Pharmacological Research 63 (2011) 190–198

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Mechanisms underlying the diabetes-induced hyporeactivity of the rabbit carotid artery to atrial natriuretic peptide

Vannina G. Marrachelli [a], Francisco J. Miranda [a,c,*], José M. Centeno [a,c], Ignacio Miranda [a], María Castelló-Ruiz [a,b,c], María C. Burguete [a], Teresa Jover-Mengual [a,c], Juan B. Salom [a,b,c], Germán Torregrosa [a,b,c], Enrique Alborch [a,b,c]

[a] Departamento de Fisiología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain
[b] Centro de Investigación, Hospital Universitario La Fe, Valencia, Spain
[c] Unidad Mixta de Investigación Cerebrovascular, IIS-Fundación Investigación Hospital La Fe – Universidad de Valencia, Valencia, Spain

A R T I C L E I N F O

Article history:
Received 7 September 2010
Received in revised form 1 October 2010
Accepted 19 October 2010

Keywords:
Atrial natriuretic peptide
Diabetes
Endothelium
Natriuretic peptide receptors
Prostanoids
Carotid artery

A B S T R A C T

Atrial natriuretic peptide (ANP) plays an important role in the pathophysiology of the vascular complications in diabetes. The working hypothesis was that diabetes might modify the vascular actions of ANP in isolated rabbit carotid arteries and the mechanisms involved in these actions. ANP (10^{-12}–10^{-7} M) induced a relaxation of precontracted carotid arteries, which was lower in diabetic than in control rabbits. In arteries from both groups of animals, endothelium removal increased the ANP-induced relaxation. Isatin inhibited the relaxation to ANP both in arteries with and without endothelium. Carotid arteries from diabetic rabbits showed a decreased natriuretic peptide receptor (NPR)-A expression and an enhanced NPR-C expression. Inhibition of NO-synthesis did not modify ANP-induced relaxation in control rabbits but inhibited it in diabetic rabbits. In arteries with endothelium indomethacin enhanced the relaxation to ANP in control rabbits but did not modify it in diabetic rabbits. In endothelium-denuded arteries indomethacin inhibited the relaxation to ANP in both groups of animals. In KCl-depolarised arteries, relaxation to ANP was almost abolished both in control and diabetic rabbits. Tetraethylammonium inhibited the relaxation to ANP, and this inhibition was higher in diabetic than in control rabbits. These results suggest that diabetes produces hyporeactivity of the rabbit carotid artery to ANP by a mechanism that at least includes a reduced expression of NPR-A, an enhanced expression of NPR-C and a reduced participation of K^+-channels. Furthermore, diabetes enhances endothelial NO release and diminishes the ratio thromboxane A_2/prostacyclin. This increase of vasodilators could result from compensatory mechanisms counteracting the arterial hyporeactivity to ANP.

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1. Introduction

Hyperglycaemia is the primary causal factor in the development of diabetic vasculopathy and several experimental and epidemiological data demonstrate that the atrial natriuretic peptide (ANP) plays an important role in the pathophysiology of the development of the cardiovascular disease [1,2]. Increased plasma concentrations of ANP have been reported in patients with both type 1 [3] and type 2 [4] diabetes mellitus, as well as in rats [5] and rabbits [6] with experimental diabetes and, in addition, elevated plasma concentrations of ANP are related to a poor glycaemic control [3]. During the last decade the natriuretic peptides have received increasing attention as potential markers of cardiovascular disease. Specifically, increased plasma levels of ANP could be useful to predict early diastolic dysfunction in type 1 diabetes [7] and seem to be also potentially useful in the risk stratification of patients with acute stroke [8,9]. Furthermore, several evidence revealed that specific sequence mutations in ANP gene are associated with increased risk of stroke [10,11].

ANP is synthesised, stored, and released by atrial myocytes in response to atrial distension, as well as to angiotensin II, endothelin, and beta-adrenoceptor mediated sympathetic stimulation. In addition to ANP, the natriuretic peptide system includes two other members, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) which are involved in the regulation of blood pressure and fluid homeostasis in humans. Three distinct natriuretic peptide receptors (NPR) have been identified. Two of these receptors (NPR-A, NPR-B) are membrane guanylate cyclases with a tyrosine kinase domain, whereas NPR-C lacks guanylate cyclase activity. NPR-A binds to both ANP and BNP, NPR-B binds to CNP and NPR-C binds with similar affinity to all three natriuretic peptides and participates in the clearance mode, capturing and

* Corresponding author at: Departamento de Fisiología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain. Tel.: +34 963543814; fax: +34 963543395.
E-mail address: francisco.j.miranda@uv.es (F.J. Miranda).

1043-6618/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phrs.2010.10.015

degrading natriuretic peptides in the extracellular compartment [12,13].

ANP causes a variety of biological activities including effects on endothelial function and inflammation, augmentation of urinary volume and urinary sodium excretion and relaxation of vascular smooth muscle [13] which may contribute to the pathogenesis of diabetic cardiovascular disease. 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, most commonly associated with endothelial dysfunction which results in an elevation in vascular tone [14]. Injection of carperitide, a human recombinant atrial natriuretic peptide, accelerates blood flow recovery with increasing capillary density in ischemic legs in diabetic mice and is useful in the treatment of peripheral arterial disease in diabetic patients [15]. In addition, ANP prevents diabetes-induced endothelial dysfunction and may contribute to ameliorate the vascular complications of diabetes [16]. In our laboratory, we have previously reported that diabetes alters the responsiveness of the rabbit carotid artery to several vasoconstrictors [17,18] and vasodilators [19,20]. ANP is well known to elicit direct vasodilatation but information on the direct endothelial or vascular actions of ANP in brain supplying arteries is lacking, even more so in the diabetic state. Thus, the carotid arteries are of particular importance in this regard because of their role in maintaining cerebral blood flow and oxygen supply. This deficiency forms a rationale for the present study, which aim was: (1) to find out how alloxan-induced diabetes alters the response of the rabbit carotid artery to ANP; (2) to examine the role of natriuretic peptide receptors, NO, prostanoids, K^+ efflux and Ca^{2+} influx in the vascular action of ANP; and (3) to determine whether diabetes modifies these mechanisms.

2. Materials and methods

Twenty-eight male New Zealand white rabbits were used in the present study. Animals were randomly divided into two experimental groups: 14 in the control group and 14 destined for induction of 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 (RD 1201/2005).

2.1. Induction of diabetes

Rabbits weighing 2.64 ± 0.05 kg were sedated with intramuscular injection of ketamine (40 mg; Ketolar) after fasting 24 h. Diabetes was induced by injecting i.v. alloxan ($100\ mg\ kg^{-1}$) as previously described [20]. Control rabbits (2.63 ± 0.06 kg) were maintained under the same conditions for the same time period. Control and diabetic animals were well matched with no significant differences at baseline for body weight and glycaemia measures. Plasma glucose concentrations were weekly measured by the glucose oxidase method with a glucose analyser (Glucometer Elite, Bayer).

2.2. Isometric tension recording

Six weeks after diabetes induction, rabbits were anaesthetised with 2% i.v. sodium pentothal (Tiobarbital Braun) and killed by injection of potassium chloride (10 mequiv., 0.5 ml/kg, i.v.). The common carotid arteries were dissected free and cut into cylindrical segments measuring 3–4 mm in length. Each segment was prepared for isometric tension recording in an organ bath as previously described [20]. After a period of 60–90 min of equilibration, the reactivity of the arterial segments was checked by depolarisation with 50 mM KCl. There was no significant difference in the response to KCl between arteries from control and diabetic rabbits. Then, the functional integrity of endothelium was checked by examining the relaxant action of acetylcholine (10^{-5} M) in arteries precontracted with phenylephrine (10^{-7} M).

2.3. Concentration–response curves

The experiments were carried out with carotid arteries from both control and diabetic rabbits. Concentration–response curves to ANP (10^{-12}–10^{-7} M) were obtained cumulatively in carotid arteries previously contracted with phenylephrine (10^{-7} M). The active tone induced by phenylephrine in carotid arteries from control rabbits (1736 ± 185 mg) was similar to that obtained in carotid arteries from diabetic rabbits (1877 ± 161 mg). Control responses to ANP were compared to the responses obtained after the following treatments: (1) endothelium removal by rubbing the intimal surface with a scored stainless steel rod (rubbed arteries) to assess the influence of the endothelium on the effect of ANP; (2) incubation (20 min) with the antagonist of the natriuretic peptide receptors isatin (10^{-3} M) to examine the participation of these receptors in the action of ANP; (3) incubation (20 min) with the NOS inhibitor N^G-nitro-L-arginine (L-NOArg, 10^{-5} M) to check the participation of NO in the effects of ANP; (4) incubation (20 min) with indomethacin (10^{-5} M), an inhibitor of cyclooxygenase, to examine the possibility that arachidonic acid derivatives could modulate the arterial response to ANP; and (5) incubation (20 min) with tetraethylammonium (TEA, 10^{-3} M), an inhibitor of large conductance calcium-activated (BK_{Ca}) and voltage-sensitive (K_V) K^+ channels, to explore the participation of these channels in the arterial response to ANP. In other experiments, concentration–response curves to ANP (10^{-12}–10^{-7} M) were obtained in KCl 50 mM depolarised arteries to study the participation of K^+ channels. Finally, to investigate the possible Ca^{2+}-channel antagonistic effect of ANP, concentration–response curves to $CaCl_2$ (10^{-5}–3×10^{-2} M) were obtained in the absence and in the presence of ANP (10^{-8} M). For this purpose, the rings were washed three times at 10-min intervals with Ca^{2+}-free Ringer–Locke solution containing 1 mM ethylene glycol tetraacetic acid (EGTA). The rings were then bathed with Ca^{2+}-free, high KCl Ringer–Locke solution with or without ANP (10^{-8} M).

Each concentration–response curve to ANP was expressed as a percentage of the active tone. $CaCl_2$ induced contractions were expressed as a percentage of the previous depolarisation induced by 50 mM KCl.

2.4. Western blotting

To assess the expression of NPR-A and NPR-C in rabbit carotid arteries, frozen samples from nine rabbits (four control and five diabetic) were pulverised in liquid nitrogen and subsequently homogenised in lysis buffer (ProteoJetTM Mammalian Cell Lysis Reagent, Fermentas) containing a protease inhibitor cocktail (1%, Sigma). Tissue powder was resuspended by vortexing and incubated for 10 min at room temperature on a shaker (1200 rpm), and the lysate was clarified by centrifugation at 8255 rpm for 15 min. The resulting whole-cell lysate was stored at −80 °C until further use. Protein concentration of samples was determined by BCA protein assay kit (Pierce, Rockford, IL). Proteins samples (80 μg) were dissolved in sample buffer (NuPAGE LDS, Invitrogen), loaded on a gradient gel (NuPAGE 4-12% Bis-TRIS GEL, Invitrogen), subjected to electrophoresis and transferred to nitrocellulose membranes for immunolabeling. Membranes were blocked in 5% skim milk in TBS plus 100 μl of Tween 20 for 1 h at room temperature and then incubated overnight (4 °C) with the following primary polyclonal antibodies (Santa Cruz): goat anti-NPR-A (1:500) and goat anti-NPR-C (1:500). Membranes were then incubated with a sec-

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