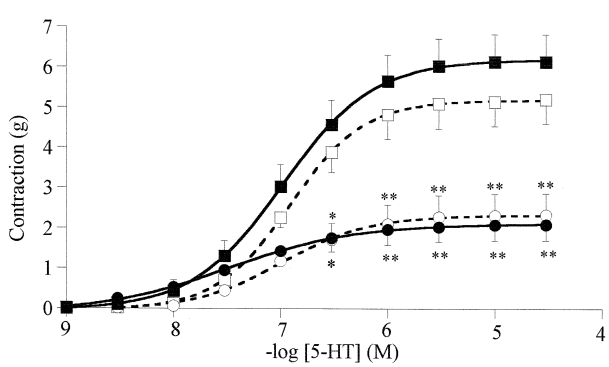

Fig. 2. Effects of collar and FK409 treatment on 5-HT-induced contractions. Concentration–response relationships for 5-HT-induced contraction obtained from placebo-treated and sham-operated (□); placebo-treated and collared (○); FK409-treated and sham-operated (■); and FK409-treated and collared (●) arteries are shown. Shown are means ± S.E.M. (placebo group, $n = 5$; FK409 group, $n = 6$). $^{*}P < 0.01$, $^{**}P < 0.001$, sham vs. collared in placebo- or FK409-treated group (ANOVA).

Table 1
Effects of collar and FK409 (10 mg kg^{-1} day^{-1}) on pD_2 values for 5-HT- and phenylephrine-induced contractions

	Placebo	FK409
5-HT	($n=5$)	($n=6$)
Sham	6.92 ± 0.06	6.96 ± 0.10
Collared	7.22 ± 0.15	7.38 ± 0.13
Significance of factors in analysis of variance		
Collar	$P = 0.026$	
FK409	N.S.	
Interaction: FK409 by collar	N.S.	
Phenylephrine	($n=6$)	($n=6$)
Sham	6.24 ± 0.15	6.31 ± 0.17
Collared	5.88 ± 0.21	5.76 ± 0.15
Significance of factors in analysis of variance		
Collar	$P = 0.018$	
FK409	N.S.	
Interaction: FK409 by collar	N.S.	

Shown are means ± S.E.M. n represents the number of animals in each group.
N.S., not significant.

1.1 ± 0.41 g; $P < 0.001$, sham vs. collared, ANOVA, $n = 5$). FK409 treatment did not significantly affect KCl-induced contractions in either sham or collared artery (sham 4.6 ± 0.43 g, collared 0.5 ± 0.12 g; $P < 0.001$, sham vs. collared, ANOVA, $n = 6$).

5-HT induced concentration-dependent contractions in both sham-operated and collared arteries. In collared arteries, the maximal contractile response (E_{max}) was significantly diminished (Fig. 2). Collar placement significantly increased the sensitivity to 5-HT, as indicated by higher pD_2 values (Table 1). Treatment with FK409 did not influence the pD_2 and the E_{max} values (Table 1, Fig. 2).

Phenylephrine induced concentration-dependent contractions in both sham-operated and collared arteries. The pD_2 and the E_{max} values were significantly diminished in collared arteries as compared to those in sham-operated arteries (Table 1, Fig. 3). FK409 treatment did not significantly influence either the pD_2 or the E_{max} values (Table 1, Fig. 3).

The initial phenylephrine (10^{-6} M) contractions prior to acetylcholine and nitroglycerine were reduced in collared-arteries. FK409 did not affect these contractions (Figs. 4 and 5).

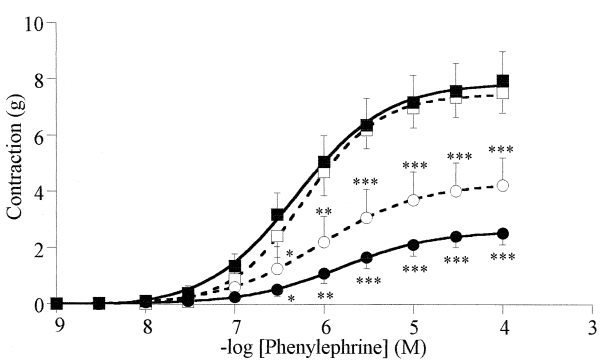

Fig. 3. Effects of collar and FK treatment on phenylephrine-induced contractions. Concentration–response relationships for phenylephrine-induced contractions obtained from placebo-treated and sham-operated (□); placebo-treated and collared (○); FK409-treated and sham-operated (■); and FK409-treated and collared (●) arteries are shown. Shown are means ± S.E.M. (placebo group, $n = 6$; FK409 group, $n = 6$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, sham vs. collared in placebo- or FK409-treated group (ANOVA).

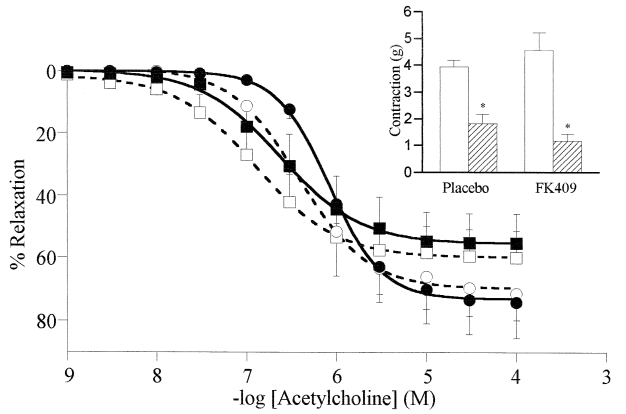

Fig. 4. Effects of collar and FK409 treatment on acetylcholine-induced relaxations. Concentration–response relationships for acetylcholine-induced relaxations obtained from placebo-treated and sham-operated (□); placebo-treated and collared (○); FK409-treated and sham-operated (■); and FK409-treated and collared (●) arteries precontracted with 10^{-6} M phenylephrine are shown. Shown are means ± S.E.M. (placebo group, $n = 5$; FK409 group, $n = 6$). Relaxation data are expressed as percentage of the 10^{-6} M phenylephrine-induced initial contractions. Insert: initial contractions elicited by 10^{-6} M phenylephrine in sham (open columns) and collared arteries (hatched columns) are shown. $^*P < 0.001$, sham vs. collared in placebo- or FK409-treated group (ANOVA).

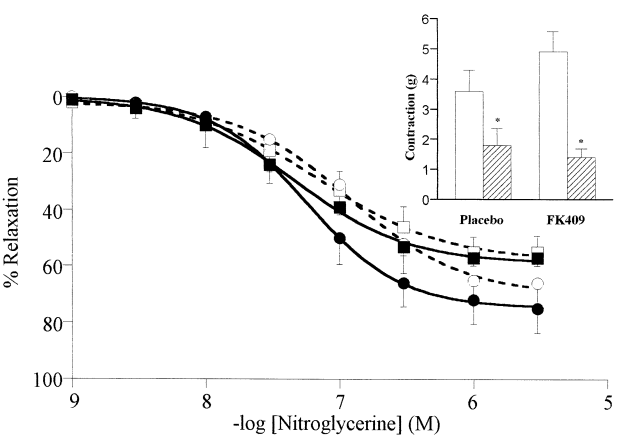

Fig. 5. Effects of collar and FK409 treatment on nitroglycerine-induced relaxations. Concentration–response relationships for nitroglycerine-induced relaxations obtained from placebo-treated and sham-operated (□); placebo-treated and collared (○); FK409-treated and sham-operated (■); and FK409-treated and collared (●) arteries precontracted with 10^{-6} M phenylephrine are shown. Shown are means ± S.E.M. (placebo group, $n = 5$; FK409 group $n = 7$). Relaxation data are expressed as percentage of the 10^{-6} M phenylephrine-induced initial contractions. Insert: initial contractions elicited by 10^{-6} M phenylephrine in sham (open columns) and collared arteries (hatched columns) are shown. $^*P < 0.001$, sham vs. collared in placebo- or FK409-treated group (ANOVA).

Table 2
Effects of collar and FK409 (10 mg kg^{-1} day^{-1}) on pD_2 values for acetylcholine- and nitroglycerine-induced relaxations

	Placebo	FK409
Acetylcholine	($n=5$)	($n=6$)
Sham	6.95 ± 0.18	6.56 ± 0.17
Collared	6.24 ± 0.25	6.12 ± 0.07
Significance of factors in analysis of variance		
Collar	$P = 0.014$	
FK409	N.S.	
Interaction: FK409 by collar	N.S.	
Nitroglycerine	($n=5$)	($n=7$)
Sham	7.29 ± 0.09	7.32 ± 0.03
Collared	7.04 ± 0.26	7.19 ± 0.10
Significance of factors in analysis of variance:		
Collar	N.S.	
FK409	N.S.	
Interaction: FK409 by collar	N.S.	

Shown are means ± S.E.M. *n* represents the number of animals in each group.
N.S., not significant.

3.3.2. Relaxations

Acetylcholine induced concentration-dependent relaxations in both sham-operated and collared arteries precontracted with 10^{-6} M phenylephrine (Fig. 4). The maximal acetylcholine relaxation was not significantly altered whereas the sensitivity to acetylcholine was significantly decreased in collared arteries (Fig. 4, Table 2). FK409 treatment did not significantly alter the pD_2 and the E_{max} values for acetylcholine-induced relaxations in both sham-operated and collared arteries (Table 2, Fig. 4).

Nitroglycerine induced concentration-dependent relaxations in both sham-operated and collared arteries precontracted with 10^{-6} M phenylephrine (Fig. 5). Neither collar nor FK409 treatment significantly influenced the E_{max} and the pD_2 values for nitroglycerine-induced relaxations (Fig. 5, Table 2).

4. Discussion

4.1. Inhibition of intimal thickening

The present study clearly demonstrated that FK409 treatment prevents the collar-induced intimal thickening in the rabbit carotid artery. This result is consistent with the observation that SPM-5185 [*N*-nitratopivaloyl-*S*-(*N'*-acetylalanyl)-cysteine ethylester], another NO donor, also inhibits thickening of the intima in rabbits (De Meyer et al., 1995) and in rats (Guo et al., 1994). Furthermore, FK409 suppresses intimal thickening following balloon injury of the rat carotid artery (Seki et al., 1995). Additionally, local or systemic administration of nitric oxide synthase inhibitors, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) (McNamara et al., 1993) and N^{ω}-nitro-L-arginine (L-NNA) (Cayatte et al., 1994) aggravated intimal thickening whereas chronic supplementation with L-arginine, a precursor of NO, has been shown to reduce intimal hyperplasia in rabbit thoracic aorta (McNamara et al., 1993) and rat carotid artery (Taguchi et al., 1993).

In the present study, this inhibitory effect of FK409 was likely to have resulted from its NO generating capacity since NO and nitrovasodilators have been shown to inhibit proliferation of smooth muscle cells (Garg and Hassid, 1989; Newby et al., 1992; Sarkar et al., 1997). However, SPM-5185 was shown not to reduce smooth muscle cell replication in the media (De Meyer et al., 1995). Therefore, the inhibition of intimal thickening by a nitric oxide donor may be due to inhibition of migration rather than proliferation of smooth muscle cells.

On the other hand, with regard to the possible mechanism(s) of collar-induced intimal thickening, it has been concluded that placing the collar leads to retention of toxic metabolites and/or cytokines within the segment (De Meyer et al., 1997). Various cytokines, such as interleukins-1 and -2 are powerful stimulators of inducible nitric oxide synthase gene expression (Iyengar et al., 1987; Drapier et al., 1988; Stuehr and Nathan, 1989). Induction of this enzyme results in the formation of NO (Stuehr and Marletta, 1987). Consistent with this, in a recent study, Arthur et al. (1997) have shown that NO is produced by the inducible isoform of nitric oxide synthase in modified smooth muscle cells of the developing intimal thickening. In this context, regarding the possibility that NO was involved in the development of intimal thickening in the present study, the inhibitory effect of the NO donor, FK409, on intimal thickening seems intriguing. A conceivable explanation may be that the chronic exposure to NO donor, FK409, may down-regulate NO production in response to collaring, an assumption that is consistent with Bult et al. (1995).

Finally, the present results demonstrated that FK409 inhibited intimal thickening in the collar model. However, the mechanism(s) by which FK409 inhibits thickening of the intima awaits further study.

4.2. FK409 and vascular reactivity

4.2.1. Contractile responses

Consistent with the previous results (De Meyer et al., 1994; Üstünes et al., 1996), the collar placement suppressed the KCl, 5-HT and phenylephrine-induced maximal contractions in rabbit carotid artery. The possible mechanisms of collar-induced decreases in response to contractile agonists were discussed in detail in previous reports (Manderson et al., 1989; De Meyer et al., 1991, 1994; Beesley et al., 1992; Kockx et al., 1992). In addition, the idea that the collar may cause mechanical damage to medial vascular smooth muscle cells would be unlikely to apply in the present study since no mechanical damage or necrosis was found on the medial layer on histological

examination. Moreover, the medial cross-sectional area did not change in collared arteries with or without FK409.

From recent work by Arthur et al. (1997), it was reported that nitric oxide was produced by the inducible isoform of nitric oxide synthase in modified smooth muscle cells of the developing intimal thickening. However, the possibility that an increase in nitric oxide release might prevent the contractile agonist-induced contractions in collared arteries was also eliminated previously since contractility studies failed to demonstrate the induction of inducible nitric oxide synthase in collared arteries (De Meyer et al., 1994).

Chronic treatment of FK409 did not significantly affect the KCl-, 5-HT- and phenylephrine-induced contractions in either sham-operated or collared arteries, suggesting that the maximal contractile response of collared vessels does not depend on the inhibition of intimal thickening.

In accordance with previous results, collaring typically increased the sensitivity to 5-HT whereas it decreased the sensitivity to phenylephrine (De Meyer et al., 1994; Üstünes et al., 1996). As observed with SPM-5185 (De Meyer et al., 1995), treatment with FK409 did not affect sensitization to 5-HT and desensitization to phenylephrine, suggesting that FK409 does not interfere with the collar-induced vascular reactivity changes in the rabbit carotid artery.

4.2.2. Relaxations

Acetylcholine was used to induce relaxations in collared and sham-operated arteries precontracted with phenylephrine (10^{-6} M) by stimulation of the endothelial arginine–NO system. Consistent with the previous reports, the collar decreased the sensitivity of the collared artery to acetylcholine without affecting E_{max} values (De Meyer et al., 1991, 1992; Üstünes et al., 1996). Although it has been shown that inducible nitric oxide synthase is expressed in intimal layers of collared arteries, it is unclear whether or not this is directly related to the abnormal endothelium-dependent relaxations in this model (Arthur et al., 1997). Neither the pD_2 values nor the E_{max} of acetylcholine were significantly affected by the FK409 treatment in collared and sham arteries, confirming the results with SPM-5185 (De Meyer et al., 1995). Thus, it can be suggested that chronic treatment with this NO donor did not alter the capacity of the endothelium of the carotid artery to generate NO in response to muscarinic stimulation.

In addition, nitroglycerine was used to induce cGMP-mediated relaxations thus directly acting on smooth muscle cells. Consistent with De Meyer et al. (1991), neither the pD_2 nor the E_{max} value for nitroglycerine-induced relaxations was affected in collared arteries. FK409 treatment did not significantly affect nitroglycerine-induced relaxations in either sham-operated or collared arteries, suggesting that the NO donor did not desensitise the arteries to cGMP-mediated responses (Isono et al., 1993; De Meyer et al., 1995).

In conclusion, the combined data demonstrated that, in rabbit carotid artery, FK409 treatment prevented the collar-induced intimal thickening without affecting the changes in vascular reactivity. The ineffectiveness of FK409 on accompanying vascular reactivity changes suggests that different mechanism(s) may be involved in the formation of intimal thickening and altered vascular responses observed in collared arteries. Indeed, inhibition of intimal thickening by various agents did not affect the vascular reactivity changes (Van Put et al., 1995; Kerry et al., 1999). On the other hand, the inhibitory effect of FK409 on thickening of the intima may have further applications, not only as therapeutic agent in early atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty but possibly for our further understanding of the mechanism(s) involved in the early atherosclerotic lesions.

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