Mechanisms Underlying the Vascular Actions of Endothelin 1, Angiotensin II and Bradykinin in the Rat Carotid

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Key Words

Carotid artery, rat • Angiotensin II • Bradykinin • Endothelin 1

Abstract

The carotid artery has a pivotal role in the body since it supplies the head and neck with oxygenated blood. Alterations in the functional and structural integrity of these vessels can decrease blood flow to the brain. For this reason, it is important to understand how the carotid artery responds to various stimuli. The organ bath is a traditional experimental set-up that has been used extensively to investigate the (patho)physiology and pharmacology of in vitro tissue preparations including the rat carotid artery. Molecular biology developed from related fields such as biochemistry, genetics and biophysics is now considered an important tool for understanding physiological pathways in a variety of tissues. Several local and systemic factors regulate carotid reactivity, including vaso-active peptides, such as endothelin 1 (ET-1), angiotensin II (Ang II) and bradykinin (BK). These vaso-active peptides play a fundamental role in controlling the functional and structural integrity of the arterial wall and may be important in physiological processes and in pathological mechanisms underlying vascular diseases. In the rat

carotid, these peptides induce vasoconstriction or relaxation by the release of endothelium-derived relaxing factors, such as nitric oxide and prostacyclin. Identification of such signal transduction processes is essential for understanding the mechanisms that regulate vascular smooth muscle cell function, both physiologically and pathophysiologically. The present review discusses the mechanisms of action, distribution of ET-1, Ang II and BK and their receptors in the rat carotid. With this purpose, data obtained in functional studies using classical pharmacological approaches as well as data obtained in molecular biology experiments are discussed.

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Introduction

Measurements of circulatory system function in laboratory animal models play an important role in pharmacological evaluations. For pharmacologists, parameters such as blood pressure, heart rate and vascular reactivity provide valuable tools for predicting whether a new compound will be safe and effective in human beings. Traditionally, a variety of techniques have been employed to

obtain these measurements. The organ bath is a traditional experimental set-up that has been used extensively to investigate the (patho)physiology and pharmacology of in vitro tissue preparations including the rat carotid artery [1–4]. Since the 1940s, molecular biology has developed from the related fields of biochemistry, genetics and biophysics, and is considered an important tool in the understanding of physiological pathways in a variety of tissues.

The common carotid artery is a paired structure, meaning that there are two in the body, one for each half. The left and right common carotid arteries follow the same course with the exception of their origin. The right common carotid originates in the neck from the brachiocephalic trunk. The left arises from the aortic arch in the thoracic region. The carotid artery branches in the neck to form the external and internal carotid arteries. The main function of these arteries is to supply the head and neck with oxygenated blood. Alterations in the functional and structural integrity of these vessels can decrease blood flow to the brain. For this reason, it is important to understand how the carotid artery responds to several stimuli.

Blood vessels are composed of different cells: endothelial, smooth muscle, fibroblasts and other, less characterised, cell types. Each of these cell types is subjected to different chemical and haemodynamic stimuli. The components of the arterial wall dynamically change shape, increase, decrease or reorganise, in response to physiological and pathological stimuli [5]. Vascular smooth muscle cells are the final common pathway for many of these dynamic changes in vascular wall structure. These cells are responsible for structural and functional characteristics of the vessel wall, including contraction and relaxation. Several local and systemic factors regulate vascular smooth muscle cell function, including vaso-active peptides, such as endothelin 1 (ET-1), angiotensin II (Ang II) and bradykinin (BK). These peptides induce vasoconstriction or relaxation by the release of endothelium-derived vasorelaxing factors, such as nitric oxide (NO) and prostacyclin (PGI₂). Vaso-active peptides play a fundamental role in controlling the functional and structural integrity of the arterial wall and may be important in physiological processes regulating blood pressure and in pathological mechanisms underlying vascular diseases. Vaso-active peptides are particularly important because they have been identified as potential therapeutic targets in cardiovascular diseases. The actions of ET-1, Ang II and BK are mediated via specific, highly complex intracellular signalling pathways that are

stimulated following initial binding of peptides to their respective extracellular receptors. Intracellular signalling pathways are composed of interconnected molecular cascades that transmit information from the receptors to intracellular proteins that regulate cell activities such as contraction and relaxation. Identification of such signal transduction processes is essential for understanding mechanisms that regulate vascular smooth muscle cell function, both physiologically and pathophysiologically.

The present review will discuss the mechanisms underlying the physiological action of ET-1, Ang II and BK in the rat carotid. This review will focus on the local pathways involved in the production of these vaso-active peptides, the receptors that mediate the response induced by these peptides and the mechanisms underlying such responses. With this purpose, data obtained from functional studies using classical pharmacological approaches as well as data obtained in molecular biology experiments are discussed.

The Endothelineric Pathway

Synthesis and Expression of ET in the Rat Carotid

In the adult rat, ET-1 expression is observed in vascular endothelium and smooth muscle [6]. A study of ET gene expression using reverse transcription polymerase chain reaction (RT-PCR) indicated that mRNA for prepro-ET-1 and prepro-ET-3 is detectable in the rat carotid [7]. The constitutive expression of mRNA for prepro-ET-1 in the rat carotid is also described in other studies [4, 8]. It should be pointed out that under physiological conditions, ET-1 is synthesised in the endothelium and smooth muscle of the rat carotid as demonstrated by immunostaining assays [6]. In endothelial cells from rat carotid arteries, immunoreactivity for ET-1 is distributed in the cytoplasm and in association with the membranes of intracellular organelles and structures, such as mitochondria, endoplasmic reticulum and cytoplasmic vesicles [9]. The data describing the expression and synthesis of ET-1 in the rat carotid clearly show that this tissue possesses the ability to synthesise the peptide in both the endothelium and the vascular smooth muscle. More recently, we found that low yet detectable ET-1 levels are produced and released physiologically by the rat carotid [4]. ET-1 is produced in a variety of tissues and cell types. Indeed, mRNA for prepro-ET-1 is also detected in other blood vessels such as the rat aorta [10] and porcine vena cava and pulmonary artery [11] as well as human renal, gastric and internal thoracic arteries [12].

The 3 ET isoforms are synthesised as larger prepro-isoforms which are processed by dibasic amino acid endopeptidase to the respective big ET. ET-1 is generated from the 38-amino-acid precursor, big ET-1, by cleavage of the Trp²¹-Val²² bond by an ET-converting enzyme (ECE). Two different ECE forms, termed ECE-1 and ECE-2, have been isolated, but their chemical nature and tissue distribution suggest that ECE-1 is responsible for the conversion of big ET-1 to ET-1 in vascular tissue [13]. In the rat carotid, the expression of ECE-1 was described by Wang et al. [7] using RT-PCR. In the following year, the same group of researchers confirmed the expression of ECE-1 in the rat carotid and suggested that this enzyme is expressed by the vascular media in this artery [6]. Using immunohistochemical assays, the authors detected a strong immunoreactive signal to ET-1 in intimal smooth muscle cells, an observation that strengthened the hypothesis that ET-1 is produced by ECE-1 in the vascular smooth muscle of the rat carotid.

The approach to verify vessel wall ECE activity by measuring the vasomotor effects induced by big ET-1 using functional assays has been extensively employed [14–16]. In the rat carotid, we recently observed that big ET-1 induces concentration-related contraction in both endothelium-intact and -denuded rings [4]. Interestingly, the onset of contraction and the increase in tone are slow upon application of big ET-1 when compared to ET-1. This observation suggests the involvement of an enzyme-mediated step in the contractile action of exogenously applied big ET-1 as previously observed in other vascular tissues such as the rabbit aorta [16], the human umbilical vein [17], the human placental artery [18] and the rat basilar artery [15]. Additionally, our results show that phosphoramidon, a non-selective inhibitor of ECE and neutral endopeptidase, produces rightward displacements of the big ET-1 concentration-response curves with a reduction in the maximal response, further indicating that this peptide must be converted to exert its biological activity. Functional ECE activity seems to be a constitutively expressed feature in the media and possibly in the adventitia of the rat carotid artery wall since big ET-1 induces the contraction of endothelium-denuded carotid rings. In fact, immunohistochemical analysis of the radial artery revealed the presence of ECE-1 not only in the endothelium, but also in the underlying intima as well as in smooth muscle cells of the media [14]. Finally, our functional assay with big ET-1 corroborates the initial proposal of Wang et al. [6] that ECE-1 is constitutively expressed in the media of rat carotid arteries. A system for the local production of ET-1 has also been described in

other blood vessels such as porcine vena cava and pulmonary artery [11] as well as human renal, gastric and internal thoracic arteries [12].

Vasocontractile ET Receptors in the Rat Carotid

ETs produce their physiological effects by binding to receptors named ET_A and ET_B. Initial indirect evidence for the existence of ET_A and ET_B receptors in the rat carotid was provided by Douglas et al. [19], who observed that administration of the dual ET_{A/B} receptor antagonist SB209670 ameliorated neo-intimal formation induced by rat carotid artery balloon angioplasty. The presence of ET_A and ET_B receptor mRNA in the rat carotid was first identified by Wang et al. [6, 7]. Simultaneously to their findings, De Oliveira et al. [20] demonstrated the presence of ET_A receptors in the medial layer of rat carotid arteries using autoradiography. The authors also suggested that a small amount of ET_B receptors could be expressed in this artery. Later, Viswanathan et al. [21] confirmed the presence of ET_A receptors in the medial layer of rat carotid arteries and also verified that ET_A receptor expression is increased in the media of these arteries after balloon angioplasty.

More recently, the mRNA and protein expression of both ET_A and ET_B receptors, evaluated by RT-PCR and Western immunoblotting, were detected in carotid segments [3]. Interestingly, we verified that in endothelium-denuded vessels, the levels of ET_B receptor mRNA were reduced, suggesting that these receptors are expressed in both the endothelium and the vascular smooth muscle. As expected, RT-PCR as well as the Western blotting allow the identification of mRNA and protein levels for ET_A and ET_B receptors in tissue homogenates. On the other hand, the immunohistochemical technique allows the identification of ET receptors on the endothelium and tunica media of blood vessels. Interestingly, the latter approach has not been used for the identification of ET receptors in the rat carotid. Using specific antibodies for both ET_A and ET_B receptors, we identified anatomically distinct localisations of the 2 above-mentioned receptors in cells [3]. Our data showed that the ET_A receptor is localised mainly in the vascular smooth muscle whereas the ET_B receptors are present in the endothelium as well as in the vascular smooth muscle. Thus, ET_B receptors are abundant in the rat carotid and play an important role in the control of vascular tone in this artery, as it will be further discussed in this review.

In addition to molecular biology identification, we functionally characterise ET receptors in the rat carotid artery. Vascular reactivity experiments, using standard

muscle bath procedures, showed that ET-1 induces slowly developing and long-lasting contraction in endothelium-intact and -denuded carotid rings in a concentration-dependent manner [3, 22]. A pharmacological characterisation of the receptors mediating the contractile effects of ET-1 in rat carotid arteries was attempted using classical criteria recommended by Schild, namely the order of potency of agonists and the affinities of competitive antagonists. The selective antagonist for ET_A receptors BQ123 produced a rightward displacement of ET-1-induced curves in a concentration-dependent manner resulting in a Schild plot slope less than unity (0.36 ± 0.10). The most plausible explanation for a slope less than unity is a heterogeneous population of ET receptors mediating contraction. In support of this proposal, BQ788, a selective antagonist for ET_B receptors, produced a rightward displacement of the concentration-response curves for ET-1. Furthermore, a combination of BQ123 and BQ788 resulted in greater antagonism than that exerted by the antagonists when added individually, further supporting the idea that ET_A and ET_B receptors are involved in ET-1-induced contraction.

An interesting finding of our investigation was that although ET_A and ET_B receptors mediate ET-1-induced contraction in the rat carotid, the contribution of these receptors to this response is different. IRL1620, a selective agonist for ET_B receptors, produced about 10% of the total contraction induced by ET-1 in both endothelium-intact and endothelium-denuded carotid rings. A smaller contribution of ET_B receptors in mediating the contraction induced by ET-1 was also reported in rat mesenteric small arteries [23] and porcine coronary arteries [24]. Our data indicate that ET-1 acts predominantly on the ET_A receptor to induce contraction while ET_B receptors play a minor role. Interestingly, ET receptors possess different distribution patterns and functional consequences depending on the species and blood vessel. Caló et al. [25] demonstrated that the rabbit pulmonary artery contains contractile ET_A and ET_B receptors, which contribute approximately 20 and 80%, respectively, of the contraction induced by ETs. Moreover, it has been demonstrated previously that the human saphenous vein [26] and human umbilical vein [27] contain both ET_A and ET_B contractile receptors.

Like several other vasoconstrictor agents, the vascular contraction induced by ET-1 is accompanied by an increase in cytosolic free Ca²⁺. Antagonists of L-type Ca²⁺ channels were found to markedly inhibit ET-1-induced contraction in several arterial tissues such as the rat aorta [28] and human forearm circulation [29]. Recent data

from our laboratory have described the involvement of extracellular Ca²⁺ influx in the contraction induced by ET-1 in the isolated rat carotid [30]. In this same study, the influence of ET-1 in Ca²⁺ release from intracellular stores was also analysed. ET-1 induced a rapid and transient increase in tension in a Ca²⁺-free medium that was observed in endothelium-intact and -denuded rat carotid rings. Several studies in isolated blood vessels [28, 31] demonstrated that one of the early events after ET-1 stimulation is a rapid transient increase in cytosolic Ca²⁺ that is not sensitive to removal of extracellular Ca²⁺ [32] or L-type Ca²⁺ channel blockers [32, 33], but can be completely abolished by previous treatment of tissues with caffeine or ryanodine, an inhibitor of Ca²⁺ release from the sarcoplasmic reticulum [33]. Thus, extracellular Ca²⁺ influx and mobilisation of Ca²⁺ from intracellular stores participate in signal transduction mechanisms involved in ET-1-induced contraction of the rat carotid.

De Nucci et al. [34] provided the first line of evidence that ET-1 can stimulate arachidonic acid metabolism, when the authors demonstrated that ET-1 induced the release of PGI₂ and thromboxane A₂ (TxA₂) from isolated perfused rat and guinea pig lung preparations. In the rat carotid, no evidence was found supporting the participation of vasoconstrictor prostanoids in the contractile response induced by ET-1 [30]. In fact, we observed that neither AH6809, an antagonist of prostaglandin F_{2α} (PGF_{2α}) receptors, nor SQ2954, an antagonist of prostaglandin H₂ (PGH₂)/TxA₂ receptors, affects the contractile response induced by ET-1. Moreover, ET-1-induced contraction was not inhibited by SC-560, a selective inhibitor of cyclooxygenase (COX) 1, or SC-236, a selective inhibitor of COX-2, further discarding the possibility that activation of contractile ET-1 receptors induces the metabolism of arachidonic acid. Finally, ELISA experiments for TxA₂ measurements showed that ET-1 does not increase the production of this prostanoid in comparison with basal levels. Thus, it seems unlikely that vasoconstrictor prostanoids mediate the contractile response induced by ET-1 in the rat carotid. Although no evidence for the participation of vasoconstrictor prostanoids is found in physiological circumstances, we must be aware of the participation of these arachidonic acid metabolites in the contraction induced by ET-1 in pathological states. For example, ET-1 stimulates the release of TxA₂ in carotid rings from hyperhomocysteinaemic rats. Moreover, the contractile response of this peptide is inhibited by COX inhibitors and antagonists for PGF_{2α} and TxA₂ [30].

Relaxation Mediated by ET_B Receptors in the Rat Carotid

De Nucci et al. [34] showed that low doses of ET-1 provoke dose-dependent vasodilatation in the rat isolated perfused mesentery. Moreover, endothelial removal abolished the vasodilatation induced by ET-1. Such findings indicated that apart from its vasoconstrictor activity, ET-1 is also able to induce vascular relaxation. Later, similar endothelium-dependent and ET_B-receptor-mediated vascular relaxation was observed in other vascular tissues such as the rat aorta and the rabbit mesenteric artery [35, 36]. In rat carotids precontracted with phenylephrine, low concentrations of ET-1 induced endothelium-dependent relaxation that was increased in the presence of BQ123, but reduced by BQ788, indicating that the receptor involved in the relaxation induced by ET-1 is of the ET_B subtype [3]. IRL1620-induced relaxation was reduced in the presence of BQ788, confirming that the endothelium-dependent relaxation induced by IRL1620 is mediated by ET_B receptors. Interestingly, IRL1620 induces relaxation in low concentrations when compared to that necessary to induce contraction. A possible explanation for this observation is that ET_B receptors found on vascular smooth muscle possess far less affinity for ET_B-receptor-selective agonists than the supersensitive receptor moiety located on the endothelium [37].

ET-1 was described to stimulate the release of NO from rabbit blood vessels [38], rat mesenteric bed [34] and the rat aorta [39]. Moreover, the relaxation induced by ETs in the rat aorta [28] and in the rat mesenteric bed [34] was described to involve the activation of the guanylyl cyclase enzyme. In the rat carotid, L-nitroarginine methylester (L-NAME), a non-selective NO synthase (NOS) inhibitor and oxadiazole quinoxalinone, a selective inhibitor of guanylyl cyclase, partially, but significantly, reduced ET_B-mediated relaxation, further confirming the participation of the NO-cGMP pathway in ET_B-mediated vasorelaxation [3].

In addition to NO, ETs and big ET-1 were also described to stimulate PGI₂, a vasodilator prostanoid from bovine vascular endothelial cells, from isolated rat mesenteric arteries and from perfused rat lung [40–44]. In the rat carotid, indomethacin, a non-selective COX inhibitor, significantly reduced ET_B-mediated relaxation further, indicating that vasodilator COX product(s) play a role in ET_B-mediated relaxation [3]. The relaxant response was not completely abolished in the presence of both L-NAME and indomethacin, indicating that factors unrelated to the production of vasodilator COX product(s) or NOS activation also participate in this response.

A role for K⁺ channels in ET_B-mediated vasorelaxation has already been described by Hasunuma et al. [45], in pulmonary arteries. In the rat carotid, similar results were found where ET_B-mediated relaxation involves the activation of voltage-dependent K⁺ channels. The mechanisms underlying ET_B-mediated relaxation in the rat carotid differ from those of other types of blood vessels. In the rat aorta, the relaxation induced by ET-1 is mainly mediated by the NO-cGMP pathway and does not involve the opening of K⁺ channels [39]. In rat pulmonary arteries, ATP-sensitive K⁺ channels are involved in ET_B-induced relaxation [45].

In summary, the rat carotid artery contains a heterogeneous population of contractile ET_A and ET_B receptors located in the vascular smooth muscle and endothelial ET_B receptors that mediate vasorelaxation via the NO-cGMP pathway, vasodilator COX product(s) and the activation of voltage-dependent K⁺ channels. Considering the anatomical location and the function of ET receptors in the rat carotid, activation of ET_A and ET_B receptors by ET-1 induces some type of physiological antagonism where endothelial ET_B receptors, which mediate relaxation, counteract the contractile response induced by the activation of ET_A and ET_B receptors located on the vascular smooth muscle (fig. 1). Although the functions of ET receptors are well characterised, the present discussion shows that these receptors possess different distributions and functions depending on the species and blood vessel.

The Angiotensin System

Synthesis and Expression of Ang II in the Rat Carotid

In the classical renin-angiotensin system, circulating renin produced by the kidney cleaves hepatic-derived angiotensinogen to form a decapeptide, the so-called angiotensin I (Ang I). Ang I is converted by the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) in the lungs to the physiologically active Ang II [46–49]. However, some studies suggest the existence of a local tissue renin-angiotensin system since many of its components are localised in tissues such as the heart and blood vessels [50]. With the exception of renin, all components of the renin-angiotensin system are produced in the vasculature. The rat carotid also produces ACE. Nakamura et al. [51] detected ACE activity in the rat carotid that was strongly inhibited by cilazapril, an ACE inhibitor. Some years later, Schiffers et al. [52] verified that Ang I as well as Ang II induce contraction of isolated carotid rings with

Fig. 1. Schematic illustration of the intracellular signalling pathways involved in the response of ET-1 in the rat carotid. AA = Arachidonic acid; eNOS = endothelial NOS; GC = guanylate cyclase; EC = endothelial cell; BL = basal lamina; SR = sarcoplasmic reticulum.

Ang I being less potent than Ang II. The authors also described that the ACE inhibitors captopril and lisinopril produce a rightward shift of the concentration-response curve to Ang I. These findings indicated the existence of a functional pathway for Ang II generation in rat carotid artery that involves the catalytic action of ACE. Similar results were found in our laboratory as presented in figure 2. Our findings show that Ang I concentration dependently constricts the rat carotid with pD_2 values of 8.0 ± 0.07 ($n = 5$). However, this peptide is significantly less potent than Ang II (pD_2 : 8.9 ± 0.1 ; $n = 11$; $p < 0.05$, Student's t test), but the maximal responses for these peptides were not different (Ang I: 0.96 ± 0.07 g mg^{-1} ; Ang

II: 0.95 ± 0.09 g mg^{-1}). The onset of contraction and the increase in tone were slow upon application of Ang I when compared to Ang II, suggesting the involvement of an enzyme-mediated step in the contractile action of exogenous applied Ang I as previously observed by Schiffers et al. [52].

ACE is constitutively expressed in the rat carotid [53]. More recently, ACE mRNA was measured by quantitative RT-PCR in the rat carotid [54]. In fact, previous findings from Caputo et al. [55] suggested that the vessel wall, in the absence of exogenous substrates of the renin-angiotensin system, is capable of generating Ang II, whose formation or action can be inhibited by lisinopril, an ACE

inhibitor, or losartan, a selective Ang II type 1 (AT₁) receptor antagonist.

The existence of alternative pathways to ACE for Ang II generation in tissues of different species has been proposed. More recently, Becari et al. [56] provided evidence supporting a functional role for elastase 2 in the generation of Ang II in the rat carotid. In this study, chymostatin (an elastase 2 and chymase inhibitor) induced a rightward shift of the vasoconstrictor effect of Ang I, thus indicating the participation of a non-ACE pathway in the conversion of this peptide to Ang I. The synergistic inhibitory effect of captopril and chymostatin on the constrictor effect induced by Ang I suggests a marked participation of a non-ACE pathway for Ang II generation in the rat carotid. Furthermore, Ac-AAPL-CK (an elastase 2 inhibitor) completely abolished the contraction induced by [Pro¹¹-D-Ala¹²]Ang I, a biologically inactive precursor that selectively yields Ang II when incubated with chymases but not with ACE. Finally, rat elastase 2 mRNA could be detected in the rat carotid. Taken together, these findings suggest the presence of a functional alternative pathway to ACE for Ang II generation in the rat carotid. Moreover, these results represent strong evidence of a physiological role for elastase 2 as an alternative pathway as previously demonstrated in the rat mesenteric bed [57–59]. Functional chymase and a non-ACE pathway have also been demonstrated in dog carotid artery [60] and in human vascular tissue [50]. The data presented support the notion that a locally generated Ang II system is functional in the carotid artery since several components of the renin-angiotensin system are present in the wall of the carotid artery (fig. 3).

Contraction Induced by Angiotensin II in the Rat Carotid

Two receptor subtypes – AT₁ and AT₂ –, first distinguished on a pharmacological basis, have been identified by expression cloning from various species. Both receptors have a 7-transmembrane domain topology as shown by de Gasparo et al. [61]. In the rat carotid artery, Ang II likely activates the AT₁ receptor to induce contraction. Boulanger et al. [62] demonstrated that in carotid rings without endothelium, losartan, a selective antagonist for AT₁ receptors, causes a parallel rightward displacement of the concentration-response curve to Ang II without affecting the maximal response to the peptide. The authors also found that the slope of the Arunlakshana-Schild plot is not different from unity (slope = 1.003), an observation that suggests that the contractile response induced by Ang II is not mediated by a heterogeneous

Fig. 2. Concentration-response curves for Ang I and Ang II obtained from endothelium-intact rat carotid rings. The concentration-response curves were obtained by a stepwise increase in the concentration of Ang I (from 10^{-12} to 3×10^{-8} mol l⁻¹) and Ang II (from 10^{-12} to 3×10^{-7} mol l⁻¹). Values are means $\pm$ SEM of 5–11 independent preparations.

population of receptors [63, 64]. This interpretation is reinforced further by the absence of an effect of PD123319, a preferential AT₂ receptor antagonist. Moreover, losartan displayed a competitive antagonism toward the response to Ang II, with a pA₂ value (9.5 ± 0.2) in the range reported for AT₁ receptor subtypes [65]. The results from Boulanger et al. [62] were recently corroborated by Becari et al. [56] and Zyzdorzcyk et al. [66], who identified the AT₁ receptor as the sole receptor implicated in carotid artery vasoconstriction response to Ang II. The authors discarded the participation of either AT₂ or Mas, the Ang(1–7) receptor [67], on the vasocontractile action displayed by Ang II. Moreover, by using Western blot assays, this study provided direct evidence for the existence of AT₁ protein expression in the rat carotid. In fact, the functional evidence provided by Boulanger et al. [62] and Zyzdorzcyk et al. [66] demonstrating the weak effect of AT₂ receptors in mediating Ang II contraction in the rat carotid was strengthened by molecular biology experiments recently performed by Barker et al. [67], who demonstrated that AT₂ receptors are weakly expressed in the intima of the rat carotid. However, the Ang II system has been shown to have marked regional differences. The constrictor effect of Ang II in rat mesenteric venules and portal vein involves the activation of both AT₁ and AT₂ receptors [68]. Interestingly, overexpression of the AT₂

Fig. 3. Schematic illustration of the intracellular signalling pathways involved in the response of ET-1 in the rat carotid. eNOS = Endothelial NOS; GC = guanylate cyclase; EC = endothelial cell; BL = basal lamina; SR sarcoplasmic reticulum.

receptor has been shown in balloon-injured rat carotid tissue [67, 69]. Because the expression of AT_2 receptors is normally low in adults, its increased expression in pathological situations is likely an important mechanism by which this receptor contributes to injury responses in tissue [70].

Ang II elicits complex highly regulated cascades of intracellular signal transduction that lead to long-term biological effects, such as cell growth, migration, extracellular matrix deposition and inflammation. In addition, it can also have short-term vascular consequences such as contraction. Recent observation from our group provid-

ed evidence that in the rat carotid the AT_1 -mediated increase in tension depends on the influx of extracellular Ca^{2+} and the mobilisation of intracellular Ca^{2+} [71].

Thromboxanes are involved in Ang-II-induced contraction, while vasorelaxant prostaglandins, such as PGE_2 and PGI_2 , attenuate Ang-II-mediated vasoconstriction in some vascular beds [72, 73]. We previously observed that indomethacin, a non-selective COX inhibitor, reduces the contraction induced by Ang II in the rat carotid [1, 74], further implicating the participation of vasoconstrictor prostanoids in the contraction induced by this peptide.

Some studies demonstrated that the contractions evoked by Ang II are impaired by endothelium-derived NO [75, 76]. However, whether this impairment is due to the basal release of NO or to stimulation of endothelial cells by Ang II remains inconclusive. The hypothesis that Ang II causes the release of endothelium-derived NO in the rat carotid artery was tested by Boulanger et al. [62], who showed that Ang-II-induced contractions are decreased by an AT₁-stimulated release of endothelium-derived NO. The conclusion was based on the observation that in the rat carotid artery the presence of the endothelium significantly shifts the concentration-response curve to Ang II to the right and decreases the maximal response to the peptide. Moreover, these data were supported by the observations that endothelial cells express angiotensin receptors [77, 78]. In fact, this group previously described a simultaneous opposing dose-dependent action of Ang II in the rat carotid: constriction of the carotid artery and a concurrent elevation of carotid wall cGMP content, the second messenger classically involved in vasodilatation [55]. The authors found that the AT₁ receptor subtype involved in smooth muscle cell contraction is simultaneously involved in Ang-II-induced cGMP production, since neither Ang-II-induced vessel constriction nor the increase in cGMP wall content is affected by blockade of Ang II AT₂ receptors with a specific antagonist of AT₂ receptors, PD 123319. Moreover, the mechanism by which Ang II increases cGMP levels appears to be via the endothelium, since the effect is abolished after endothelium removal. Thus, the response of rat carotid arteries to Ang II results from the combined activation of endothelial and smooth muscle AT₁ receptors. Distribution of the AT₁ receptor subtype both on the smooth muscle cells and on endothelial cells could simultaneously result in direct vasoconstriction and endothelium-mediated NO-cGMP production. The endothelium-dependent production of NO and cGMP on the stimulation of AT₁ receptors likely contributes to the impairment of direct vasoconstriction caused by the peptide. Endothelial dysfunction reported in hypertension, atherosclerosis and aging could impair this modulating effect of Ang II on the endothelium and increase the hypertensive action of Ang II.

Relaxation Induced by Ang II in the Rat Carotid

Apart from its potent vasoconstrictor responses, which are known to be mediated by AT₁ receptors, Ang II has also been shown to produce relaxation. Activation of AT₂ receptors leads to relaxation in some types of blood vessels [79–82]. The relaxation induced by Ang II involves the

production of vasodilator substances such as prostaglandins, NO, kinins and the opening of K⁺ channels [82, 83].

In the rat carotid, we observed that concentrations of Ang II ranging from 10⁻¹⁰ to 10⁻⁷ mol l⁻¹ induce a sustained contraction [1, 2, 84] whereas concentrations above this range induce a decrease in tension. Thus, Ang II added in a cumulative fashion induces a bell-shaped concentration-response curve in the rat carotid. This profile was also observed by Becari et al. [56]. Experimental evidence suggests that in conditions where the levels of Ang II are high, the vasorelaxant action of this peptide might provide a brake for the peptide signal, counteracting the contractile effects of Ang II [58, 75, 76]. Experiments using standard muscle bath procedures showed that Ang II concentration (0.01–3 μmol l⁻¹) dependently induces relaxation of phenylephrine-precontracted rat carotid rings [85]. The Ang-II-induced relaxation was strongly inhibited by saralasin, the non-selective antagonist of Ang II receptors, but not by the selective antagonists of AT₁ and AT₂ receptors, losartan and PD123319, respectively, indicating that this response is mediated by an atypical angiotensin-type receptor. This observation raised the possibility that Ang II fragments participate in this response. On the other hand, A-779, an Ang(1–7) receptor antagonist, inhibits the relaxation induced by Ang II. Santos et al. [86] reported that Ang(1–7) is an endogenous ligand for the G-protein-coupled receptor Mas. In Mas-receptor-deficient mice, there is no binding of radiolabelled Ang(1–7) to renal tissue, and the aortas from these mice do not relax in response to exogenously administered Ang(1–7) [86] or AVE0991, a nonpeptide mimic of Ang(1–7) [87]. These observations corroborate the findings in Mas-transfected Chinese hamster ovary cells, where Ang(1–7) causes a concentration-dependent increase in NO release [88], which is blocked by A-779 but not by AT₁ or AT₂ antagonists. ACE-2, a novel ACE homologue, is a type I integral membrane protein that efficiently hydrolyses Ang II to Ang(1–7) [89]. This enzyme is expressed in the media of rat carotid [90]. Thus, the current knowledge of the actions of Ang(1–7) suggests the existence of the ACE-2-Ang(1–7)-Mas axis. Based on these observations, the participation of Ang(1–7) in the relaxant action displayed by Ang II in the rat carotid can be inferred. On the other hand, it is important to consider that Ang II might also stimulate the Ang(1–7) receptor [91]. The possibility that, at the high concentrations employed in our study, Ang II non-specifically activates the receptors of Ang(1–7) to promote its vasodilator effect cannot be discarded [85]. In fact, our results are in accordance with that observed by Soares de Moura et al. [92] describing

that the vasodilator effect of Ang II on the rat mesenteric vascular bed is partially attenuated by A-779. On the other hand, Ang-II-induced relaxation has been shown to have marked regional differences. The relaxation induced by this peptide is mediated by AT₂ receptors in the fowl aorta [79], dog renal and cerebral arteries [80] and rat aorta [82]. The relaxation mediated by Ang II in the rat carotid involves the activation of the NO-cGMP pathway [85]. These results are in agreement with previous studies, which have demonstrated that NO is released and accompanied by an increase in the cGMP content in endothelium-denuded vessels from rats [81, 93].

In conclusion, in the rat carotid Ang II is able to induce relaxation via activation of the NO-cGMP pathway. Different from other vascular beds, where Ang II exerts its vasodilator effect by traditional receptors [79, 80], the relaxant actions displayed by this peptide in the rat carotid artery are also dependent on the activation of the Ang(1–7) receptor [85].

The Bradykinin System

Local Kallikrein-Kinin System in the Rat Carotid

The kallikrein-kinin system (KKS) consists of precursor kininogens, the proteolytic kallikrein enzymes and the kinin peptides (which are produced by cleavage of kininogens by kallikreins). Kallikreins are a distinct group of serine proteases, which are capable of generating vasoactive kinins from kininogens in vitro. The nonapeptide BK is a potent vaso-active agent that is formed in the plasma by the proteolytic cleavage of a larger precursor termed high-molecular-weight kininogen. Two G-protein-coupled receptors, termed the B₁ and B₂ receptors, mediate the biological effects of BK.

Vascular tissue contains kininogen and kallikrein mRNA, suggesting the existence of a local source of the KKS. The tissue KKS is present in both endothelial and smooth muscle cells of various vascular territories and is different from the circulating plasma KKS [94, 95]. Moreover, vascular endothelial cells take up kininogen HK from the plasma [96].

The presence of kininogens and kallikrein-like activities in the vessel wall suggests the existence of an endogenous vascular kinin-generating system, different from the circulating plasma KKS [94, 95]. Bergaya et al. [97] showed that tissue kallikrein plays an essential role in kinin synthesis within the vascular wall of mouse carotid arteries. Oza et al. [95] reported that kininogen is synthesised in cultured rat aortic smooth muscle cells.

Expression of endogenous rat tissue kallikrein, bradykinin B₂ receptor and kininogen mRNAs in the carotid artery was previously described by Murakami et al. [98]. Furthermore, expression of the B₁ receptor has been detected in the rat carotid [99]. Together, these data demonstrate that all of the key components of the kallikrein-kinin system are present in the rat carotid, where they may function by discrete autocrine/paracrine mechanisms.

As mentioned before, ACE is a dipeptidyl carboxypeptidase that catalyses the conversion of Ang I to Ang II. Although ACE is a member of the renin-angiotensin system and contributes to the production of the vasopressor Ang II, this enzyme (also termed kininase II) hydrolyses a carboxyl-terminal dipeptide from BK, which leads to fragments that are inactive on either BK receptor type. ACE is constitutively expressed in the rat carotid [53] and contributes to the local degradation of BK. Thus, the local KKS described in the rat carotid plays an important role in the control of vascular tone and also in smooth muscle cell proliferation. ACE inhibitors comprise one of the most successful drug classes for cardiovascular applications, and these compounds can inhibit the major degradation pathway of BK, pointing to the possibility that kinins mediate the beneficial vasodilator effect and other cardioprotective actions of these drugs [100]. The role of BK is mediated at least in part by NO release in endothelial cells, which has antiproliferative effects on vascular smooth muscle cells [101]. In fact, Farhy et al. [102] suggested that the beneficial effects of ACE inhibitors on neo-intima formation can be explained by both the stimulation of kinins and NO, and the reduction in Ang II production. Moreover, kallikrein gene delivery inhibits vascular smooth muscle cell growth and neo-intima formation in the rat carotid artery after balloon angioplasty [98]. On the other hand, the increased ACE activity observed in rat carotid artery after balloon catheter placement could therefore contribute to the enhanced degradation of BK and a reduction of NO production induced by this peptide [54]. ACE inhibitors increase vascular relaxation induced by BK [103], which in turn affects the reactivity of blood vessels. Recent data from our laboratory showed that treatment with the ACE inhibitor tran-dolapril for 6 days decreased the potency of phenylephrine (a selective α_1 -receptor agonist) and Ang II in the isolated rat carotid [74]. Interestingly, HOE-140, a selective antagonist for B₂ receptors, prevents the decrease in potency for both agonists, further suggesting the participation of BK in this response. These results corroborate previous experimental evidence suggesting that locally

formed kinins can participate in the regulation of vascular tone [104, 105].

Function of BK Receptors in the Rat Carotid

BK is a potent agonist in numerous organs, and its actions are mediated by the activation of two main BK receptor subtypes named B_1 and B_2 , both of which are members of the family of 7 transmembrane G-protein-coupled receptors [106]. Results from our laboratory showed that BK induces relaxation of the rat carotid artery [2], and this response involves the release of NO [22]. Based on such findings, we pursued the mechanisms underlying BK-induced relaxation in the rat carotid. BK concentration-dependently relaxed isolated rat carotid rings precontracted with phenylephrine vasorelaxant, and endothelial denudation strongly abolished BK-induced relaxation, further indicating that its vasodilator effect is dependent on the integrity of the vascular endothelium [107]. A role for the vascular endothelium on BK-induced relaxation was previously described in other vascular tissues such as bovine middle cerebral arteries [108] and porcine coronary arteries [109]. Immunocytochemistry [110] and real-time PCR [111] in human vascular tissue have demonstrated the expression of endothelial B_2 receptors. In the rat carotid, HOE-140, a selective antagonist for B_2 receptors, reduces BK-induced relaxation, further indicating the participation of endothelial B_2 receptors in the vasorelaxant effect of BK [107]. Several studies on carotid arteries from different species also revealed that B_2 receptors mediate the relaxation induced by BK. D'Orléans-Juste et al. [112] showed that BK is able to abolish the contraction of the dog carotid artery induced by noradrenaline. These authors suggest that the response of the dog carotid artery to kinins is therefore exclusively dependent on the activation of endothelial B_2 receptors. Regoli et al. [113] confirmed the presence of B_2 receptors in the endothelium of the dog carotid artery. Bergaya et al. [97] verified that the vascular relaxation induced by BK in the murine carotid involves the activation of endothelial B_2 receptors.

BK-induced relaxation involves a number of endothelium-derived relaxing factors, such as NO and vasorelaxant prostanoids. Inhibition of NO synthesis by L-NAME abolishes the vasodilator effect induced by BK in the rat carotid [22]. The vasorelaxation induced by BK in this artery involves NOS activation, in particular constitutive NOS, endothelial (eNOS) and neuronal NOS [85]. Gambillara et al. [114] showed that BK-induced vasorelaxation is completely abrogated by the concomitant use of L-NAME, a non-selective NOS inhibitor in pig carotid ar-

teries. These authors suggest that decreased BK-induced vasorelaxation is related with the down-regulation of eNOS gene expression and impairment of eNOS enzyme activation. Cherry et al. [115] verified that BK modulates vascular smooth muscle tone by stimulating endothelial B_2 receptors and releasing NO and other mediators. Using endothelial cell cultures, Wiemer et al. [116] verified that kinins activate B_2 receptors and stimulate the release of NO. In the mouse carotid, Bergaya et al. [97] showed the inhibitory effect of L-NAME in the dilation induced by BK and suggested that this effect is mediated by the release of endothelial NO. Regoli et al. [113] verified that the endothelium promotes the production of NO, which diffuses into the smooth muscle and induces relaxation by activation of guanylate cyclase and production of cGMP which mediates relaxation of the vascular smooth muscle.

By using animal and human arteries, several studies demonstrated that BK-induced hypotension involves a number of relaxing factors, such as NO and vasorelaxant prostanoids, which are released from endothelial cells [105, 117]. In the rat carotid, indomethacin surprisingly enhances the relaxation induced by BK, suggesting that a vasoconstrictor prostanoid(s) counteracts BK-induced relaxation [85]. In the process of analysing any possible prostanoid(s) involved in this process, we found that SQ29548, a competitive antagonist of PGH_2/TxA_2 receptors, or AH6809, an antagonist of $PGF_{2\alpha}$ receptors, altered the relaxant response induced by BK. Interestingly, the combination of SQ29548 and AH6809 induced a significant increase in BK-induced relaxation. Based on these observations, it is possible to conclude that vasoconstrictor prostanoids, such as $PGF_{2\alpha}$, PGH_2 and TxA_2 , could have an inhibitory action on BK-induced relaxation by counteracting the vasorelaxant action displayed by this peptide. Vasoconstrictor prostanoids were also found to counteract the vasodilator response to BK on renal vascular resistance vessels from cats [118].

B_1 receptors are not normally functional, and this only becomes evident after certain types of tissue injury, such as the injection of bacterial materials or under pathological conditions [119]. In some vascular tissues, B_1 receptors were described to mediate contraction [41, 119]. In the rat carotid, B_1 receptors are weakly expressed constitutively [67]. However, there are no data describing the role of these receptors in the control of vascular tonus in the rat carotid. Whether B_1 receptors alter the contraction or relaxation of the rat carotid remains to be determined.

Interplay between Peptide Systems

Several studies support the existence of cross-talk between different peptidergic systems. Emerging experimental evidence suggests interplay between the Ang II and ET-1 endocrine/paracrine systems. Ang II and ET-1 interplay has been extensively studied due to its relevance in the physiological regulation of vascular tone and blood pressure, as well as in hypertension and vascular remodeling. Ang II potentiates the production of ET-1, and conversely ET-1 augments the synthesis of Ang II. Similarly, Ang II was described to increase prepro-ET-1 mRNA expression in endothelial cells [120] and to stimulate the release of ET-1 from endothelial cells, a response that involves the activation of AT₁ receptors [121]. Infusion of Ang II increases rat aortic ET-1 content, and this response is blocked by losartan, an antagonist of the AT₁ receptor [122]. Conversely, ET-1 stimulates the conversion of Ang I to Ang II in pulmonary endothelial cells [123]. Moreover, BQ123, a selective ET_A receptor antagonist, shifts the Ang-II-induced contraction response curves of rabbit aortas to the right [124], and ET_A receptor antagonism inhibits vasoconstriction induced by Ang II [125]. The interaction between Ang II and ET-1 is an important process since human essential hypertension involves the participation of these two powerful and well-characterised vasoconstrictors. Clinical trials with ACE inhibitors and AT₁ receptor blockers have demonstrated improved morbidity and mortality in hypertension, congestive cardiac failure and myocardial infarction, and support the significance of Ang II in the pathogenesis of cardiovascular disease. Because Ang II and ET-1 similarly cause hypertension and copromote the other's actions, it is plausible that concomitant blockade of both Ang II and ET-1 endocrine/paracrine pathways may lead to enhanced blood pressure reduction. Thus, the understanding of the interplay between Ang II and ET-1 opens new perspectives in the concomitant use of antagonists for both peptides in the treatment of cardiovascular disorders, such as hypertension. Based on the studies discussed above, it is likely that the cross-talk between these two systems occurs in different types of blood vessels. However, whether this interplay takes place in the rat carotid remains elusive.

Conclusions

The study of the physiological expression and function of peptidergic receptors may provide valuable information in the understanding of the arterial response to

vaso-active agents. In the rat carotid, a locally generated ET-1 system is utilised since several components of the endothelinergic system such as prepro-ET-1 and ECE are present in the wall of this artery. The vascular actions of this peptide are mediated by contractile ET_A and ET_B receptors located in the smooth muscle and endothelial ET_B receptors that induce relaxation via NO, vasodilator prostanoid and the opening of K⁺ channels. The rat carotid also generates Ang II locally through ACE-dependent and -independent pathways, the latter involving elastase 2. AT₁ and AT₂ receptors are expressed in the rat carotid, and Ang II likely activates the AT₁ receptor to induce contraction. Finally, BK induces relaxation of the rat carotid via B₂ receptors located in the endothelium, a response that involves the activation of the NO pathway. These vaso-active peptides play fundamental roles in controlling the functional and structural integrity of the arterial wall and may be important in physiological processes. Moreover, ET-1 and Ang II influence arterial tone and remodelling in some vascular diseases by stimulating vascular smooth muscle cell contraction, augmenting cell growth, increasing deposition of the extracellular matrix, inducing cell migration and promoting inflammation. This further illustrates the important role of these peptides in pathological mechanisms underlying vascular diseases. For this reason, the function of peptidergic receptors in blood vessels could have important implications for the design of antagonists for use in vascular diseases in which these peptides are implicated. With pharmacological and molecular tools that allow understanding of specific signal transduction pathways, identification of physiological pathways or distinct abnormalities in intracellular signalling in vascular diseases should be possible.

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