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Review Article

Angiotensin-(1–7): beyond the cardio-renal actions

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Abstract

It is well known that the RAS (renin–angiotensin system) plays a key role in the modulation of many functions in the body. AngII (angiotensin II) acting on AT_1R (type 1 AngII receptor) has a central role in mediating most of the actions of the RAS. However, over the past 10 years, several studies have presented evidence for the existence of a new arm of the RAS, namely the ACE (angiotensin-converting enzyme) 2/Ang-(1–7) [angiotensin-(1–7)]/Mas axis. Ang-(1–7) can be produced from AngI or AngII via endo- or carboxy-peptidases respectively. ACE2 appears to play a central role in Ang-(1–7) formation. As described for AngII, Ang-(1–7) also has a broad range of effects in different organs and tissues which goes beyond its initially described cardiovascular and renal actions. Those effects are mediated by Mas and can counter-regulate most of the deleterious effects of AngII. The interaction Ang-(1–7)/Mas regulates different signalling pathways, such as PI3K (phosphoinositide 3-kinase)/AKT and ERK (extracellular-signal-regulated kinase) pathways and involves downstream effectors such as NO, FOXO1 (forkhead box O1) and COX-2 (cyclo-oxygenase-2). Through these mechanisms, Ang-(1–7) is able to improve pathological conditions including fibrosis and inflammation in organs such as lungs, liver and kidney. In addition, this heptapeptide has positive effects on metabolism, increasing the glucose uptake and lipolysis while decreasing insulin resistance and dyslipidaemia. Ang-(1–7) is also able to improve cerebroprotection against ischaemic stroke, besides its effects on learning and memory. The reproductive system can also be affected by Ang-(1–7) treatment, with enhanced ovulation, spermatogenesis and sexual steroids synthesis. Finally, Ang-(1–7) is considered a potential anti-cancer treatment since it is able to inhibit cell proliferation and angiogenesis. Thus the ACE2/Ang-(1–7)/Mas pathway seems to be involved in many physiological and pathophysiological processes in several systems and organs especially by opposing the detrimental effects of inappropriate overactivation of the ACE/AngII/AT_1R axis.

Key words: angiotensin-(1–7), angiotensin-converting enayme 2 (ACE2), Mas receptor, renin–angiotensin system (RAS)

INTRODUCTION

The textbook-like view of the RAS (renin–angiotensin system) as a linear limited proteolysis pathway toward the production of a single active end product, AngII (angiotensin II), has changed dramatically over the past 10 years with the identification of novel components [ACE (angiotensin-converting enzyme) 2, Mas and renin/pro-renin receptor] and actions of this system [1–6]. A major shift in our understanding of the RAS was the proposition of the concept of the RAS as a dual axis system: one axis represented by ACE/AngII/AT_1R (type 1 AngII receptor) and the other by ACE2/Ang-(1–7) [angiotensin-(1–7)]/Mas [7,8]. The identification of the renin/pro-renin receptor [4] and its physiological actions, which go beyond its role in the RAS [9], and more recently of the other ACE2 product Ang-(1–9) [angiotensin-(1–9)] as a biologically active member of the RAS [10,11], suggest that we are still far from understanding the complexity of this fascinating system.

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; AIA, antigen-induced arthritis; Ang-(1–7), angiotensin-(1–7); Ang-(1–9), angiotensin-(1–9); AngI etc., angiotensin I etc.; AP2, adipose lipid-binding protein 2; ApoE, apolipoprotein E; AT_1R, type 1 AngII receptor; AT_2R, type 2 AngII receptor; BDL, bile-duct ligation; COX, cyclo-oxygenase; CTGF, connective tissue growth factor; ERK, extracellular-signal-regulated kinase; FOXO1, forkhead box O1; GnRH, gonadotropin-releasing hormone; HAEC, human aortic endothelial cell; HDAC1, histone deacetylase 1; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MMP, metalloproteinase; mTOR, mammalian target of rapamycin; NEFA, non-esterified 'free' fatty acid; NEP, neutral endopeptidase; NF-κB, nuclear factor κB; NOS, NO synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; PEP, prolyl-endopeptidase; PI3K, phosphoinositide 3-kinase; PI3KC2A, class II PI3Kα; PlGF, placental growth factor; PPARγ, peroxisome-proliferator-activated receptor γ; PRAS40, proline-rich Akt substrate of 40 kDa; RAS, renin–angiotensin system; RASIP1, Ras-interacting protein 1; TGF, transforming growth factor; TGR, transgenic; TNF, tumour necrosis factor; TOP, thimet oligopeptidase; VEGF, vascular endothelial growth factor.

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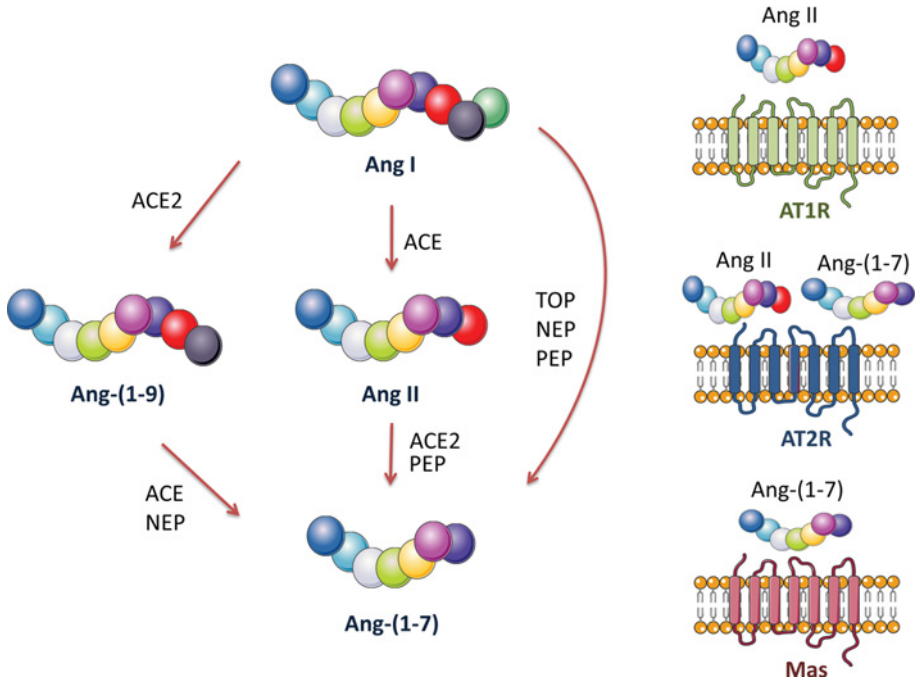

Figure 1 **Simplified view of the different pathways of Ang-(1–7) formation and the receptors for Ang-(1–7) and AngII**

The starting point in the canonical and non-canonical enzymatic pathways of the RAS is the glycoprotein AGT (angiotensinogen), which is produced and secreted into the circulation by the liver. Most organs, including the brain, vasculature, kidney, heart, and lungs, can also produce AGT. The limited proteolysis cascade starts with renin, which plays a central role in the formation of the inactive decapeptide AngI by cleavage of AGT. AngI (angiotensin I) can be cleaved by ACE or chymase to form the octapeptide AngII, and by many other peptidases, especially PEP (prolyl-endopeptidase), NEP (neutral endopeptidase) and TOP (thymet oligopeptidase), which can generate Ang-(1–7) by the cleavage of its Pro^{7}-Phe^{8} bond. Ang-(1–7) can also be formed by hydrolysis of AngII via ACE2 [12]. Furthermore, this heptapeptide can be generated via the hydrolysis of AngI by ACE2 to form Ang-(1–9), which is subsequently cleaved by NEP or ACE [13]. However, it is important to stress that this pathway seems to be catalytically less efficient than the ones mentioned above [13]. A simplified view of the enzymatic pathways involved in the formation of Ang-(1–7) is shown in Figure 1.

Accumulating evidence suggests that a balance between the activation of the ACE/AngII/AT_1R axis and the ACE2/Ang-(1–7)/Mas receptor axis plays an important role in the function of different organs and systems and that an imbalance in these opposing pathways toward the ACE/AngII/AT_1R axis predispose to cardiovascular diseases and other disorders. Several recent reviews have focused on the cardiovascular and renal actions of the novel axis of the RAS and its implication in cardiovascular and renal diseases [6,14–16]. However, this axis has pleiotropic effects going far beyond its cardio-renal and vascular actions (Figure 2). In the present paper, we aim to briefly review some recent aspects related to the non-classical actions of ACE2/Ang-(1–7)/Mas, especially Ang-(1–7).

GLUCOSE AND LIPID METABOLISM

Both, the pancreas [17,18] and adipose tissue [19], contain RAS components and this local RAS is highly regulated by food intake. Although a high-sugar diet increases the expression levels of AGT, ACE and AT_1R in the pancreas [20], a diet rich in fat or sugar increases the concentration of ACE2 and Ang-(1–7) in adipose tissue [21,22]. The RAS is currently being considered as a potential target for the treatment of the metabolic syndrome, a disorder characterized by the association of obesity, insulin resistance, hypertension, diabetes, hyperinsulinaemia and dyslipidaemia [23,24].

The observation that insulin resistance is frequently associated with cardiovascular impairment has drawn the attention of the scientific community to the possible interplay between the RAS and insulin. The possibility of such a relationship was prompted by clinical trials [25–28] and experimental studies [20,29–35] in which the overall observation was an improvement in hyperglycaemia either by inhibiting the ACE/AngII/AT_1R axis or by activating the ACE2/Ang-(1–7)/Mas axis. These observations also suggest that, together with bradykinin [36,37], Ang-(1–7) could have an important anti-hyperglycaemic effect, whereas AngII acts in the opposite way. Indeed, there are a number of publications reporting that Ang-(1–7) prevents the metabolic syndrome, increases glucose uptake and protects cells against the oxidative stress that can induce insulin resistance [31,34,35] (Figure 2B).

The events behind the positive regulation of insulin by Ang-(1–7) are now being revealed. Ang-(1–7) and insulin have some common downstream signalling effectors in HAECs (human aortic endothelial cells) [38] and in the rat heart [39] (Figure 2A). By using Western-blot-based techniques to interrogate Ang-(1–7) signalling, it has been reported that Ang-(1–7) phosphorylates the

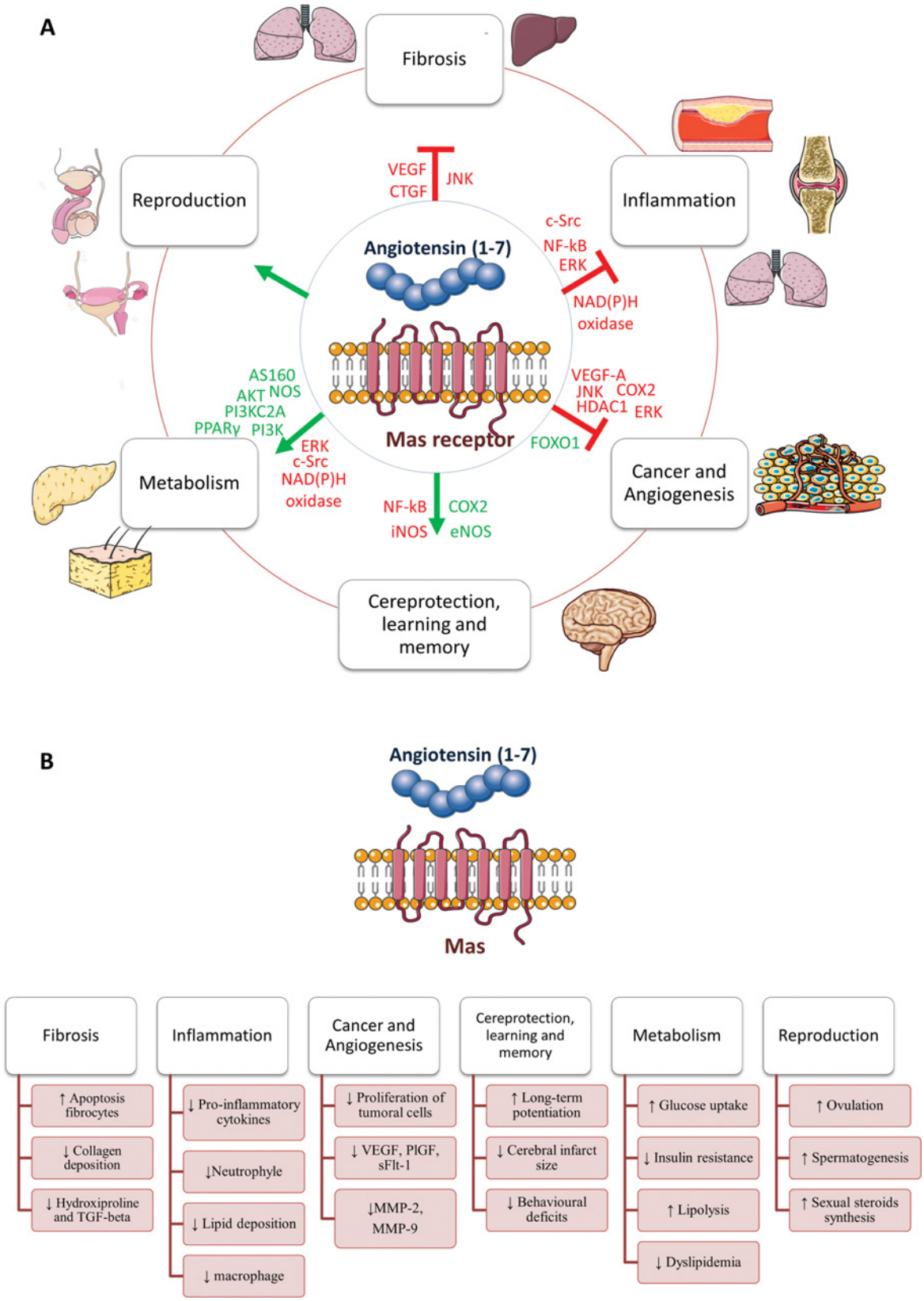

Figure 2 **Main non-cardiovascular effects of Ang-(1–7) via Mas in different tissues and processes**

(**A**) Signalling molecules involved in the action of Ang-(1–7). The molecules in green and red indicate activation or inhibition by Ang-(1–7)/Mas respectively. The green arrows indicate activation or positive effects, and the red lines indicate inhibition of the process. (**B**) The main non-cardiovascular consequences of Ang-(1–7) on each process.

insulin downstream effectors PI3K (phosphoinositide 3-kinase) and AKT via the Mas receptor in HAECs [38,39], and IRS1 (insulin receptor substrate 1) and JAK2 (Janus kinase 2) via the AT_1R in rat hearts [39]. Moreover, Ang-(1–7)/Mas negatively regulates AngII/AT_1R signalling in HAECs by promoting dephosphorylation of c-Src and ERK (extracellular-signal-regulated kinase) 1/2, and inhibition of NADPH oxidase activity [40]. Recently, Muñoz and co-workers [41] used FFRs (fructose-fed rats), a model of the metabolic syndrome, to study whether Ang-(1–7) positively modulates insulin signalling via Mas and attenuates the inhibitory effect of AngII on this signal transduction pathway. Indeed, the authors observed that, in a Mas-dependent manner, the downstream effectors of insulin including AKT, GSK-3β (glycogen synthase kinase-3β) and AS160 (AKT substrate of 160 kDa) were positively modulated by Ang-(1–7) in insulin-target tissues (skeletal muscle, liver and adipose tissue). In the same way, the inhibitory effect of AngII on these effectors was also attenuated by Ang-(1–7) through Mas [41].

Western-blot-based studies have several advantages including high sensitivity and specificity. However, one needs to rely on commercially available specific antibodies with proven quality. Moreover, this type of technique is hypothesis-driven. In other words, the downstream effectors of a specific signalling cascade need to have been defined previously. Therefore it is not possible to identify unknown targets of signalling networks by Western blotting. In order to overcome these technical limitations and to gain more insights into the Mas signalling pathway, we recently applied an MS-based approach to study the HAEC phosphoproteome treated with Ang-(1–7) [42]. In that study, we were able to detect 79 phosphoproteins that had their phosphorylation state significantly changed over the observed time frame (0–20 min). Among these phosphoproteins, eight known downstream effectors of the insulin signal transduction cascade had their phosphorylation levels differentially regulated by Ang-(1–7): AKT1 (RAC-α serine/threonine protein kinase), PRAS40/AKT1S1 (proline-rich Akt substrate of 40 kDa/proline-rich AKT1 substrate 1), CAV1 (caveolin-1 isoform α), FOXO1 (forkhead box O1), MAPK1 (mitogen-activated protein kinase 1)/ERK2, PXN (paxillin isoform 1), PI3KC2A (class II PI3Kα) and VIM (vimentin) [42]. Ser^{124} on the AKT1 was phosphorylated after 3 min of Ang-(1–7) treatment. The phosphorylation of this residue, together with the phosphorylation of Thr^{308} and Ser^{473}, activates this kinase [43,44]. This finding is in agreement with previous publications reporting that AKT is rapidly activated by Ang-(1–7) [38,39,45]. The class I PI3K plays an important role in the Ang-(1–7) signalling and it is an upstream kinase that mediates AKT phosphorylation in HAECs and rat hearts [38,39]. However, our phosphoproteome study revealed that PI3KC2A also plays a role in this signal transduction cascade, as Mas activation induced the phosphorylation of Ser^{338} on this kinase [42]. Diverging from class I PI3K, PI3KC2A is not an upstream kinase of the AKT signalling branch [46,47]. Interestingly, this kinase induces translocation of GLUT4 (glucose transporter 4) to the plasma membrane in response to insulin stimulation [47]. PRAS40 regulates insulin-induced mTOR (mammalian target of rapamycin) activity. When Thr^{246} on PRAS40 is dephosphorylated, it binds to the mTOR complex to inhibit it. On the other hand, AKT1 can phosphorylate PRAS40 to induce its dissociation from mTOR, activating this complex [48,49]. The transcriptional factor FOXO1 is another component of the insulin signalling pathway that is a downstream target of AKT1 [50]. Once activated, AKT1 translocates to the nucleus and phosphorylates FOXO1 at Thr^{24}, Ser^{256} and Ser^{319}, inactivating this transcriptional factor [50]. Strikingly, Ang-(1–7) induced the dephosphorylation of Thr^{246} on PRAS40 and Ser^{256} on FOXO1. These data are consistent with mTOR inactivation and FOXO1 activation. Indeed, we have also demonstrated that Ang-(1–7) induces FOXO1 activation with its translocation to the nucleus [42]. These findings suggest that Ang-(1–7) signalling is a highly controlled system, possibly with more regulatory inputs than previously suspected. Concerning the AKT/FOXO relationship, the possibility that Ang-(1–7) modulates the equilibrium between FOXO1 activation, which is predominant during the fasting period, and AKT activation, which predominates after feeding [51], should be explored in future studies (Figure 3).

Lipid metabolism is also regulated by Ang-(1–7) (Figure 2). When treated with this heptapeptide, rats with diabetic cardiomyopathy [32] and diabetic nephropathy [52] have a significant reduction in dyslipidaemia in a Mas-dependent way (Figure 2b). Moreover, Mas-knockout mice on the FVB/N background have impaired lipid metabolism, leading to dyslipidaemia, lower glucose tolerance and insulin sensitivity, hyperinsulinaemia, hyperleptinaemia, lower adiponectin secretion, decreased glucose uptake and increased abdominal fat mass when compared with the wild-type phenotype [34]. On the other hand, TGR (transgenic) animals with increased plasma levels of Ang-(1–7) had reduced fat mass, decreased triacylglycerols (triglycerides) and cholesterol levels, despite normal food intake [53]. In addition, the expression levels of adiponectin and AP2 (adipose lipid-binding protein 2) were increased, whereas there was a remarkable decrease in AGT expression in TGR animals. Adiponectin is a key adipokine that regulates insulin sensitivity and tissue inflammation, and its plasma level is inversely proportional to body fat content. AP2 is an important protein in the adipose tissue metabolism involved in fatty acid esterification [53]. In the same way, ACE-knockout mice had reduced fat mass due to an increase in lipid metabolism and energy expenditure as a consequence of higher expression levels of key genes involved in the hydrolysis of lipids into NEFAs (non-esterified 'free' fatty acids) [LPL (lipoprotein lipase)], translocation of fatty acids to the mitochondria [CPT-1 (carnitine palmitoyltransferase-1)], and β-oxidation inside mitochondria and peroxisomes [LCAD (long-chain acyl-CoA dehydrogenase)] [54].

Recently, two groups have shed more light on the molecular mechanisms behind the beneficial modulation of lipid metabolism by Ang-(1–7). Mario et al. [55] demonstrated that the expression of the PPARγ (peroxisome-proliferator-activated receptor γ) is compromised in $Mas^{-/-}$ mice (Figure 2A). The transcription factor PPARγ is believed to have a beneficial effect in insulin-resistant patients, as its activation leads to the expression of target genes involved in fatty acid metabolism, triacylglycerol storage and reduction in plasma NEFA supply [56,57]. In the same way, Oh et al. [58] reported that Ang-(1–7) stimulates lipolysis via Mas. Interestingly, these authors observed that an AKT inhibitor

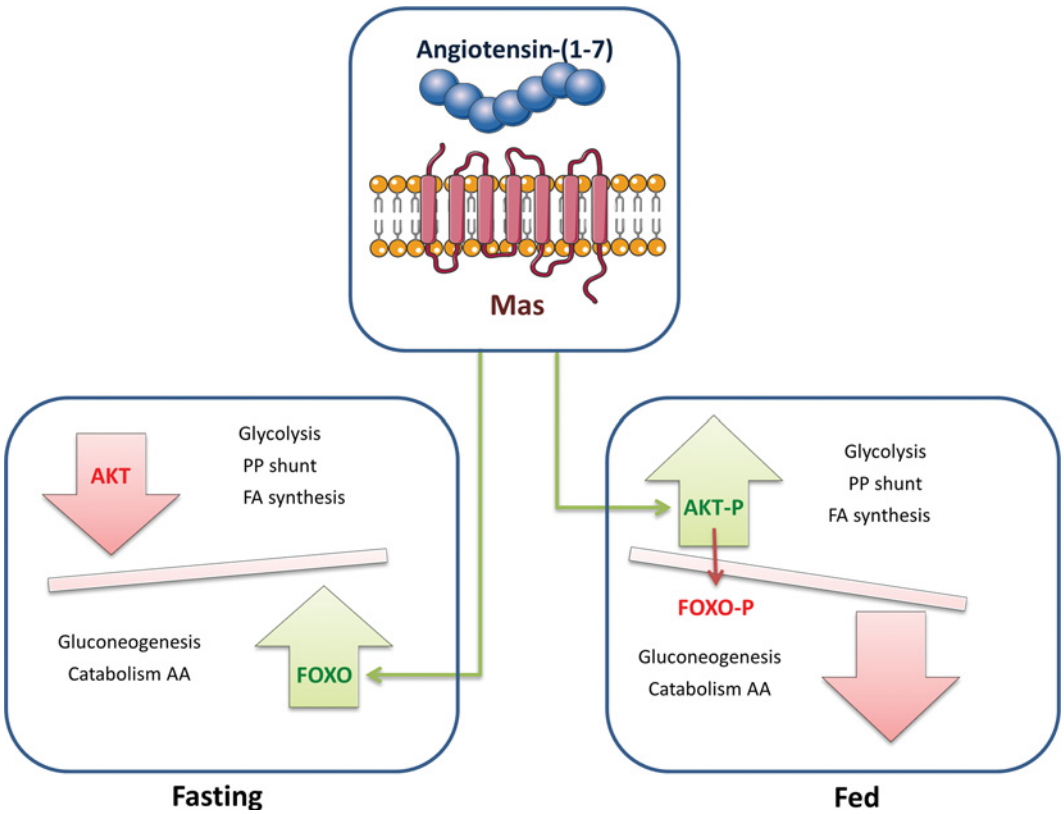

Figure 3 **Putative mechanism by which Ang-(1–7) could modulate the balance of FOXO1/AKT in the fasting/feeding cycle**

In the fasting period, the inhibitory effect of AKT on FOXO1 is decreased as a consequence of the low insulin levels, which leads to a reduction in the AKT phosphorylation (AKT-P) state. After feeding, insulin levels increase Akt phosphorylation, which then phosphorylates Ser^{256} on FOXO1 (FOXO-P), preventing its translocation to the nucleus. Ang-(1–7) could amplify/modulate both phases due to its ability to induce the dephosphorylation of FOXO1 at Ser^{256}, as well as AKT phosphorylation via a PI3K-dependent pathway. Activation is represented in green and inhibition in red. AA, amino acids; FA, fatty acid; PP, pentose pathway.

[API-2 (Akt/protein kinase B signalling inhibitor-2)] did not attenuate Ang-(1–7)-induced lipolysis, whereas the inhibitor of PI3K (wortmannin) and eNOS [endothelial NOS (NO synthase)] [L-NAME (N^G-nitro-L-arginine methyl ester)] significantly reduced Ang-(1–7)-induced lipolysis. These findings suggest that Ang-(1–7)-induced lipolysis is via a Mas/PI3K/eNOS signalling pathway in an AKT-independent manner [58].

CELLULAR PROLIFERATION AND ANGIOGENESIS

Studies *in vivo* and *in vitro* have suggested that Ang-(1–7) has both anti-proliferative and anti-angiogenic properties, conferring a potential alternative to cancer treatment (Figure 2B). The growth of lung cancer cells, such as SK-LU-1, A549 and SK-MES-1 cells, as well as human lung tumour xenografts, was inhibited by treatment with Ang-(1–7) [59]. This phenotype was explained, at least in part, as a consequence of the inhibition of the ERK (MAPK) signal transduction pathway [59] or reduction in COX (cyclo-oxygenase)-2 [60] (Figure 2A), as both have a central role in lung cancer cell proliferation. Treatment with Ang-(1–7) also reduced the volume of orthotopic human breast tumours, probably by decreasing the growth of cancer associated fibroblasts and fibrosis [61]. This later study also showed an increase in the MAPK phosphatase DUSP1 (dual-specificity phosphatase 1), reinforcing a possible role of Ang-(1–7) in the reduction of MAPK/ERK signalling [61]. Indeed, our group has recently shown that HAEC MAPK1 is dephosphorylated following Ang-(1–7) treatment, which probably leads to MAPK1 inactivation [42].

HDAC1 (histone deacetylase 1) is one of the proteins that has a long-lasting inactivation through dephosphorylation of its regulatory sites (Ser^{421} and Ser^{423}) after Ang-(1–7) treatment [42]. This protein is overexpressed in solid tumours and it has a role in tumorigenesis and cancer progression. On the other hand, Ang-(1–7) is also able to induce activation of the transcriptional factor FOXO1 through dephosphorylation of its regulatory site (Ser^{256}) and consequently its translocation to the nucleus of endothelial and tumour cells [42]. FOXO1 is responsible for triggering the activation of genes involved in apoptosis, cell-cycle arrest and oxidative stress resistance [62]. Recently, it is also been suggested that FOXO1 is a critical negative regulator of endothelial

angiogenic behaviour [63], indicating a possible pathway by which Ang-(1–7) can inhibit angiogenesis and, overall, tumorigenesis. RASIP1 (Ras-interacting protein 1) also seems to be a downstream effector of Ang-(1–7), as it was phosphorylated on Ser^{331} after 3 min of Ang-(1–7) treatment [42]. RASIP1 acts in blood vessel morphogenesis; however, it is yet to be established whether this phosphorylation modulates the activity of this protein [64]. The role of Ang-(1–7) in angiogenesis inhibition was shown in a mouse sponge model of angiogenesis [65]. Daily injections of Ang-(1–7) significantly inhibited angiogenesis, probably through interaction with Mas, since its antagonist A779 blocked this effect [65]. On the other hand, the AT_1R and AT_2R (type 2 AngII receptor) do not appear to be involved in this mechanism, since their antagonists did not affect the Ang-(1–7) effect [65]. The mechanism of the anti-angiogenic effect of Ang-(1–7) in mice is related to NO release [65]. Anton et al. [66] also showed *in vitro* inhibition of angiogenesis by Ang-(1–7) in the tube formation assay using endothelial cells. This inhibition was also abolished by A779 [66]. Similarly, tube formation of A549 tumour cells was also inhibited after Ang-(1–7) treatment [67]. A reduction in vessel density in Ang-(1–7)-treated human A549 lung tumours was also observed. The mechanism of the anti-angiogenic property of Ang-(1–7) in this condition seems to involve VEGF (vascular endothelial growth factor)-A, a primary pro-angiogenic protein, which was reduced after treatment with this heptapeptide [67]. In addition, Ang-(1–7) decreased proliferation markers and intratumoral vessel densities of a xenograft prostate tumour in mice [68]. The treatment also reduced angiogenic factors such VEGF and PlGF (placental growth factor), and increased the soluble fraction of VEGF receptor 1 (sFlt-1), preventing the triggering of the angiogenesis signalling pathway [68]. Data from *in vivo* and *in vitro* studies are in line with the results obtained in patients with advanced cancer that were treated with Ang-(1–7) in Phase I clinical trials [69]. Patients treated with Ang-(1–7) had reduced circulating levels of PIGF, a placental growth factor involved in angiogenesis. In this trial, treatment with Ang-(1–7) led to disease stabilization [69]. Finally, recent results have shown that Ang-(1–7) treatment also has anti-metastatic properties in lung and prostate cancer cells [70]. Ang-(1–7)-treated A549 human lung adenocarcinoma cells reduced the expression and activity of the MMPs (metalloproteinases) MMP-2 and MMP-9, which are involved in migration and invasion of cells. The mechanism for this effect includes the inhibition of PI3K/AKT, p38 and JNK (c-Jun N-terminal kianse) signalling pathways [71] (Figure 2A). Therefore separate studies indicate that Ang-(1–7) is a potential candidate for cancer treatment as it presents both anti-proliferative and anti-angiogenic properties.

REPRODUCTION

Several tissues of the reproductive system express a local RAS. Besides its vascular actions, the local RAS plays an important role during spermatogenesis, follicle maturation and ovulation, endometrium function and pregnancy [72–80] (Figure 2).

In female mammals, the ACE2/Ang-(1–7)/Mas axis has been detected in the ovary, endometrium and placenta [72–76]. The ACE2/Ang-(1–7)/Mas axis is highly regulated by GnRH (gonadotropin-releasing hormone) in the rat ovary [72]. This hormone induces the expression of ACE2, leading to increased levels of Ang-(1–7). Furthermore, GnRH positively regulates the expression of Mas [72]. In the same way, the ACE2/Ang-(1–7)/Mas axis also seems to play a role in the ovulation process in cattle. Following 24 h of GnRH stimulation, Ang-(1–7) levels increase in the follicular fluid, probably due to ACE2, NEP and PEP up-regulation in granulosa cells [81]. In addition, Ang-(1–7) increases during pro-oestrus and oestrus, and after eCG (equine chorionic gonadotropin) treatment [76,82]. Furthermore, perfusion of Ang-(1–7) in immature rat ovaries leads to increasing progesterone and oestradiol synthesis in a Mas-dependent manner [76]. The evidence for a role of the ACE2/Ang-(1–7)/Mas axis in the female reproductive system was recently reinforced by the observation that LH (luteinizing hormone) up-regulates the ACE2/Ang-(1–7)/Mas axis and that Ang-(1–7) promotes meiotic resumption, possibly as a gonadotrophin intermediate [83]. Therefore there is substantial evidence suggesting an important role for the ACE2/Ang-(1–7)/Mas axis in the ovulation process.

The ACE2/Ang-(1–7)/Mas axis is also found in the uterus. Although the expression of ACE2 is constant during the menstrual cycle, the levels of Ang-(1–7) and Mas in the glandular endometrium increase during the mid- and late secretory phase [74]. This local RAS may be regulated by sex steroids as increased expression of local renin following progesterone stimulation has been reported previously [77]. Recently, Brosnihan et al. [84] reported that Ang-(1–7) is present in the luminal and glandular epithelial cells of pseudopregnant ovariectomized rats submitted to hormonal treatment. Ang-(1–7) levels were found to be reduced in the decidualized horn when compared with the non-decidualized horn, and was irregular in the luminal epithelium, although persistent in the glandular epithelium [84]. In another study using ovariectomized rats, Vaz-Silva et al. [85] also evaluated the tissue distribution of Ang-(1–7) in the uterus, but compared hormone-treated animals with non-treated ones. The authors observed that Ang-(1–7) levels were significantly decreased in the glandular epithelium and the circular myometrium of hormonally treated animals, suggesting that Ang-(1–7) production is negatively modulated by steroids in the glandular compartment. It is important to mention that Ang-(1–7) levels did not change in the luminal epithelium, endometrial stroma and serosa following the hormonal treatment [85]. In the same way, Mas expression did not change in all uterine tissues of hormone-treated animals [85]. Neves et al. [86] studied the uterine tissue distribution of Ang-(1–7) and ACE2 during gestation in Sprague–Dawley rats. During early pregnancy, Ang-(1–7) and ACE2 were found in the luminal and glandular epithelial cells and in the primary and secondary decidual zone, whereas, during late gestation, these RAS components were detected in the labyrinth placenta and amniotic and yolk sac epithelium [86].

The placenta has a pivotal role in the reproductive tract as it allows nutrients uptake and waste elimination by the fetus via the mother circulation, as well as gas exchange. The local RAS plays an important role in the placentation, leading to angiogenesis and

embryo development by modulating trophoblast proliferation and invasion [75,79,80]. In the human placenta, ACE2 is markedly up-regulated during early gestation (6–16 weeks) when compared with term gestation (37–41 weeks), whereas ACE is differentially expressed in the term placenta. Moreover, the localization of these isoforms in the early gestation placentae is also divergent; while ACE is only found in the fetal endothelium of the placental villi, ACE2 is predominantly detected in the syncytiotrophoblast and villous stroma [75]. Decreased Mas expression in the placenta has been observed in pre-eclampsia, a condition in which the plasma concentration of Ang-(1–7) is also lower than that present in normotensive pregnant women [87,88].

The male human tract and the testis express all components of the classical RAS [89,90]. Regarding the ACE2/Ang-(1–7)/Mas axis, its components have been detected in the testis of rats [91], mice [92], and humans [93]. In humans, Ang-(1–7) and Mas are mainly found in the interstitial compartment and in the cytoplasm of the Leydig cells, and are also detected with less intensity in the seminiferous tubules [93]. As the Leydig cells are involved in the synthesis of sex steroid hormones (e.g. testosterone), the presence of Ang-(1–7) and Mas indicates that the ACE2/Ang-(1–7)/Mas axis may modulate the production of testosterone by the human testis. Even though it has been reported that Mas-deficient mice remain fertile [94], Leal et al. [78] found that $Mas^{-/-}$ mice exhibited a significant reduction in testis weight, although the total number of Sertoli and Leydig cells were comparable in both wild-type and knockout animals. Moreover, in $Mas^{-/-}$ animals, the authors observed a significant number of apoptotic cells during meiosis, giant cells and vacuoles in the seminiferous epithelium, and a striking reduction in daily sperm production due to disturbed spermatogenesis [78]. Accordingly, humans with severe spermatogenesis impairment have lower levels of ACE2, Ang-(1–7) and Mas when compared with fertile subjects [93]. Taken together, these data clearly indicate that the ACE2/Ang-(1–7)/Mas axis modulates spermatogenesis.

FIBROSIS

Growing evidence suggests a protective role of Ang-(1–7) against fibrosis. This pathological condition is characterized by the excessive deposition of extracellular matrix such as collagen, by fibroblasts [95]. The proliferation of circulating fibrocytes, precursors of mature fibroblasts, is described to be involved in the progression of tissue fibrosis and can be a predictor of myocardial fibrosis [96]. Recently, a study showed that Ang-(1–7) causes apoptosis of these cells, inhibits proliferation and diminishes the secretion of collagen, leading to a regression of the cardiac fibrosis [97]. Indeed, Ang-(1–7) protects against myocardial fibrosis, counter-regulating the effects of AngII, as has been shown by different studies reviewed recently by Oudit and Penninger [98]. Moreover, the deletion of its receptor Mas in mice resulted in a pro-fibrotic profile of the extracellular matrix proteins [99], whereas rhACE2 (recombinant human ACE2) administration inhibited the development of cardiac fibrosis, lowering the levels of AngII and increasing Ang-(1–7) [100].

Besides its cardiac function, Ang-(1–7) can also reduce fibrosis in different organs, such as lung, kidney and liver (Figure 2). Indeed, a study using an experimental model of liver fibrosis, the BDL (bile-duct ligation) rat model, showed that Ang-(1–7) administration improved different aspects of fibrosis, such as reduction in collagen and hydroxyproline content and diminishment of gene expression of collagen IA1, α-SMA (smooth muscle actin), VEGF and CTGF (connective tissue growth factor) [101] (Figure 2A). A different study using the same model demonstrated that incubation with the Mas antagonist A779 increased the collagen deposition in the liver of BDL rats [102]. Other factors involved in fibrosis such as TGF (transforming growth factor)-β1 and hydroxyproline were also enhanced after A779 treatment [102]. In addition, genetic deletion of ACE2 worsened fibrosis in a chronic liver injury model, as demonstrated by increased fibrosis markers such as collagen and TNF (tumour necrosis factor)-α [103]. On the other hand, the administration of recombinant ACE2 attenuated fibrosis in mice [103]. In addition, data obtained in a rat fibrosis model showed elevated ACE2 activity in chronically injured liver and consequently enhanced Ang-(1–7) plasma levels [104,105]. In agreement, the levels of circulating Ang-(1–7) are also increased in human liver diseases, such as hepatitis C [104].

In order to demonstrate a possible action of Ang-(1–7) in pulmonary fibrosis, studies used rats treated with bleomycin to induce the development of fibrosis in the lung. Using this model, it was demonstrated that overexpression of an Ang-(1–7)-producing fusion protein or ACE2, using lentivirus-packaged Ang-(1–7) administration into lungs of rats, decreased collagen content and pro-inflammatory cytokines [106]. In addition, Mas blockade with A779 abolished this effect, indicating that Mas mediates this action [106]. Furthermore, pre-incubation with Ang-(1–7) prevents the AngII- or bleomycin-induced apoptosis of alveolar epithelial cells, a distinct feature of pulmonary fibrosis, as measured by caspase 3 and 9 activation and nuclear fragmentation [107]. The anti-apoptotic effect of Ang-(1–7) in these cells involved inhibition of JNK phosphorylation [107] (Figure 2A). These observations suggest activation of the ACE2/Ang-(1–7)/Mas axis as a potential strategy for antifibrotic therapy.

INFLAMMATION

The classical RAS component AngII via the AT_1R contributes to the inflammatory process, increasing the expression of pro-inflammatory cytokines, chemokines and cell adhesion molecules [108–111]. In contrast, recent studies have shown that Ang-(1–7), through interaction with Mas, has an anti-inflammatory action (Figure 2). Mouse peritoneal macrophages treated with Ang-(1–7) had a reduction in pro-inflammatory cytokines expression, including TNF-α and IL (interleukin)-6 after LPS (lipopolysaccharide) stimulation [112] (Figure 2A). Indeed, the Mas transcripts increased 8-fold after LPS exposure and the Mas antagonist A779 abrogated the anti-inflammatory effect, indicating that Mas mediates the Ang-(1–7) anti-inflammatory effect in these cells [112]. The molecular mechanism of this effect involves c-Src, which is

known to modulate inflammation, as it is dephosphorylated after Ang-(1–7) treatment as shown by Western blot analysis [112]. Another study using two experimental models, AIA (antigen-induced arthritis) in mice and AdIA (adjuvant-induced arthritis) in rats, also showed an anti-inflammatory effect following Ang-(1–7) treatment. Arthritic joints express Mas, and treatment with Ang-(1–7) or the orally active Mas agonist AVE0991 ameliorates arthritis in both experimental models [113]. Indeed, AIA-induced neutrophil accumulation was reduced, as well as hypernociception and the production of pro-inflammatory cytokines, such as TNF-α and IL-1b, and the chemokine CXCL1 (CXC chemokine ligand 1). Leucocyte rolling and adhesion were also reduced after AVE0991 treatment. Moreover, the knockout of Mas in mice increased different inflammatory features in arthritis, indicating an endogenous property of Mas in modulating the inflammatory process [113]. Thus Ang-(1–7) leads to an overall reduction in inflammation in models of arthritis, supporting a novel therapeutic approach for arthritis [113].

Another inflammatory condition that has been reported to be affected by Ang-(1–7) is atherosclerosis, which is characterized by endothelial dysfunction, vascular inflammation and the accumulation of lipids within the intima of the vessel wall [114]. Chronic treatment with Ang-(1–7) of the atherosclerotic model ApoE (apolipoprotein E)$^{-/-}$ knockout mice improved endothelial function and attenuated the lesion progression as shown by a reduction in fatty deposits (Oil Red O) and intima/media ratio [115]. The same model treated with the Ang-(1–7) agonist AVE 0991 also ameliorated atherogenesis. This is probably a consequence of a decrease in inflammation as Ang-(1–7) inhibited NADPH oxidase expression, which is involved in production of ROS (reactive oxygen species), and diminished the expression of co-stimulatory molecules on antigen-presenting cells, both involved in inflammation development [116]. In addition, overexpression of ACE2 reduced atherosclerosis in ApoE$^{-/-}$ mice, with less macrophage infiltration and lipid deposition [117] and stabilized the atherosclerotic plaques [118].

The effects of Ang-(1–7) have also recently been evaluated in inflammatory conditions of the respiratory tract. A mouse model of allergic asthma challenged with ovalbumin and treated with Ang-(1–7) resulted in attenuation of ovalbumin-induced perivascular and peribronchial inflammation and fibrosis [119]. One possible mechanism of the anti-inflammatory property of Ang-(1–7) in the airways is through the ERK and NF-κB (nuclear factor κB) signalling pathways, as Ang-(1–7) diminished the phosphorylation of ERK1 and IκBα (inhibitor of NF-κB) induced by ovalbumin [119] (Figure 2A). These effects are mediated by Mas, as its antagonist A779 abolished the majority of these outcomes [119]. In addition, ACE2 and Ang-(1–7) levels were diminished after intratracheal administration of LPS in rats, a model of ARDS (acute respiratory distress syndrome) [120]. However, after treatment with a protease-resistant form of Ang-(1–7), the lung injury scores diminished and lung function was improved through attenuation of inflammation [120]. Thus these results indicate that the local levels of Ang-(1–7) are crucial in avoiding the development and progression of this syndrome [120].

Taken together, these studies have shown that Ang-(1–7) has anti-inflammatory properties that can be useful in the treatment of different inflammatory conditions such as arthritis, atherosclerosis and asthma. However, in the kidney, conflicting results have been observed ([121]; reviewed in [122]).

CEREBROPROTECTION, LEARNING AND MEMORY

Ang-(1–7) is the main angiotensin peptide in the CNS (central nervous system) and is expressed in different regions of the brain, including the hypothalamus, hippocampus, amygdala and many others [123]. Besides its effects as a critical neuromodulator of cardiac baroreflex mechanisms ([124]; reviewed in [125]), Ang-(1–7) influences different non-cardiovascular functions in the brain such as learning, memory and cerebroprotection against ischaemia (Figure 2). Indeed, early in 1992, Holy et al. [126] showed that administration of Ang-(1–7) had psychotropic effects.

The hippocampus is one of the regions with the highest expression of Ang-(1–7) [91]. The knockout or blockage of Mas leads to a deficit in object recognition memory, indicating that the Ang-(1–7)/Mas axis is an important modulator of learning and memory [127]. One of the possible molecular pathways involved in this mechanism is the synthesis of NO, a crucial factor for object recognition memory that is modulated by Ang-(1–7). Indeed, Yang et al. [128] have shown that Ang-(1–7), via Mas activation, increases NO levels through nNOS (neuronal NOS) in catecholaminergic neurons [128]. Besides that, Ang-(1–7) through its interaction with Mas is also able to increase LTP (long-term potentiation) in the hippocampus and amygdala [129]. LTP is the basis of learning and memory [129] (Figure 2B) and it is increased by Ang-(1–7) in the amydgala through changes in COX-2 and NO [130] (Figure 2A).

Ang-(1–7) may also confer protection against cerebral ischaemic stroke. Central administration of Ang-(1–7) upregulates eNOS expression and consequently increases NO release in ischaemic tissues in rats [131]. Interestingly, ACE2 overexpression also increased NOS (both eNOS and nNOS) and NO levels in cerebrospinal fluid of mice [132]. During the early stages of cerebral ischaemia, eNOS-derived NO is beneficial as it promotes collateral circulation and microvascular flow [133]. However, the induction of iNOS (inducible NOS) expression in neurons produces toxic levels of NO, contributing to neuronal death elicited by cerebral ischaemia ([134]; reviewed in [135]). A recent study using an endothelin-induced model of cerebral ischaemia showed that central administration of Ang-(1–7) or an ACE2 activator diminished the expression of iNOS (Figure 2B), leading to a decrease in cerebral infarct size and behavioural deficits (Figure 2A). Blockade of Mas with A779 reversed this phenotype [136]. Thus Ang-(1–7) appears to modulate the NO release from different sources, contributing to improving the protection against cerebral ischaemia. Another recent study added a new mechanism for the cerebroprotective action of Ang-(1–7) [137]. Ang-(1–7) infusion into the cerebroventricular region attenuated neurological deficits and diminished infarct volume. This effect was mediated by its anti-inflammatory effects through a reduction

of oxidative stress and pro-inflammatory cytokines, in addition to NF-κB suppression [137] (Figure 2B). Therefore Ang-(1–7) is a potential viable treatment to prevent individuals with a high risk of stroke suffering greater damage caused by ischaemia. However, as pointed out by Jiang et al. [137], new studies are needed to establish whether this peptide can improve cerebroprotection in a post-ischaemia scenario.

ADDITIONAL MECHANISMS

Ang-(1–7) has been detected in glial cells of human retina [138] and its receptor Mas has been described in different parts of the eye, such as the retina and cilliary body, in rats [139]. Interestingly, intravitreal treatment with Ang-(1–7) in rabbits diminished the intraocular pressure without modifying aqueous humour outflow [140], indicating a possible direct action/function of this peptide in the eye. In addition, intraocular administration of AAV (adeno-associated virus)-mediated gene delivery of ACE2 or Ang-(1–7) to diabetic rats and mice diminished diabetes-induced retinal vascular leakage, infiltrating inflammatory cells and oxidative damage, conferring protection against diabetic retinopathy [141].

Ang-(1–7) also seems to be involved in several other processes. For example, this heptapeptide increases the jejunal absorption of water when administered to rats. This effect occurs through interaction with the Mas receptor and it is mediated by NO and COX [142]. Ang-(1–7) also plays a role in the regulation of haematopoiesis and progenitor cells, an action that was recently reviewed by Durik et al. [143]. Baykan et al. [144] evaluated the effects of Ang-(1–7) on skin ischaemia induced by subcutaneous administration of nicotine in female Sprague–Dawley rats. The authors observed that the group treated with Ang-(1–7) had a decrease in the ischaemic area due to increased angiogenesis [144]. Finally, Ang-(1–7) is highly expressed in the gastric mucosa and this heptapeptide has a potent gastroprotective effect (reviewed in [145]).

CONCLUSIONS AND PERSPECTIVES

As observed for AngII, the effects of Ang-(1–7) in the body are not restricted to the cardiovascular or renal system. Although it is difficult in many instances to differentiate between cardiovascular and non-cardiovascular actions, it is clear that Ang-(1–7) can influence many organs and systems. With advances in our understanding related to its signalling mechanisms, novel insights about the overall physiological and pathophysiological role of Ang-(1–7) will soon be available. In general, despite some controversial findings in the kidney [122], ACE2/Ang-(1–7)/Mas seems to exert protective effects in several systems and organs, opposing the detrimental effects of the overactivation of the ACE/AngII/AT_1R axis. What are the mechanisms governing the fine tuning of the equilibrium between these two RAS axes? When and how do the angiotensin-mediated actions via the AT_2R take place in this balance? What is the role of Ang-(1–9)-mediated effects in the overall function of the RAS [10,11]? These are just a few questions waiting for enlightenment in order to advance our knowledge of this fascinating system.

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