

Available online at www.sciencedirect.com

European Journal of Pharmacology 486 (2004) 43–51

www.elsevier.com/locate/ejphar

Different role of endothelin ET_A and ET_B receptors and endothelial modulators in diabetes-induced hyperreactivity of the rabbit carotid artery to endothelin-1

Silvia Lloréns^a, Francisco J. Miranda^{a,*}, José A. Alabadí^a,
Vannina G. Marrachelli^a, Enrique Alborch^{a,b}

^aDepartamento de Fisiología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, Burjassot 46100, Valencia, Spain

^bResearch Centre, Hospital La Fe, Valencia, Spain

Received 3 July 2003; received in revised form 4 December 2003; accepted 5 December 2003

Abstract

The influence of diabetes on regulatory mechanisms and specific receptors implicated in the contractile response of isolated rabbit carotid arteries to endothelin-1 was examined. Endothelin-1 induced a concentration-dependent contraction that was greater in arteries from diabetic rabbits than in arteries from control rabbits. Endothelium removal or *N*^G-nitro-L-arginine enhanced contractions in response to endothelin-1 only in control arteries, without modifying the endothelin-1 response in diabetic arteries. Indomethacin, furegrelate (thromboxane A₂ inhibitor), or cyclo-(D-Asp-Pro-D-Val-Leu-D-Trp) (BQ-123; endothelin ET_A receptor antagonist) inhibited the contractions in response to endothelin-1, the inhibition being greater in diabetic arteries than in control arteries. 2,6-Dimethylpiperidinecarbonyl-γ-methyl-Leu-N_{in}-(methoxycarbonyl)-D-Trp-D-Nle (BQ-788; endothelin ET_B receptor antagonist) enhanced the contraction elicited by endothelin-1 in control arteries and displaced to the right the contractile curve for endothelin-1 in diabetic arteries. In summary, diabetes induces hyperreactivity of the rabbit carotid artery to endothelin-1 by a mechanism that at least includes: (1) enhanced activity of muscular endothelin ET_A receptors; (2) impairment of endothelin ET_B receptor-mediated nitric oxide (NO) release; and (3) enhancement of the production of thromboxane A₂. © 2004 Elsevier B.V. All rights reserved.

Keywords: Diabetes; NO (Nitric oxide); Endothelin-1; Endothelin ET_A receptor; Endothelin ET_B receptor; Endothelium; Carotid artery

1. Introduction

Epidemiologic and pathologic data indicate that diabetes mellitus is a risk factor for ischemic stroke. The prevalence of cerebrovascular disease, the severity of ischemia, and the mortality of stroke are higher in diabetic patients than in nondiabetic patients (Caplan, 1996; Lukovits et al., 1999). Carotid artery occlusive disease is directly linked to 20–30% of strokes that occur each year in the United States (Eugene et al., 1999). The Epidemiology of Diabetes Interventions and Complications (EDIC) (1999) research group has reported that in patients with type I diabetes, intimal–medial thickness is increased in the common and internal carotid arteries. Moreover, these carotid morpho-

logical changes are positively related to indexes of diabetic angiopathy and to risk factors for atherosclerosis, such as enhanced blood pressure and raised urinary concentrations of albumin, endothelin-1, and free cortisol (Peppas-Patrikiou et al., 1998).

The endothelium has a decisive role in regulating both the tone and the growth of the vessel wall by maintaining a critical balance between different constrictor (endothelin-1, angiotensin II, thromboxane A₂, and prostaglandin H₂) and relaxant [nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor] substances. Accordingly, the vasoconstrictor and mitogenic actions of endothelin-1 are opposed to those of vasodilator and antigrowth factors such as NO and prostacyclin secreted by endothelial cells. Diabetes alters the responsiveness of different vascular beds to several vasoconstrictors and vasodilators, and it has been hypothesised that endothelial dysfunction could partially explain many of these altered responses (Haller, 1997; De

* Corresponding author. Tel.: +34-963544903; fax: +34-963543395.
E-mail address: francisco.j.miranda@uv.es (F.J. Miranda).

Vriese et al., 2000). In patients with diabetes mellitus, the ratio of vasodilating to vasoconstricting substances in the vessel wall is markedly shifted towards those with a vasoconstricting action (Haller, 1997; De Vriese et al., 2000).

Since the discovery of endothelin-1 as the most potent vasoconstrictor and pressor substance (Yanagisawa et al., 1998), several events have suggested its implication in various cardiovascular disorders, including those related to diabetes mellitus. It is clear that many of the well-known metabolic abnormalities encountered in diabetes mellitus, such as those affecting plasma insulin, glucose, and lipid levels, contribute individually and synergistically to alterations in the release and action of endothelin-1 (Hopfner and Gopalakrishnan, 1999). The endothelin system is altered in both vascular and neuronal components of the retina in early diabetic retinopathy (De Juan et al., 2000). The increased plasma levels of endothelin-1 in diabetic patients (Sarman et al., 2000; Seligman et al., 2000; Schneider et al., 2002), together with increased free radical levels and a deficiency of prostacyclin, have been related to carotid atherosclerosis in diabetic patients (Kalogeropoulou et al., 2002). Finally, current experimental evidence suggests that endothelin antagonism may potentially represent an adjuvant therapeutic tool in the treatment of chronic diabetic complications (Chakrabarti et al., 2000; Mather et al., 2002).

We have previously reported that cerebral arteries exhibit hyperreactivity to endothelin-1 after subarachnoid haemorrhage via a mechanism that involves the absence of the modulatory role of endothelial NO (Alabadi et al., 1997). In two recent studies, we have reported that diabetes changes endothelial modulatory mechanisms in the rabbit carotid artery (Miranda et al., 2000a,b). The aim of the present study was to analyse diabetes-induced changes in the reactivity of the rabbit carotid artery to endothelin-1, including the study of the specific receptors and the effects of the disease on the endothelial mechanisms that regulate this response.

2. Materials and methods

Forty-three male New Zealand white rabbits were used in the present study. Animals were randomly divided into two experimental groups: 21 in the control group and 22 destined for induction of experimental diabetes. Housing conditions and experimental procedures were in accordance with the European Union regulations on the use of animals for scientific purposes (86/609/EEC, Article 5, Appendix II) and as promulgated by Spanish legislation on March 14, 1988 (RD 223/1988).

2.1. Induction of diabetes and control animals

For induction of experimental diabetes, rabbits weighing 2.0–3.2 kg were sedated with intramuscular injection of ketamine (40 mg; Ketolar®). Diabetes was induced by

injecting alloxan (100 mg kg^{-1}) into the lateral ear vein. To prevent hypoglycaemia, 10 ml of glucose 5% was injected intravenously after alloxan, and drinking water was supplemented with 10% glucose for the first 24 h after the alloxan injection. Thereafter, the animals were maintained on tap water and regular food ad libitum for 6 weeks. A second group of rabbits (2.1–3.0 kg) was maintained under the same conditions for the same time period to serve as age-matched controls (henceforth, “control rabbits”). Diabetic rabbits showed a marked increase in serum glucose and a failure to increase their body weight when compared with control rabbits. Table 1 shows the mean values of body weight and glycaemia before and 6 weeks after diabetes induction for the rabbits in the diabetic group and for the rabbits in the control group.

2.2. Isometric tension recording

Six weeks after diabetes induction, the diabetic and the age-matched control rabbits were anaesthetised with sodium thiopental (sodium pentothal, 2%, i.v.) and killed by injection of potassium chloride (10 mEq , 0.5 ml kg^{-1} , i.v.). The common carotid arteries were dissected free and cut into cylindrical segments measuring 4 mm in length. Each segment was prepared for isometric tension recording in an organ bath. Two stainless steel L-shaped pins (diameter, $125 \mu\text{m}$) were introduced through the arterial lumen. One pin was fixed to the organ bath wall and the other pin was connected to a strain gauge for isometric tension recording (isometric tension transducer models Panlab UF-1 and Letica TRI 201). The organ bath contained 5 ml of Ringier–Locke solution that was bubbled continuously with 95% O_2 and 5% CO_2 to provide a pH of 7.3–7.4. Temperature was kept at 37°C . A resting tension of 2 g was applied to the arterial segments, and they were allowed to equilibrate for a period of 60–90 min before the experiments were started. Tension was readjusted when necessary and the bath fluid was changed every 15 min. After this period of equilibration, the reactivity of the arterial segments was checked by depolarisation with 50 mM KCl. There were not significant differences in the response to KCl between arteries from control and diabetic rabbits.

Table 1
Body weight and glycaemia in control and diabetic rabbits

	Body weight (kg)	Glycaemia (mmol/l)	n
<i>Control rabbits</i>			
Initial time	2.53 ± 0.08	5.8 ± 0.2	21
6 weeks after	3.49 ± 0.07	5.9 ± 0.2	21
<i>Diabetic rabbits</i>			
Initial time	2.50 ± 0.08	5.9 ± 0.2	22
6 weeks after	$2.98 \pm 0.09^*$	$20.4 \pm 0.6^*$	22

Results are mean \pm S.E.M.

*Significantly different from corresponding value in control rabbits ($P < 0.01$).

2.3. Concentration–response curves for endothelin-1

The experiments were carried out with carotid arteries from both control and diabetic rabbits. Concentration–response curves for endothelin-1 (10^{-12} – 3×10^{-8} M) were obtained by its cumulative addition to the organ bath. To assess the influence of the endothelium on the effect of endothelin-1, concentration–response curves were obtained with arteries from which the endothelium had been removed by rubbing the intimal surface with a scored stainless steel rod (rubbed arteries). The absence of endothelium was checked either by silver staining or by testing the absence of a relaxant response to acetylcholine. To assess the participation of NO in the response of carotid artery to endothelin-1, concentration–response curves for this peptide were obtained after incubation (20 min) of the carotid arteries with the inhibitor of nitric oxide synthase (NOS), *N*^G-nitro-L-arginine (L-NOARG; 10^{-4} M). To examine the possibility that an arachidonic acid derivative could modulate the arterial response to endothelin-1, we obtained concentration–response curves for endothelin-1 after incubation (20 min) of the arterial segments with either indomethacin (10^{-5} M), an inhibitor of cyclooxygenase, or furegrelate (10^{-5} M), an inhibitor of thromboxane A₂ synthesis. In addition, we also obtained concentration–response curves for endothelin-1 in the presence of both L-NOARG (10^{-4} M) and indomethacin (10^{-5} M). To verify the endothelial origin of the arachidonic acid derivative, concentration–response curves for endothelin-1 after incubation with indomethacin (10^{-5} M) were obtained for rubbed arteries. To examine the participation of specific endothelin ET_A receptors, concentration–response curves for endothelin-1 were obtained in the presence of the selective endothelin ET_A receptor antagonist, cyclo-(D-Asp-Pro-D-Val-Leu-D-Trp) (BQ-123; 10^{-6} M). Finally, to examine the participation of specific ET_B receptors, concentration–response curves for endothelin-1 were obtained, in unrubbed and rubbed arteries, in the presence of the selective endothelin ET_B receptor antagonist, 2,6-dimethylpiperidinecarbonyl- γ -methyl-Leu-N_{in}-(methoxycarbonyl)-D-Trp-D-Nle (BQ-788; 10^{-6} M).

2.4. Drugs and solutions

Alloxan, endothelin-1, indomethacin, furegrelate, BQ-123, and BQ-788 were obtained from Sigma and L-NOARG was from Peptide Institute. Alloxan was dissolved in saline solution. Endothelin-1 was dissolved in 0.1% aqueous acetic acid and diluted in a mixture of phosphate-buffered saline solution (150 mM NaCl+10 mM NaH₂PO₄) and 0.05% bovine serum albumin. Furegrelate, BQ-123, BQ-788 (sodium salt), and L-NOARG were dissolved in twice-distilled water; the L-NOARG solution required sonication. Indomethacin was dissolved in ethanol and diluted in saline solution. The composition of the Ringer–Locke solution is as follows (in mM): NaCl, 120; KCl, 5.4; CaCl₂, 2.2;

MgCl₂, 1.0; NaHCO₃, 25; and glucose, 5.6. To prepare the KCl-depolarising solution, NaCl was replaced by an equimolar amount of KCl in the normal Ringer–Locke solution.

2.5. Statistical analysis

Comparisons of body weight and glycaemia between control and diabetic rabbits were made by using unpaired Student's *t* test. Endothelin-1-induced contractions are expressed as a percentage of the previous depolarisation induced by 50 mM KCl. For each concentration–response curve, the maximum effect (E_{\max}) and the concentration of endothelin-1 that produced half of E_{\max} (EC₅₀) were calculated. Maximum effects are expressed as mean \pm S.E.M. and EC₅₀ as the geometric mean with its confidence limits (95%) for repeated experiments. Statistical comparisons of E_{\max} and $-\log$ EC₅₀ (pD₂) values between arteries from control and diabetic rabbits receiving the same experimental treatment were made by using unpaired Student's *t* test. Comparisons between the values of E_{\max} and pD₂ of the concentration–response curves for endothelin-1 obtained with the different treatments in the arteries from the control rabbits were made using analysis of variance (ANOVA) followed by the Newman–Keuls test. The same tests were used to compare the E_{\max} and pD₂ values of the response curves for the different treatments in the arteries from diabetic rabbits. A probability value of less than 5% was considered significant.

3. Results

Endothelin-1 (10^{-12} – 3×10^{-8} M) induced a concentration-dependent contraction of the carotid artery from either control or diabetic rabbits (Fig. 1). The E_{\max} of the concen-

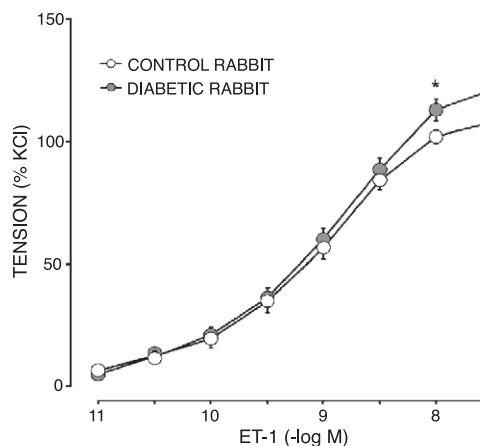


Fig. 1. Concentration–contraction response curves for endothelin-1 in carotid arteries isolated from control and diabetic rabbits. Values represent mean \pm S.E.M. *Significantly different from corresponding value in control rabbit, $P < 0.05$.

Table 2
Maximum effect (E_{\max}) and EC_{50} values for concentration–response curves for ET-1 in rabbit carotid artery

	Control rabbit		<i>n</i>	Diabetic rabbit		<i>n</i>
	E_{\max}	EC_{50} (M)		E_{\max}	EC_{50} (M)	
Control	108 ± 4	6.5 (5.4–7.8) × 10 ⁻¹⁰	32	121 ± 4*	8.7 (7.2–10.5) × 10 ⁻¹⁰	34
Rubbed	124 ± 6**	9.3 (7.9–11.0) × 10 ⁻¹⁰	21	137 ± 8	8.3 (6.8–12.6) × 10 ⁻¹⁰	30
L-NOARG (10 ⁻⁴ M)	133 ± 5**	9.5 (7.9–11.5) × 10 ⁻¹⁰	18	123 ± 4	1.2 (1.0–1.4) × 10 ⁻⁹	23
Indomethacin (10 ⁻⁵ M)	89 ± 4***	2.9 (2.4–3.4) × 10 ⁻⁹ ***	13	88 ± 5***	5.7 (5.0–6.8) × 10 ⁻⁹ ***	18
L-NOARG (10 ⁻⁴ M)+ indomethacin (10 ⁻⁵ M)	105 ± 5	1.7 (1.4–2.1) × 10 ⁻⁹ **	14	114 ± 3	1.9 (1.7–2.2) × 10 ⁻⁹ ***	16
Rubbed indomethacin (10 ⁻⁵ M)	113 ± 6	8.3 (6.0–11.5) × 10 ⁻¹⁰	15	126 ± 14	6.8 (5.7–7.9) × 10 ⁻¹⁰	14
Furegrelate (10 ⁻⁵ M)	81 ± 5***	8.7 (7.8–8.9) × 10 ⁻¹⁰	12	85 ± 4***	1.3 (1.2–1.4) × 10 ⁻⁹ *	16
BQ-123 (10 ⁻⁶ M)	104 ± 7	7.2 (6.6–7.9) × 10 ⁻⁹ **	15	97 ± 3**	6.8 (6.3–7.2) × 10 ⁻⁹ **	12
BQ-788 10 ⁻⁶ M	133 ± 7**	8.5 (6.8–10.7) × 10 ⁻¹⁰	15	129 ± 5	1.8 (1.4–2.2) × 10 ⁻⁹ **	10
Rubbed BQ-788 (10 ⁻⁶ M)	128 ± 4**	2.7 (2.3–3.1) × 10 ⁻⁹ ***	14	143 ± 6*	2.9 (2.7–3.1) × 10 ⁻⁹ ***	14

E_{\max} values are expressed as a percentage of depolarisation obtained with 50 mM KCl. E_{\max} values are mean ± S.E.M. and EC_{50} values are means and confidence limits.

n = number of arterial segments.

* Significantly different from corresponding value in control rabbits ($P < 0.05$).

** Significantly different from corresponding control value ($P < 0.05$).

*** Significantly different from corresponding rubbed value ($P < 0.05$).

tration–response curve was significantly higher in arteries from diabetic rabbits than in arteries from control rabbits, without there being significant differences between EC_{50} values (Table 2). In arteries from control rabbits, mechanical removal of the endothelium significantly increased the maximal contraction induced by endothelin-1 without significantly changing the EC_{50} value (Fig. 2, Table 2). In arteries from diabetic rabbits, removal of the endothelium did not significantly affect the endothelin-1-induced contraction both in terms of E_{\max} and EC_{50} values (Fig. 3, Table 2). The concentration–response curve for endothelin-1 obtained in rubbed arteries from diabetic rabbits did not show significant differences in EC_{50} and E_{\max} values with

respect to values obtained for rubbed arteries from control rabbits (Table 2).

Incubation with L-NOARG (10⁻⁴ M) significantly enhanced the maximal contraction induced by endothelin-1 in arterial segments from control rabbits (Fig. 2, Table 2). In arterial segments from diabetic rabbits, L-NOARG treatment did not significantly modify the contractile response to endothelin-1 (Fig. 3, Table 2). There were no significant differences between concentration–response curves for endothelin-1 in L-NOARG-treated arteries from control rabbits and those from diabetic rabbits (Table 2).

The incubation of carotid arteries from control (Fig. 2) or diabetic (Fig. 3) rabbits with indomethacin (10⁻⁵ M)

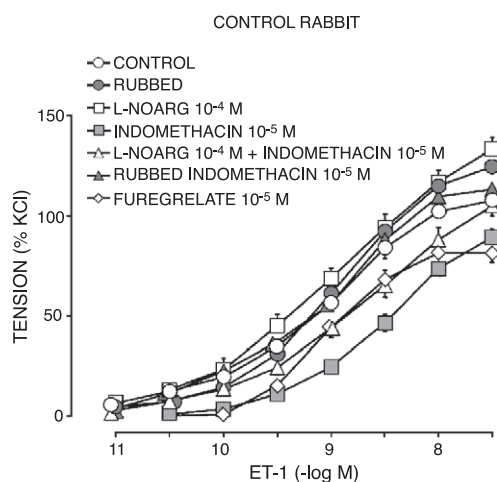


Fig. 2. Concentration–contraction response curves for endothelin-1 in carotid arteries isolated from control rabbits: control, without endothelium (rubbed), incubated with L-NOARG (10⁻⁴ M), incubated with indomethacin (10⁻⁵ M), incubated with L-NOARG (10⁻⁴ M) plus indomethacin (10⁻⁵ M), rubbed incubated with indomethacin (10⁻⁵ M), and incubated with furegrelate (10⁻⁵ M). Values represent mean ± S.E.M.

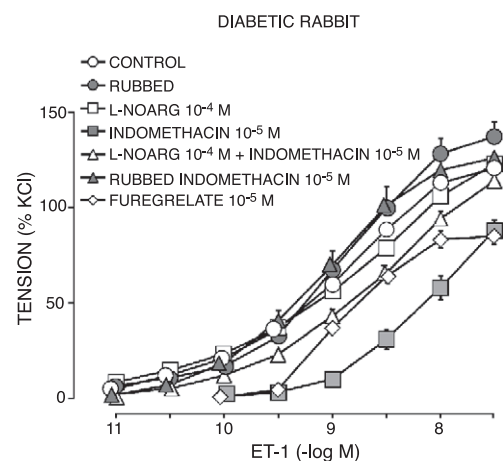


Fig. 3. Concentration–contraction response curves for endothelin-1 in carotid arteries isolated from diabetic rabbits: control, without endothelium (rubbed), incubated with L-NOARG (10⁻⁴ M), incubated with indomethacin (10⁻⁵ M), incubated with L-NOARG (10⁻⁴ M) plus indomethacin (10⁻⁵ M), rubbed incubated with indomethacin (10⁻⁵ M) and incubated with furegrelate (10⁻⁵ M). Values represent mean ± S.E.M.

significantly inhibited the E_{\max} value and increased the EC_{50} (displaced to the right) of the concentration–response curve to endothelin-1 (Table 2). The EC_{50} of the concentration–response curve for endothelin-1 obtained in indomethacin-treated arteries from diabetic rabbits was significantly higher than that obtained in indomethacin-treated arteries from control rabbits (Table 2).

The incubation of carotid arteries either from control (Fig. 2) or diabetic (Fig. 3) rabbits with both L-NOARG (10^{-4} M) and indomethacin (10^{-5} M) together significantly increased the EC_{50} (displaced to the right) of the concentration–response curve for endothelin-1, without modifying the E_{\max} value (Table 2).

The incubation of rubbed carotid arteries either from control (Fig. 2) or diabetic (Fig. 3) rabbits with indomethacin (10^{-5} M) did not significantly modify the concentration–response curve for endothelin-1 with respect to the corresponding curve obtained in rubbed arteries in the absence of indomethacin (Table 2).

The incubation of carotid arteries from control (Fig. 2) or diabetic (Fig. 3) rabbits with furegrelate (10^{-5} M) significantly inhibited the E_{\max} value of the concentration–response curve for endothelin-1, without significantly modifying the EC_{50} value (Table 2). The EC_{50} of the concentration–response curve for endothelin-1 obtained in furegrelate-treated arteries from diabetic rabbits was significantly higher than that obtained in furegrelate-treated arteries from control rabbits (Table 2).

The incubation of carotid arteries from control rabbits with BQ-123 (10^{-6} M) significantly increased the EC_{50} (displaced to the right) of the concentration–response curve for endothelin-1, without modifying the E_{\max} value (Fig. 4, Table 2). In arteries from diabetic rabbits, incubation with BQ-123 (10^{-6} M) inhibited the contractile response to endothelin-1, both in terms of EC_{50} and E_{\max} (Fig. 5, Table 2).

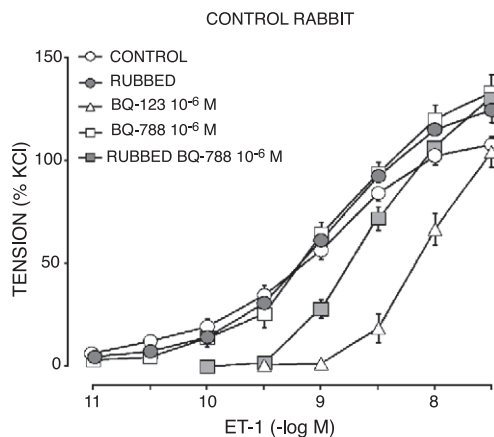


Fig. 4. Concentration–contraction response curves for endothelin-1 in carotid arteries isolated from control rabbits: control, without endothelium (rubbed), incubated with BQ-123 (10^{-6} M), incubated with BQ-788 (10^{-6} M), and rubbed incubated with BQ-788 (10^{-6} M). Values represent mean \pm S.E.M.

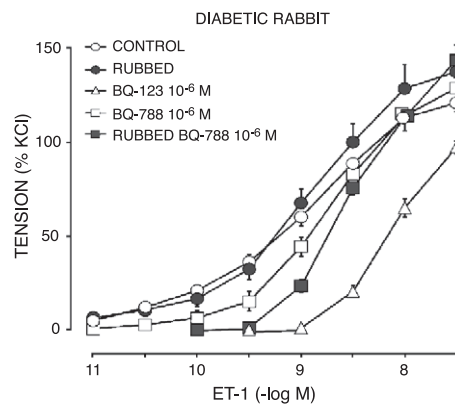


Fig. 5. Concentration–contraction response curves for endothelin-1 in carotid arteries isolated from diabetic rabbits: control, without endothelium (rubbed), incubated with BQ-123 (10^{-6} M), incubated with BQ-788 (10^{-6} M), and rubbed incubated with BQ-788 (10^{-6} M). Values represent mean \pm S.E.M.

The incubation of carotid arteries from control rabbits with BQ-788 (10^{-6} M) significantly increased the E_{\max} value of the concentration–response curve for endothelin-1, without modifying the EC_{50} (Fig. 4, Table 2). In arteries from diabetic rabbits, incubation with BQ-788 (10^{-6} M) significantly increased the EC_{50} (displaced to the right) of the concentration–response curve for endothelin-1, without modifying the E_{\max} value (Fig. 5, Table 2). The EC_{50} of the concentration–response curve for endothelin-1 obtained in BQ-788-treated arteries from diabetic rabbits was significantly higher than that obtained in BQ-788-treated arteries from control rabbits (Table 2).

The incubation of rubbed carotid arteries from either control (Fig. 4) or diabetic (Fig. 5) rabbits with BQ-788 (10^{-6} M) significantly increased the EC_{50} (displaced to the right) of the concentration–response curve for endothelin-1 with respect to the corresponding curve obtained in rubbed arteries in the absence of BQ-788 (Table 2). The E_{\max} of the concentration–response curve for endothelin-1 obtained in BQ-788-treated rubbed arteries from diabetic rabbits was significantly higher than that obtained in BQ-788-treated rubbed arteries from control rabbits (Table 2).

Table 2 summarises the E_{\max} and EC_{50} values of concentration–response curves for endothelin-1 under the different experimental conditions.

4. Discussion

The present results demonstrate that experimental diabetes enhances the contractile response of isolated rabbit carotid artery to endothelin-1. In diabetic rats, an increased contractile response to endothelin-1 was observed in renal and mesenteric arteries (Kiff et al., 1991) and aorta (Hopfner et al., 1999). The activity of endogenous endothelin-1 in the regulation of the vascular tone of resistance vessels of patients with type II diabetes mellitus is enhanced (Cardillo

et al., 2002); nevertheless, these vessels exhibit a reduced response to exogenous endothelin-1 (Cardillo et al., 2002). The vascular hyperreactivity to endothelin-1, together with the increased plasma levels of this peptide in diabetic individuals (Sarman et al., 2000; Seligman et al., 2000; Kalogeropoulou et al., 2002; Schneider et al., 2002), could play a role in the pathogenesis of cerebral ischaemia in diabetic patients. The absence of changes in the maximal contraction of the carotid artery from diabetic rabbits in response to depolarising solutions (50 mM KCl) and 5-HT (Miranda et al., 2000b) shows the selectivity of the altered response to endothelin-1 reported in the present study.

The endothelium has a decisive role in regulating the contractility of the vessel wall by secreting vasoactive substances. In the present work, endothelium denudation of arterial rings from control rabbits enhanced the contractile response of carotid artery to endothelin-1, indicating that the endothelium partially counteracts the endothelin-1-mediated vasoconstriction. The endothelial modulation of the vascular contraction in response to endothelin-1 has been reported in mesenteric arteries (Randall et al., 1989), aorta (Sakata et al., 1989), and cerebral (Kausar et al., 1990; Alabadi et al., 1997) arteries. In carotid arteries from diabetic rabbits, endothelium denudation did not significantly modify the contractile action of endothelin-1. This result would indicate that diabetes impairs the modulatory activity of the endothelium, and that this alteration could contribute, at least partially, to the hyperreactivity of the carotid artery to endothelin-1 observed in diabetic animals. Impairment of the modulatory activity of the endothelium on the vascular response to several stimuli has been reported in the aorta (Pagano et al., 1998) and renal (Costa e Forti and Fonteles, 1998) arteries of alloxan-induced diabetic rabbits and carotid arteries and aorta of women with gestational diabetes (Hu et al., 1998).

We studied the possibility that the modulatory action of the endothelium could be achieved, at least partially, through the release of NO. NO is synthesised from the amino acid, L-arginine, by the Ca^{2+} -dependent enzyme, NOS. There are three isoenzymes of NOS (Moncada et al., 1997): two constitutive isoforms [endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS)] and one inducible isoform [inducible nitric oxide synthase (iNOS)]. Incubation of carotid arteries from control rabbits with the inhibitor of the constitutive NOS, L-NOARG, significantly enhanced the contractile response of arterial segments to endothelin-1, thus indicating the participation of NO in the modulatory action of the endothelium on this response. This result is similar to that obtained previously in goat middle cerebral artery (Alabadi et al., 1997). In addition, *in vivo* studies have demonstrated that endothelin-1 stimulates the release of NO in the human forearm circulation (Cardillo et al., 2000). In arteries from diabetic rabbits, L-NOARG did not significantly enhance the contraction induced by endothelin-1. This result suggests that diabetes impairs the modulatory action of endothelial

NO on the response of the carotid artery to endothelin-1. Results previously obtained in our laboratory show that experimental diabetes enhances the modulatory activity of endothelial constitutive NO on the response of the rabbit carotid artery to 5-hydroxytryptamine (Miranda et al., 2000b). Therefore, the impairment of the modulatory action of endothelial NO on the response of the carotid artery from diabetic rabbits to endothelin-1 cannot be explained by an unspecific defect in the activity of the eNOS, but by a specific response to the contractile agonist.

In the present work, we also examined the participation of an arachidonic acid metabolite derived via cyclooxygenase in the modulation of the response of the carotid artery to endothelin-1. The results obtained show that indomethacin, an inhibitor of the enzyme cyclooxygenase, significantly decreased the sensitivity of the carotid artery to endothelin-1. This result suggests the participation of a vasoconstrictor prostanoid mediating the contractile action of endothelin-1 in the carotid artery. Because furegrelate, an inhibitor of thromboxane A_2 synthesis, inhibited the contractile response to endothelin-1, it can be assumed that this vasoconstrictor prostanoid is thromboxane A_2 . To ascertain the endothelial origin of this prostanoid, concentration–response curves for endothelin-1 were obtained in rubbed arteries in the presence of indomethacin. Our results show that the response of rubbed arteries preincubated with indomethacin to endothelin-1 was not significantly different from that obtained in the absence of the inhibitor, thus indicating the endothelial origin of the vasoconstrictor prostanoid. Endothelin-1 stimulates the production of prostaglandins and thromboxane A_2 from the metabolism of arachidonic acid via cyclooxygenase (Takayasu et al., 1989). Endothelial thromboxane A_2 modulates the response to endothelin-1 of dog basilar artery (Shirahase et al., 1991), rat hepatic vascular bed (Kurihara et al., 1992), and aorta from hypertensive rats (Taddei and Vanhoutte, 1993). Recently, we have reported that the endothelium modulates the response of the rabbit carotid artery to acetylcholine (Miranda et al., 2000a) and 5-hydroxytryptamine (Miranda et al., 2000b) and that of the rabbit renal artery to 5-hydroxytryptamine (Miranda et al., 2002) by releasing both NO and a vasoconstrictor prostanoid. Because in both indomethacin- and furegrelate-treated arteries from diabetic rabbits the EC_{50} values were increased with respect to those obtained in the corresponding arteries from control rabbits, it can be deduced that indomethacin and furegrelate were more effective at inhibiting responses in diabetic than in control arteries. This result indicates a greater modulatory role of thromboxane A_2 in the diabetic state, which could partially contribute to the hyperreactivity of the rabbit carotid artery to endothelin-1 in diabetes. The renal production of thromboxane A_2 and prostacyclin is increased in diabetic rats (Okumura et al., 2000). In contrast, other studies show a decreased formation of vasoconstrictor prostanoid in rat mesenteric arterial bed in response to methoxamine (Makino and Kamata, 1998), rabbit carotid arteries in response to

acetylcholine (Miranda et al., 2000a) or 5-hydroxytryptamine (Miranda et al., 2000b), and rabbit renal arteries in response to 5-hydroxytryptamine (Miranda et al., 2002) in diabetes. These data support the specificity of the changes in modulatory mechanisms of the arterial response to endothelin-1 mentioned above, and show that changes in vascular reactivity induced by diabetes may vary depending on the species, vascular bed, and stimuli studied. A recent study reports the existence of sex and regional differences in the alterations induced by diabetes on the reactivity of vascular smooth muscles (Sanz et al., 2003).

The vascular actions of endothelin-1 are primarily mediated by two distinct G-protein-coupled receptor subtypes designated as endothelin ET_A and ET_B (Hopfner and Gopalakrishnan, 1999). Both endothelin ET_A and endothelin ET_B receptors are located in smooth muscle cells and mediate vasoconstriction. Endothelin ET_B receptors are also located in the endothelium and mediate vasodilatation through the activation of the release of NO. In the present work, the antagonist of endothelin ET_A receptors, BQ-123, significantly inhibited the contractile response of the carotid artery to endothelin-1, both in control (increased the EC₅₀ value, without affecting the E_{max}) and diabetic (increased the EC₅₀ value and inhibited the E_{max}) rabbits. These results confirm that the contraction of the rabbit carotid artery in response to endothelin-1 is mediated by endothelin ET_A receptors, as has been previously reported in the rabbit carotid artery (White et al., 1993; Calo et al., 1996). BQ-123 inhibited the maximal contraction elicited by endothelin-1 in carotid arteries from diabetic rabbits but not in control rabbits, suggesting a greater participation of endothelin ET_A receptors in diabetes, which could partially contribute to the hyperreactivity of the rabbit carotid artery to endothelin-1 in diabetes. This would be in accordance with a recent study that shows an enhanced activity of endogenous endothelin-1 on endothelin ET_A receptors in the resistance vessels of patients with type II diabetes (Cardillo et al., 2002). In arteries from control rabbits, the incubation of unrubbed arterial segments with BQ-788 significantly enhanced the contraction in response to endothelin-1, thus suggesting the existence of endothelial endothelin ET_B receptors mediating vasodilatation. There is controversy about the presence of endothelin ET_B receptors in the rabbit carotid artery. Several studies have failed to find endothelin ET_B receptors in this artery (White et al., 1993; Lodge et al., 1995; Calo et al., 1996). Because endothelin ET_B activation mediates opposite effects depending of the endothelial or muscular location of the receptor, the sum of these effects could have masked the effect of the activation of each of them individually. However, other studies describe the presence of endothelin ET_B receptors functionally active in the rabbit carotid artery, and their possible role in several pathophysiological conditions (Lauth et al., 2000; Cattaruzza et al., 2002). In vivo studies have demonstrated that endothelin-1 contributes to regulation of the vascular tone of human resistance

vessels by stimulating NO activity, and this effect is mediated through endothelial endothelin ET_B receptors (Cardillo et al., 2000). In arteries from diabetic rabbits, BQ-788 displaced to the right the concentration–response curve of endothelin-1 in unrubbed arteries, thus indicating that vasodilatation mediated by endothelial endothelin ET_B receptors is impaired in diabetes. Therefore, the impairment of the modulatory activity of NO on the response to endothelin-1 of the carotid artery isolated from diabetic rabbits observed in the present study could be the consequence of a reduction of the number or the activity of endothelial endothelin ET_B receptors. It has been recently reported that endothelin ET_B receptor-mediated release of NO is reduced in renal perfusates in experimental models of hypertension, diabetes mellitus, and hypercholesterolemia (Kakoki et al., 1999). The incubation of rubbed arteries with the antagonist of endothelin ET_B receptors, BQ-788, significantly displaced to the right the concentration–response curve with respect to the curve obtained in untreated rubbed arteries, both in control and diabetic rabbits, thus indicating the existence of endothelin ET_B receptors located in smooth muscle cells that mediate vasoconstriction. Moreover, the maximal contraction in response to endothelin-1 obtained in BQ-788-treated rubbed arteries from diabetic rabbits was significantly greater than that obtained in BQ-788-treated rubbed arteries from control rabbits, thus supporting the above-mentioned hypothesis that the hyperreactivity of carotid artery to endothelin-1 could be associated with an increase either in the number or in the activity of endothelin ET_A receptors.

In summary, experimental diabetes induces hyperreactivity of the rabbit carotid artery to endothelin-1 by a mechanism that at least includes: (1) enhanced activity of muscular endothelin ET_A receptors; (2) impairment of endothelin ET_B receptor-mediated NO release; and (3) enhancement of the production of thromboxane A₂. This hyperreactivity may contribute to the increased risk of cerebrovascular disease in diabetic patients and drugs targeting the endothelin-1 system might prevent cerebrovascular complications in these patients.

Acknowledgements

This study was supported, in part, by a grant from University of Valencia (Ref. UV01-01). The authors are grateful to Salvador Banacloche for his technical assistance.

References

- Alabadí, J.A., Torregrosa, G., Miranda, F.J., Salom, J.B., Centeno, J.M., Alborch, E., 1997. Impairment of the modulatory role of nitric oxide on the endothelin-1-elicited contraction of cerebral arteries: a pathogenetic factor in cerebral vasospasm after subarachnoid hemorrhage? *Neurosurgery* 41, 245–253.

- Calo, G., Gratton, J.P., Telemaque, S., D'Orleans-Juste, P., Regoli, D., 1996. Pharmacology of endothelins: vascular preparations for studying ET_A and ET_B receptors. *Mol. Cell. Biochem.* 154, 31–37.
- Caplan, L.R., 1996. Diabetes and brain ischemia. *Diabetes* 45 (Suppl. 3), S95–S97.
- Cardillo, C., Kilcoyne, C.M., Cannon, R.O., Panza, J.A., 2000. Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo. *Hypertension* 35, 1237–1241.
- Cardillo, C., Campia, U., Bryant, M.B., Panza, J.A., 2002. Increased activity of endogenous endothelin in patients with type II diabetes mellitus. *Circulation* 106, 1783–1787.
- Cattaruzza, M., Berger, M.M., Ochs, M., Fayyazi, A., Fuzesi, L., Richter, J., Hecker, M., 2002. Deformation-induced endothelin B receptor-mediated smooth muscle cell apoptosis is matrix-dependent. *Cell Death Differ.* 9, 219–226.
- Chakrabarti, S., Cukiernik, M., Mukherjee, S., Chen, S., 2000. Therapeutic potential of endothelin receptor antagonists in diabetes. *Expert Opin. Investig. Drugs* 9, 2873–2888.
- Costa e Forti, A., Fonteles, M.C., 1998. Decreased endothelium dependent relaxation (nitric oxide) in diabetic kidneys. *Horm. Metab. Res.* 30, 55–57.
- De Juan, J.A., Moya, F.J., Ripodas, A., Bernal, R., Fernandez-Cruz, A., Fernandez-Durango, R., 2000. Changes in the density and localisation of endothelin receptors in the early stages of rat diabetic retinopathy and the effect of insulin treatment. *Diabetologia* 43, 773–785.
- De Vriese, A.S., Verbeuren, T.J., Van De Voorde, J., Lameire, N.H., Vanhoutte, P.M., 2000. Endothelial dysfunction in diabetes. *Br. J. Pharmacol.* 130, 963–974.
- Epidemiology of Diabetes Interventions and Complications (EDIC), 1999. Design, implementation, and preliminary results of a long-term follow-up of the Diabetes Control and Complications Trial cohort. *Diabetes Care* 22, 99–111.
- Eugene, J.R., Abdallah, M., Miglietta, M., Vernenkar, V.V., Pascual, R., Briones, R., Barnes, T., Hager, J., 1999. Carotid occlusive disease: primary care of patients with or without symptoms. *Geriatrics* 54, 24–33.
- Haller, H., 1997. Endothelial function. General considerations. *Drugs* 53, 1–10.
- Hopfner, R.L., Gopalakrishnan, V., 1999. Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42, 1383–1394.
- Hopfner, R.L., McNeill, J.R., Gopalakrishnan, V., 1999. Endothelin plasma levels and endothelin-1 evoked vascular responses at different temporal stages of diabetes in streptozotocin diabetics rats. *Eur. J. Pharmacol.* 374, 221–227.
- Hu, J., Norman, M., Wallenstein, M., Gennser, G., 1998. Increased large arterial stiffness and impaired acetylcholine induced skin vasodilatation in women with previous gestational diabetes mellitus. *Br. J. Obstet. Gynaecol.* 105, 1279–1287.
- Kakoki, M., Hirata, Y., Hayakawa, H., Tojo, A., Nagata, D., Suzuki, E., Kimura, K., Goto, A., Kikuchi, K., Nagano, T., Omata, M., 1999. Effects of hypertension, diabetes mellitus, and hypercholesterolemia on endothelin type B receptor-mediated nitric oxide release from rat kidney. *Circulation* 99, 1242–1248.
- Kalogeropoulou, K., Mortzos, G., Migdalis, I., Velentzas, C., Mikhailidis, D.P., Georgiadis, E., Cordopatis, P., 2002. Carotid atherosclerosis in type 2 diabetes mellitus: potential role of endothelin-1, lipoperoxides, and prostacyclin. *Angiology* 53, 279–285.
- Kausser, K., Rubanyi, M., Harder, D.R., 1990. Endothelium-dependent modulation of endothelin-induced vasoconstriction and membrane depolarization in cat cerebral arteries. *J. Pharmacol. Exp. Ther.* 252, 93–97.
- Kiff, R.J., Gardiner, S.M., Compton, A.M., Bennet, T., 1991. The effects of endothelin-1 and *NG*-nitro-L-arginine methyl ester on regional haemodynamics in conscious rats with streptozotocin-induced diabetes mellitus. *Br. J. Pharmacol.* 103, 1321–1326.
- Kurihara, T., Akimoto, M., Kurokawa, K., Ishiguro, H., Niimi, A., Maeda, A., Shigemoto, M., Yamashita, K., Yokoyama, I., Hirayama, Y., Ihara, M., Yano, M., 1992. Relationship between endothelin and thromboxane A_2 in rat liver microcirculation. *Life Sci.* 51, 281–285.
- Lauth, M., Berger, M.-M., Cattaruzza, M., Hecker, M., 2000. Elevated perfusion pressure upregulates endothelin-1 and endothelin B receptor expression in the rabbit carotid artery. *Hypertension* 35, 648–654.
- Lodge, N.J., Zhang, R., Halaka, N.N., Moreland, S., 1995. Functional role of endothelin ET_A and ET_B receptors in venous and arterial smooth muscle. *Eur. J. Pharmacol.* 287, 279–285.
- Lukovits, T.G., Mazzone, T., Gorelick, P.B., 1999. Diabetes mellitus and cerebrovascular disease. *Neuroepidemiology* 18, 1–4.
- Makino, A., Kamata, K., 1998. Possible modulation by endothelin-1, nitric oxide, prostaglandin I_2 and thromboxane A_2 of vasoconstriction induced by an alpha-agonist in mesenteric arterial bed from diabetic rats. *Diabetologia* 41, 1410–1418.
- Mather, K.J., Mirzamohammadi, B., Lteif, A., Steinberg, H.O., Baron, A.D., 2002. Endothelin contributes to basal vascular tone and endothelial dysfunction in human obesity and type 2 diabetes. *Diabetes* 51, 3517–3523.
- Miranda, F.J., Alabadi, J.A., Llorens, S., Ruiz De Apodaca, R.F., Centeno, J.M., Alborch, E., 2000a. Influence of experimental diabetes on regulatory mechanisms of vascular response of rabbit carotid artery to acetylcholine. *Life Sci.* 66, 2071–2080.
- Miranda, F.J., Alabadi, J.A., Lloréns, S., Ruiz De Apodaca, R.F., Centeno, J.M., Alborch, E., 2000b. Diabetes-induced changes in endothelial mechanisms implicated in rabbit carotid arterial response to 5-hydroxytryptamine. *Eur. J. Pharmacol.* 401, 397–402.
- Miranda, F.J., Alabadi, J.A., Lloréns, S., Ruiz De Apodaca, R.F., Centeno, J.M., Alborch, E., 2002. Experimental diabetes induces hyperreactivity of rabbit renal artery to 5-hydroxytryptamine. *Eur. J. Pharmacol.* 439, 121–127.
- Moncada, S., Higgs, A., Furchgott, R., 1997. XIV International Union of Pharmacology Nomenclature in nitric oxide research. *Pharmacol. Rev.* 49, 137–142.
- Okumura, M., Imanishi, M., Yamashita, T., Yamamura, Y., Kim, S., Iwao, H., Tanaka, S., Fujii, S., 2000. Renal production of thromboxane and prostaglandins in a rat model of type 2 diabetes. *Life Sci.* 66, 371–377.
- Pagano, P.J., Griswold, M.C., Ravel, D., Cohen, R.A., 1998. Vascular action of the hypoglycaemic agent gliclazide in diabetic rabbits. *Diabetologia* 41, 9–15.
- Peppas-Patrikiou, M., Scordili, M., Antoniou, A., Giannaki, M., Dracopoulou, M., Dacou-Voutetakis, C., 1998. Carotid atherosclerosis in adolescents and young adults with IDDM. Relation to urinary endothelin, albumin, free cortisol, and other factors. *Diabetes Care* 21, 1004–1007.
- Randall, M.D., Douglas, S.A., Hiley, C.R., 1989. Vascular activities of endothelin-1 and some alanyl substituted analogues in resistance beds of the rat. *Br. J. Pharmacol.* 98, 685–699.
- Sakata, K., Ozaki, H., Kwon, S.C., Karaki, H., 1989. Effects of endothelin on the mechanical activity and the cytosolic calcium level of various types of smooth muscle. *Br. J. Pharmacol.* 98, 483–492.
- Sanz, E., Fernández, N., Monge, L., Martínez, M.A., Climent, B., Diéguez, G., García-Villalón, A.L., 2003. Effects of diabetes on the vascular response to nitric oxide and constrictor prostanoids: gender and regional differences. *Life Sci.* 72, 1537–1547.
- Sarman, B., Farkas, K., Toth, M., Somogyi, A., Tulassay, Z., 2000. Circulating plasma endothelin-1, plasma lipids and complications in Type 1 diabetes mellitus. *Diabetes Nutr. Metab.* 13, 142–148.
- Schneider, J.G., Tilly, N., Hierl, T., Sommer, U., Hamann, A., Dugi, K., Leidig-Bruckner, G., Kasperk, C., 2002. Elevated plasma endothelin-1 levels in diabetes mellitus. *Am. J. Hypertens.* 15, 967–972.
- Seligman, B.G., Biolo, A., Polanczyk, C.A., Gross, J.L., Clausell, N., 2000. Increased plasma levels of endothelin 1 and von Willebrand factor in patients with type 2 diabetes and dyslipidemia. *Diabetes Care* 23, 1395–1400.
- Shirahase, H., Usui, H., Shimaji, H., Kurahashi, K., Fujiwara, N., 1991. Endothelium-independent and -dependent contractions induced by endothelin-1 in canine basilar arteries. *Life Sci.* 49, 273–281.
- Taddei, S., Vanhoutte, P.M., 1993. Role of endothelium in endothelin-evoked contractions in the rat aorta. *Hypertension* 21, 9–15.

- Takayasu, M., Kondo, K., Terao, S., 1989. Endothelin-induced mobilization of Ca^{2+} and the possible involvement of platelet activating factor and thromboxane A_2 . *Biochem. Biophys. Res. Commun.* 160, 751–789.
- White, D.G., Cannon, T.R., Garrat, H., Munding, J.W., Sumner, M.J., Watts, I.S., 1993. Endothelin ET_A and ET_B receptors mediate vascular smooth-muscle contraction. *J. Cardiovasc. Pharmacol.* 22 (Suppl. 8), S144–S148.
- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1998. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332, 411–415.