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Research Paper

Assessment of biological activity of novel peptide analogues of angiotensin IV

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Abstract

Objectives Angiotensin IV (Ang IV) is a metabolite of angiotensin II which acts on specific AT_4 receptors identified as the enzyme insulin regulated aminopeptidase (IRAP). The transduction process of these receptors is unresolved, but Ang IV inhibits the aminopeptidase activity. Ang IV improves cognition in animal models thus there is a desire to develop metabolically stable analogues for further development.

Methods Peptide analogues of Ang IV were obtained commercially or synthesised. Each peptide was tested *in vitro* for its ability to inhibit the aminopeptidase activity (IRAP) of mouse brain homogenates and for its effects on isolated rat uterine smooth muscle.

Key findings [Des-Val[1]]-Ang IV, acetylated-Ang IV-amide, Ang IV-amide and [des-His[4]]-Ang IV all inhibited IRAP. [Sar[1], Ile[8]]-Angiotensin II (10 μM) had an effect greater than that of Ang IV or any of the other analogues studied. In isolated uterine smooth muscle, angiotensins II and IV induced contractions, which could be antagonised by an AT_1-receptor antagonist. None of the novel peptides induced uterine smooth muscle contractions, but [Sar[1], des Arg[2]-Gly[8]]-angiotensin II showed significant antagonism of the contractile effects of angiotensin II and carboxyamide-terminated Ang IV-NH_2 showed antagonism of Ang IV-induced contractions.

Conclusions This study provides five novel inhibitors of IRAP worthy of assessment in behavioural models of learning and memory. The analogues are devoid of AT_1 receptor agonist properties, and the carboxyamide analogue presents an opportunity to elucidate the mechanism of action of Ang IV as, like Ang IV, it inhibits IRAP, but antagonises the effects of Ang IV on isolated smooth muscle.

Keywords angiotensin II; angiotensin IV; insulin-regulated aminopeptidase

Introduction

Angiotensin IV (Ang IV) is a metabolite of angiotensin II that has been shown to have robust effects on learning and memory in rats and mice (discussed later). The classical view of the renin-angiotensin system is that it controls blood pressure and fluid balance, and as such it has been the target for the development of a range of antihypertensive drugs. The traditional view of the system is that it consists of a precursor, angiotensinogen, which is synthesised by the liver and released into the systemic circulation and then converted to the decapeptide angiotensin I by the action of renin. Renin is released from the juxtaglomerular apparatus of the kidney in response to decreased pressure within the renal arterioles (Figure 1). Angiotensin I is then converted to the octapeptide angiotensin II, the most active component of the system, by angiotensin-converting enzyme (ACE), which is found in highest concentrations in the pulmonary vasculature. Angiotensin II ultimately produces its effects via two specific receptors, the more prevalent AT_1 receptor which is responsible for the vasoconstriction and the AT_2 receptor which is concerned with vascular and cardiac remodelling. The renin-angiotensin system is now recognised, however, as being more extensive and as playing an important role in the brain (e.g. control of gonadotrophic hormone release and modulation of behaviour).[2] Further peptidases have been identified, with further metabolic products of angiotensin II, notably angiotensin-(2-8) (also called angiotensin III), angiotensin-(1-7), angiotensin-(3-7) and angiotensin-(3-8) (also called angiotensin IV), see Table 1. All

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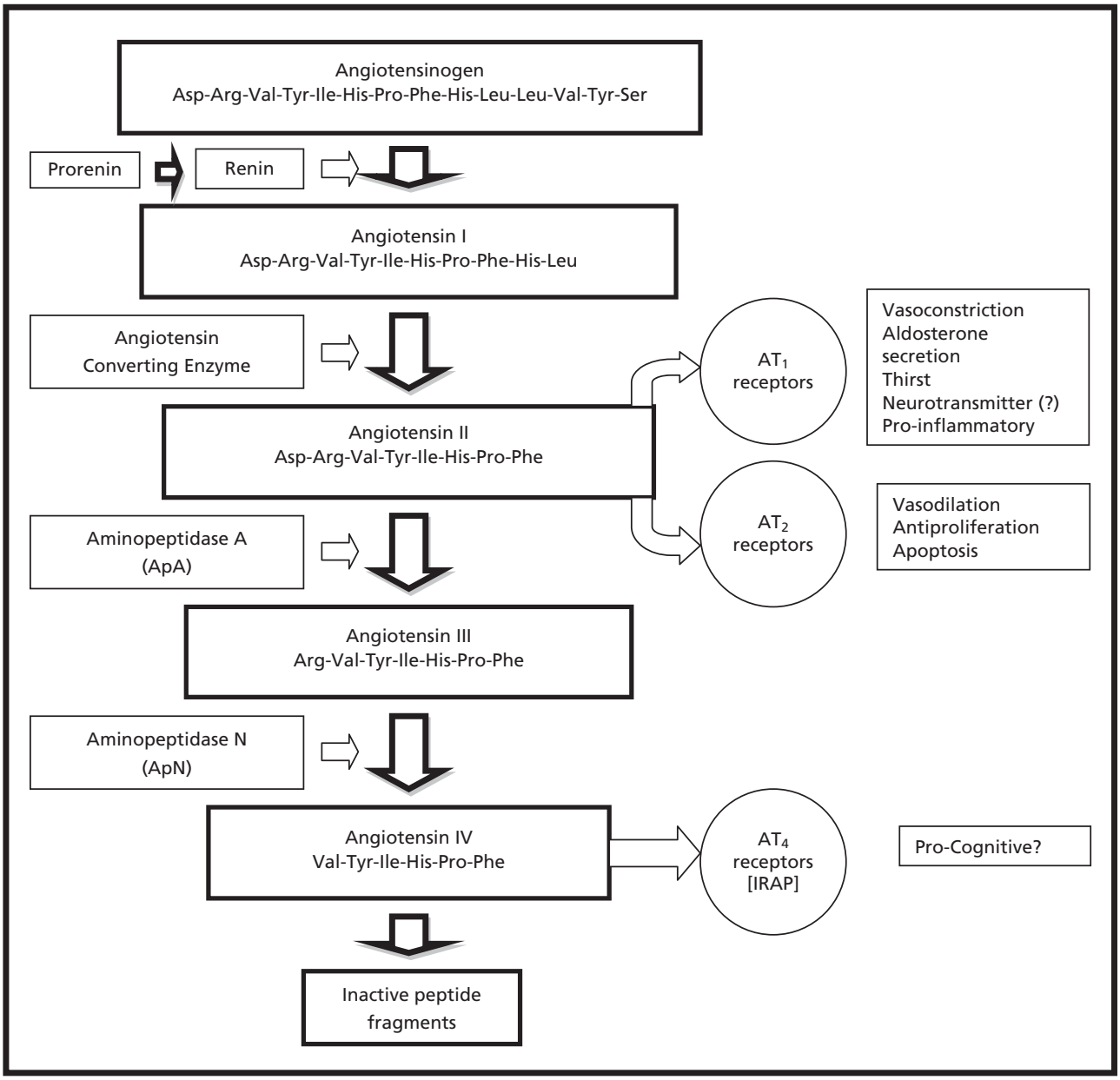

Figure 1 An outline of the components and actions of the renin-angiotensin system (reproduced from Gard[1]).

Table 1 Amino acid sequences of the angiotensin family

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Angiotensinogen	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	Ser
Angiotensin I	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu				
Angiotensin II	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe						
Angiotensin-(2-8) (AIII)		Arg	Val	Tyr	Ile	His	Pro	Phe						
Angiotensin-(1-7)	Asp	Arg	Val	Tyr	Ile	His	Pro							
Angiotensin)-(3-7)			Val	Tyr	Ile	His	Pro							
Angiotensin-(3-8) (AIV)			Val	Tyr	Ile	His	Pro	Phe						

angiotensins are pharmacologically active but have markedly different potencies and it has been demonstrated that there is a complete renin-angiotensin system within the brain, independent of the peripheral system.[3]

Early findings regarding the effects of angiotensins on learning and memory were conflicting and confusing (see Gard[2]), but it is apparent that many of the previously reported behavioural effects of angiotensin II are mediated by Ang IV. Ang IV improves memory recall in a passive avoidance task and the Morris water maze when administered centrally to rats[4] and its administration reverses scopolamine-induced amnesia and knife lesion-induced amnesia in rats.[5] Similarly LVV-hemorphin 7 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Glu-Arg-Phe), which shares little sequence homology to Ang IV but is believed to be a naturally occurring analogue, has been shown to promote memory retention and retrieval in rats.[6]

The importance of Ang IV in influencing memory and in mediating the behavioural effects of angiotensin II described above were highlighted by Harding *et al*.,[7] who demonstrated that inhibition of aminopeptidase A, which converts angiotensin II to angiotensin III, attenuated the behavioural response to angiotensin II and it has now been suggested that the cognitive effects of angiotensin II occur only 15 min after administration because of the requirement for conversion to and subsequent effect of Ang IV.[8]

Understanding of the physiology and pharmacology of Ang IV was significantly advanced by identification of the Ang IV binding site (AT_4 receptor) as insulin-regulated aminopeptidase (IRAP).[9] IRAP is a brain aminopeptidase synonymous with oxytocinase (OTase), leucyl/cystinyl aminopeptidase (Lnpep) and placental leucine aminopeptidase (P-LAP). Co-expressed with the glucose transporter GLUT4 in neurones, as well as having enzymic activity, IRAP facilitates the trafficking of GLUT4 to the cell membrane and the subsequent uptake of glucose in the presence of insulin. The mechanisms underlying the affect of Ang IV on cognition are unclear, and a matter of some ongoing debate, but as well as possibly being a substrate for IRAP, Ang IV inhibits the enzyme's catalytic activity.[10,11] Albiston *et al*.[12] speculate that the cognitive effect of Ang IV results from inhibition of IRAP, which protects bioactive peptides such as oxytocin and vasopressin from IRAP cleavage. Oxytocin and vasopressin are both behavioural modulators in their own right. Alternatively, more recently, Albiston *et al*.[13] have suggested that the pro-cognitive effects of Ang IV may be related to its actions on glucose uptake and the beneficial effects of glucose on memory.

Whatever the mechanism of the effects of Ang IV on cognition, development of analogues able to inhibit IRAP activity and that are free from agonist activity at the AT_1 receptor have the potential for use as cognition-enhancing drugs, a therapeutic area of particular importance amongst aging populations. The aim of this study was to develop a range of novel peptide analogues of Ang IV and to assess them for ability to inhibit IRAP and effect at the AT_1 receptor. Generation of novel AT4 receptor analogues will also allow further elucidation of the mechanisms of action of Ang IV.

Materials and Methods

Protocol

Peptide analogues of Ang IV were either purchased or synthesized in house. Each peptide was tested *in vitro* for its ability to inhibit the aminopeptidase activity of mouse brain homogenates (IRAP) and for its effects on isolated rat uterine smooth muscle.

Animal husbandry

Male mice, 19–30 g, of C57BL/6 strain were bred and reared in-house under identical conditions, consisting of housing in North Kent M1/M2 cages on flake sawdust bedding in an air conditioned room (19 ± 1°C; humidity 50 ± 10%) under a 14/10-h light–dark cycle, commencing 07:00 to 21:00 h. The mice had free access to food and water. Female Sprague-Dawley rats, 125–150 g, obtained from Charles River were housed under identical conditions. All animal husbandry and procedures met the requirements of the UK Animals (Scientific Procedures) Act, 1986.

Peptide synthesis

Peptides were either purchased from commercial sources (see Table 2) or were synthesized in-house using the Fmoc solid-phase peptide synthesis strategy (Fmoc-SPPS) involving sequential linking of N-protected Fmoc-amino acids to a C-terminal amino acid bound to an insoluble resin via a cleavable linking agent that would afford a peptide with a free carboxylic acid on the C terminal. Assembly of the peptides was carried out using *N,N*-dimethylformamide (DMF) as

Table 2 Peptide analogues of angiotensin IV tested for activity *in vitro* and *in vivo*

Peptide	Sequence									RMM	Source
Angiotensin II	Asp[1]	-Arg[2]	-Val[3]	-Tyr[4]	-Ile[5]	-His[6]	-Pro[7]	-Phe[8]		1046.54	Bachem
[Sar[1], Ile[8]]-Angiotensin II	Sar	-Arg	-Val	-Tyr	-Ile	-His	-Pro	-Ile		968.16	Phoenix Pharma
[Sar[1], des Arg[2]-Gly[8]]-Angiotensin II		Sar	-Val	-Tyr	-Ile	-His	-Pro	-Phe	-Gly	903.04	Phoenix Pharma.
Angiotensin III		Arg	-Val	-Tyr	-Ile	-His	-Pro	-Phe		930.51	Bachem
Angiotensin IV			Val	-Tyr	-Ile	-His	-Pro	-Phe		775.41	Bachem
Angiotensin IV-NH_2			Val	-Tyr	-Ile	-His	-Pro	-Phe	-NH_2	774.43	In-house synthesis
Ac-Angiotensin IV-NH_2		Ac	-Val	-Tyr	-Ile	-His	-Pro	-Phe	-NH_2	816.44	In-house synthesis
[des-Val[1]]-Angiotensin IV				Tyr	-Ile	-His	-Pro	-Phe		676.34	In-house synthesis
Ac-[des-Val[1]]-Angiotensin IV			Ac	-Tyr	-Ile	-His	-Pro	-Phe		718.35	In-house synthesis
[des-His[4]]-Angiotensin IV			Val	-Tyr	-Ile	-Pro	-Phe			638.35	In-house synthesis
[Lys[1], des-His[4]]-Angiotensin IV			Lys	-Tyr	-Ile	-Pro	-Phe			667.38	In-house synthesis
Ac-[Lys[1], des-His[4]]-Angiotensin IV		Ac	-Lys	-Tyr	-Ile	-Pro	-Phe			709.39	In-house synthesis

solvent with 20% piperidine in DMF as a deprotecting agent for the Fmoc protecting groups. On completion of the assembly of the required peptide sequence, removal of side-chain protecting groups and cleavage from the resin was achieved using trifluoroacetic acid (95%) and a mixture of triisopropylsilane 2%, anisole 2% and water 1% as scavengers. *N*-acetyl derivatives of the peptides were prepared using acetic anhydride in DMF after deprotection of the N-terminal amino acid. Carboxyamide terminated peptides were prepared using Rink amide linker attached to the solid phase resin support before incorporation of the C-terminal amino acid. Synthesized peptides were analysed by HPLC using a water–acetonitrile gradient containing 0.1% trifluoroacetic acid and the column eluant monitored at 218 nm. Purification was carried out by preparative HPLC using the same solvent system. Identity of the synthesized peptides was confirmed by ESI-MS using a Bruker HCT Ion trap mass spectrometer. The range of peptides and acetylated peptides investigated is presented in Table 2.

Assessment of aminopeptidase activity

To determine aminopeptidase activity and the inhibitory effects of Ang IV, a method based on the cleavage of L-leucine-*p*-nitroanilide into L-leucine and *p*-nitroaniline was employed.[14] *p*-Nitroaniline shows a characteristic absorption at 405 nm upon cleavage by aminopeptidases.[15] Male C57BL/6 mice, all bred and raised in-house, were sacrificed and whole brains removed and immediately placed in ice-cold extraction buffer (50 mM Tris, 0.25 M sucrose, 140 mM NaCl pH 7.5). Following homogenization at full speed for 1 min with a Ystral homogeniser, the homogenate was centrifuged at 4000*g* for 8 min at 4°C. The supernatant containing membrane fragments was removed and centrifuged at 100 000*g* for 40 min at 4°C. The pellet was then washed and re-suspended in assay buffer (50 mM Tris, 140 mM NaCl pH 7.4). Protein concentration was assayed using Bradford reagent (Sigma-Aldrich, Gillingham, Dorset, UK). Membrane homogenate (25 µg) was incubated at 37°C with 5 µM leucine-*p*-nitroanilide (Sigma-Aldrich UK) in a final volume of 300 µl. The absorbance was read every 10 min for 50 min using an ASYS plate reader at 405 nm. Peptide inhibition assays were carried out by addition of a range of peptide concentrations and assessment of the effects on the catalysis of leucine-*p*-nitroanilide to nitroaniline as assessed by changes in absorbance at 405 nm. All assays were carried out in duplicate.

Effect on AT_1 receptors

For determination of the effects of the peptides on angiotensin AT_1 receptors, contractile responses to angiotensin II, Ang IV and the analogues were assessed in isolated rat uterine smooth muscle in triplicate. Rat uterus was removed immediately post-mortem and separated into two uterine horns by dissection from the cervices and the ovaries. The uterine horns were suspended in De Jalon's solution at 32°C under a resting tension of 1 g. Isotonic contractile responses to angiotensin II (10^{-11} to 10^{-8} M) and Ang IV (10^{-12} to 10^{-6} M) were recorded using an 8-min dose cycle and 30-s contact time. De Jalon's solution is comprised of NaCl (154 mM), KCl (5.63 mM), $CaCl_2$ (0.648 mM), $NaHCO_3$ (5.95 mM) and glucose (2.77 mM). The angiotensin AT_1-receptor antagonist losartan (10^{-8} and 10^{-7} M, a gift from DuPont Merck, Wilmington, DE, USA) was used to characterise the site of action of the Ang IV analogues, and pre-treatment with the analogues was used to assess their antagonistic effects against angiotensin II and Ang IV. Drug effects were determined in 3–6 separate tissues. For each rat, the stage of oestrous cycle was determined by microscopic assessment of vaginal epithelial cells obtained by vaginal lavage with normal saline immediately post-mortem.

Data analysis

Changes in absorbance at 10-min intervals over 50 min were used to determine the catalytic activity of the brain homogenate. Parallel controls were carried for each assay and results of inhibition are presented as a percentage of the control. Best-fit straight lines were fitted to the data using linear regression and differences in the gradients of the lines generated were assessed using Friedman's test with drug concentration as the treatment and time as the block.

Drug effects on uterine smooth muscle were recorded as g tension. Contractile responses to Ang IV 10^{-5} M were determined in five tissues and were found not to differ significantly from normality ($P > 0.15$, Kolmogorov-Smirnoff test). The contractile effects of angiotensin II 10^{-7} M and Ang IV 10^{-5} M were tested against a theoretical mean of zero using Student's one-sample, one-tailed, *t*-test. The potential antagonistic effects of the novel analogues against angiotensin II were assessed using one-way analysis of variance followed by post-hoc testing utilising Dunnett's test. In the case of antago-

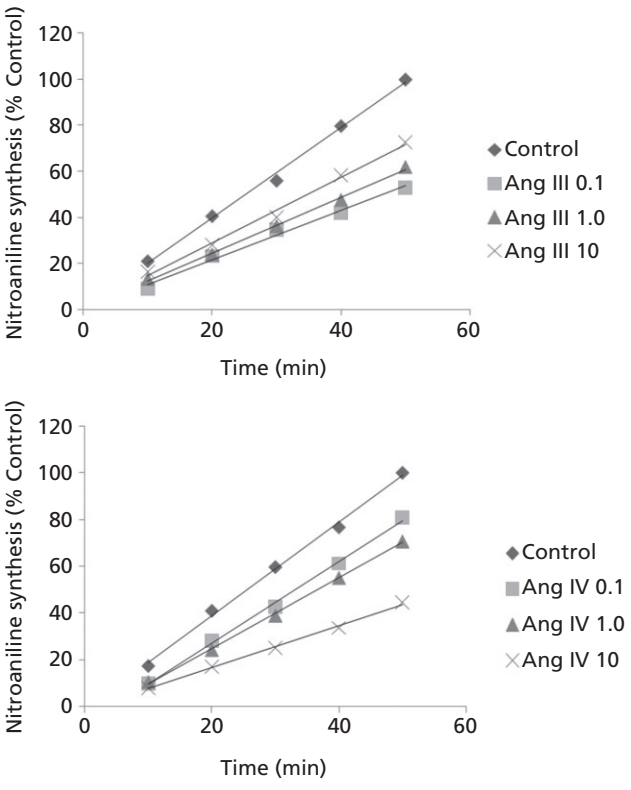

Figure 2 Inhibition of aminopeptidase activity of insulin-regulated amino peptidase by endogenous angiotensins. Activity was determined by cleavage of L-leucine-*p*-nitroanilide to leucine and nitroaniline ($P < 0.003$ in both cases, Friedman's test).

nistic effects of the peptides against Ang IV, use of analysis of variance was invalid as two of the drug combinations gave zero response with zero variance, drug effects were therefore assessed by reference to the earlier one-sample tests and use of Student's one-tailed independent *t*-test.

Results

The endogenous angiotensins Ang IV, and to a lesser extent angiotensin III, were able to inhibit the aminopeptidase activity of the brain homogenate ($P = 0.003$ and 0.002, respectively, Friedman's test) (Figure 2); angiotensin II was without significant effect at the concentrations studied. The fact that Ang IV was able to inhibit the activity by approximately 60% at a concentration of 10 μM indicates that IRAP probably accounts for the majority of the aminopeptidase activity; Ang IV is known to selectively inhibit IRAP with an IC_{50} (inhibitory concentration 50%: concentration required to reduce the response by 50%) of approximately 1 μM.[15] Of the synthetic peptides, [des-Val^1]-angiotensin IV, Ac-angiotensin IV-NH_2, angiotensin IV-NH_2 and [des-His^4]-angiotensin IV all inhibited IRAP to the same extent as Ang IV at similar concentrations ($P = 0.014$; 0.002; 0.002 and 0.002, respectively, Friedman's test). [Sar^1, Ile^8]-Angiotensin II (10 μM) had an effect greater than that of Ang IV or any of the other analogues studied ($P = 0.003$, Friedman's test, Figure 3). None of the other peptides studied consistently inhibited the aminopeptidase activity.

In rat isolated uterus, angiotensins II and IV induced significant contractions ($P = 0.001$ and 0.029, respectively, Student's one-sample, one-tailed, *t*-test); typical myograph

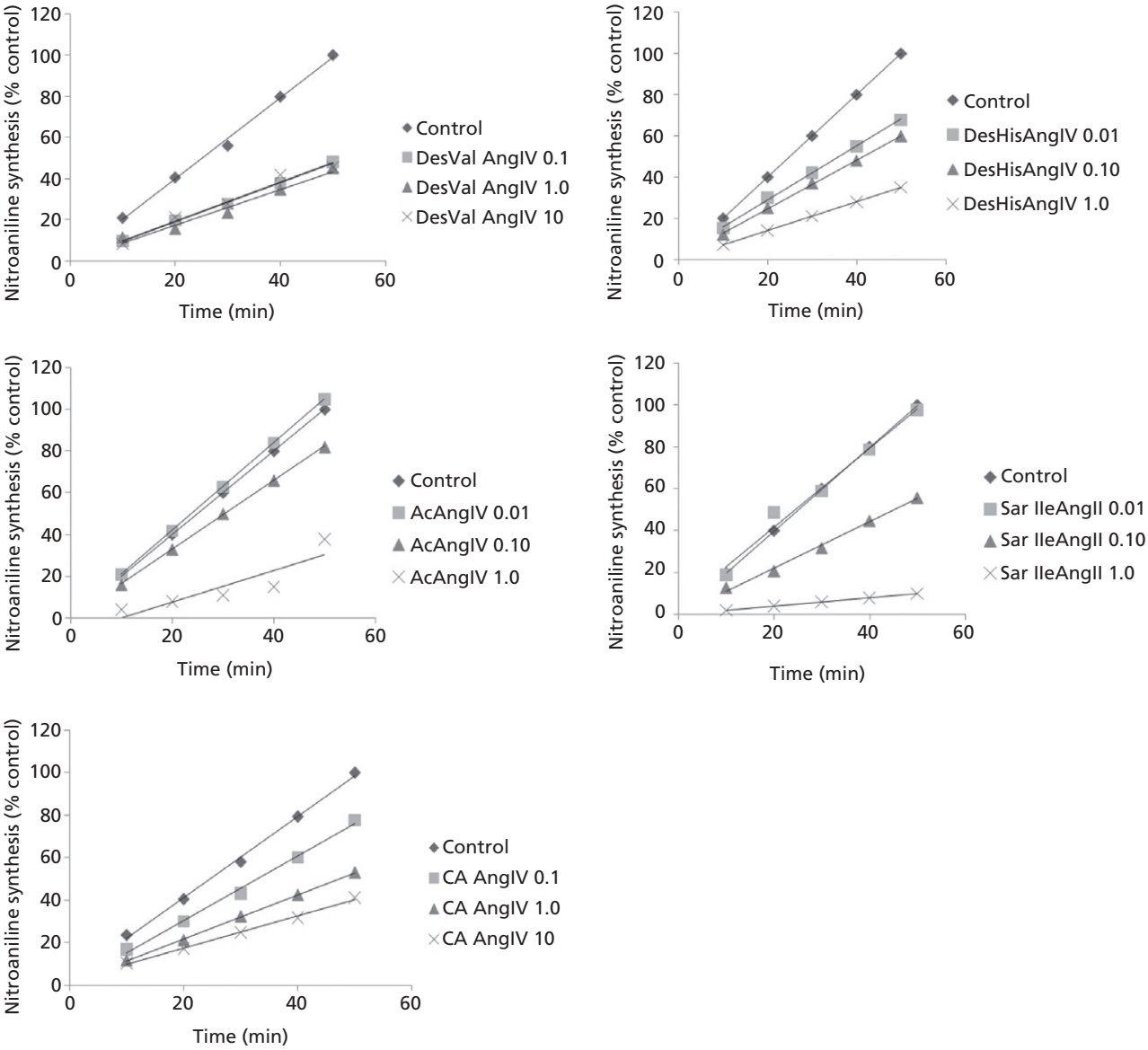

Figure 3 Inhibition of aminopeptidase activity of insulin-regulated amino peptidase by [des-Val^1]-angiotensin IV (DesVal AngIV), [des-His^4]-angiotensin IV (DesHis Ang IV), Ac AngIV; [Sar^1, Ile^8]-angiotensin II (Sar Ile Ang II) and Ac-angiotensin IV-NH_2 (CA AngIV). Activity was determined by cleavage of L-leucine-*p*-nitroanilide to leucine and nitroaniline ($P < 0.003$ in all cases, Friedman's test).

traces are shown in Figure 4. There was no effect of oestrous cycle stage on the effects of these endogenous peptides. None of the novel peptides induced uterine smooth muscle contractions but analysis of variance revealed that losartan and the novel peptides significantly reduced the contractile response to 10^{-7} M angiotensin II ($P = 0.017$, d.f. 3,10) with losartan and [Sar^1, des Arg^2-Gly^8]-angiotensin II achieving significance ($P < 0.05$, Dunnett's post-hoc test); carboxyamide-teminated Ang IV was without significant effect. Ang IV (10^{-5} M) failed to produce a contractile response in the presence of losartan and carboxyamide-terminated Ang IV, which by reference to the earlier one-sample, one-tailed *t*-tests were deemed to be significant effects. [Sar^1, des Arg^2-Gly^3] Angiotensin II had no significant antagonistic effect ($P = 0.254$, Student's independent *t*-test) (Figure 5).

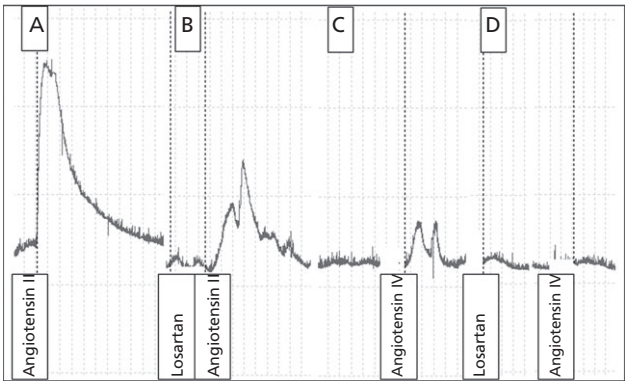

Figure 4 Typical myograph traces of the contractile effect of angiotensins II and IV in rat isolated uterus. Panel A: 10^{-5} M angiotensin II; Panel B: 10^{-5} M angiotensin II in the presence of 10^{-6} M losartan; Panel C: 10^{-5} M angiotensin IV; Panel D: 10^{-5} M angiotensin IV in the presence of 10^{-6} M losartan.

Discussion

These results raise some interesting issues. Firstly, considering the inhibition of aminopeptidase activity: inhibition of IRAP by angiotensin III is unlikely as IRAP is known to metabolise angiotensin III, the observation is thus more likely to reflect conversion of angiotensin III to Ang IV before inhibition of IRAP by Ang IV. Inhibition of IRAP by the other peptide analogues suggests that 'protection' of the terminal amino acids by acetylation or amidation has no effect on their ability to inhibit the enzyme, unless the acetyl and amide groups are cleaved by other components of the brain homogenate before release of Ang IV and subsequent inhibition, although this is unlikely. The lack of effect of Ac-[des-Val^1]-Ang IV, [Sar^1, des Arg^2-Gly^8]-angiotensin II, [Lys^1, des-His^4]-Ang IV, Ac-[Lys^1, des-His^4]-Ang IV and [des-Val^1]-Ang IV highlights the obligatory nature of the amino-terminal valine for inhibition of IRAP, but also suggests that the addition of the acetyl group has no adverse effect on the interaction with the binding site, unless prior metabolism by other components of the brain homogenate has occurred, as described above.

The greatest inhibition of IRAP was observed with [Sar^1, Ile^6]-angiotensin II raising the possibility that addition of the sarcosine has the beneficial effects of protecting the peptide from hydrolysis and maintaining a suitable configuration for interaction with the active site of the enzyme. Sarcosine has

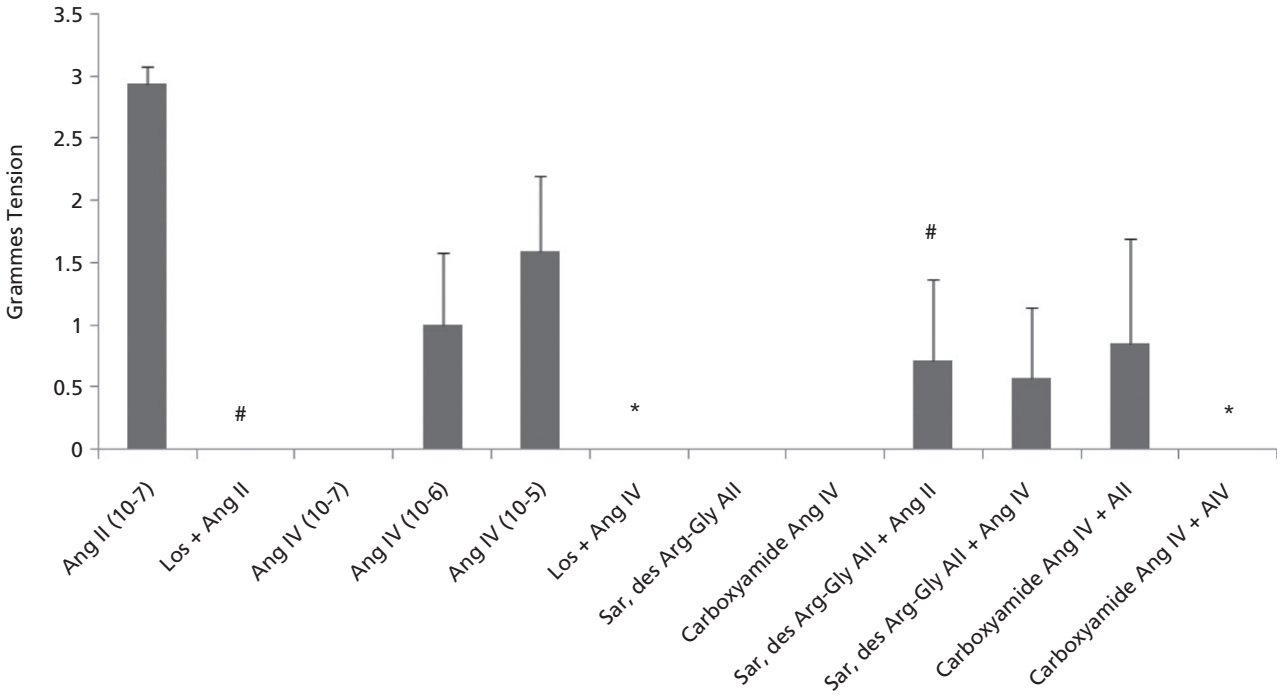

Figure 5 The agonist and antagonist effects of novel peptide analogues of angiotensin IV on contractile responses in isolated rat uterus. # and * indicate significant difference from angiotensin II and angiotensin IV controls, respectively ($P < 0.03$ analysis of variance or one-tailed *t*-test, see Methods).

been used previously in the non-selective AT_1/AT_2 receptor antagonist saralasin (Sar-Arg-Val-Tyr-Val-His-Pro-Ala).

The effects of the peptide analogues on the contractions of uterine smooth muscle also highlight conundrums. Both angiotensin II and Ang IV induced uterine contractions *in vitro* which were antagonised by losartan; losartan is recognised as a competitive, selective antagonist of angiotensin II type 1 receptors (AT_1) which does not bind to the AT4 receptor at concentrations up to 10^{-5} M.[16] Such results would suggest that Ang IV is producing the contractile effects by interaction with the AT_1 receptor. This suggestion is further supported by the fact that none of the peptides induced uterine contractions, although five of them had been seen to inhibit IRAP. Inhibition of IRAP is thus not the mechanism by which Ang IV induces uterine contraction. Importantly, however, carboxyamide Ang IV significantly antagonised the contractile effects of ang IV while having no significant effect on the effects of angiotensin II. Conversely, [Sar^1, des Arg^2-Gly^8] angiotensin II significantly antagonised the contractile actions of angiotensin II but had no significant effects on the contractile effects of Ang IV. Such a result raises the possibility of at least some partial specific actions of Ang IV on uterine smooth muscle, independent of the AT_1 receptor. The possibility of a specific AT_4 receptor in uterine smooth muscle, with an effector mechanism independent of inhibition of IRAP, fits with the proposal of Wright and Harding,[17] who reasoned that biological effects of Ang IV are apparent at concentrations below the 1 μM IC50 for inhibition of IRAP, although in the case of the uterus 1 μM was the lowest concentration seen to induce a contraction. They also reasoned that the speed of response, in the order of seconds, is unlikely to reflect accumulation of a substrate consequent to enzyme inhibition.

Conclusions

Angiotensin IV has been shown to have beneficial cognitive effects in animals models;[18] there is thus a desire to develop metabolically-stable analogues for assessment of potential anti-dementia properties. At present the mechanism of action of Ang IV on cognition is unresolved, thus whether the analogue needs to inhibit IRAP or whether it could act via another transduction process is unknown. This study has provided five novel inhibitors of IRAP worthy of assessment in behavioural models of learning and memory, such as novel object recognition.[18] The analogues are devoid of AT_1 receptor agonist properties, which lessen fears of cardiovascular adverse effects, and the carboxyamide analogue presents an opportunity to elucidate the mechanism of action of Ang IV as, like Ang IV, it inhibits IRAP, but it antagonises the effects of Ang IV on isolated smooth muscle. It is recognised, however, that the analogues represent only a starting point for future drug development as their ability to induce antibody formation and any chronic toxic effects have yet to be addressed.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Gard PR. Implications of the angiotensin converting enzyme gene insertion/deletion polymorphism in health and disease – snapshot review. *Int J Mol Epidemiol Genet* 2010; 1: 145–157.
2. Gard PR. The role of angiotensin II in cognition and behaviour. *Eur J Pharmacol* 2002; 438: 1–14.
3. Albrecht D. Physiological and pathophysiological functions of different angiotensins in the brain. *Br J Pharmacol* 2010; 159: 1392–1401.
4. Braszko JJ *et al*. Angiotensin-II-(3-8)-hexapeptide affects motor-activity, performance of passive-avoidance and a conditioned avoidance-response in rats. *Neuroscience* 1988; 27: 777–783.
5. Pederson ES *et al*. Attenuation of scopolamine-induced spatial learning impairments by an angiotensin IV analog. *Regul Pept* 1998; 74: 97–103.
6. Lee J *et al*. Effect of ICV injection of AT(4) receptor ligands, NLE1-angiotensin IV and LVV-hemorphin 7, on spatial learning in rats. *Neuroscience* 2004; 124: 341–349.
7. Harding JW *et al*. Is angiotensin-III the centrally active form of angiotensin. *Experientia* 1986; 42: 706–706.
8. Braszko JJ *et al*. Cognitive effects attributed to angiotensin II may result from its conversion to angiotensin IV. *J Renin Angiotensin Aldosterone Syst* 2006; 7: 168–174.
9. Albiston AL *et al*. Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *J Biol Chem* 2001; 276: 48623–48626.
10. Lew RA *et al*. Angiotensin AT(4) ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP). *J Neurochem* 2003; 86: 344–350.
11. Axen A *et al*. Small potent ligands to the insulin-regulated aminopeptidase (IRAP)/AT(4) receptor. *J Pept Sci* 2007; 13: 434–444.
12. Albiston AL *et al*. AT(4) receptor is insulin-regulated membrane aminopeptidase: potential mechanisms of memory enhancement. *Trends Endocrinol Metab* 2003; 14: 72–77.
13. Albiston AL *et al*. Identification and characterization of a new cognitive enhancer based on inhibition of insulin-regulated aminopeptidase. *FASEB J* 2008; 22: 4209–4217.
14. Stragier B *et al*. Involvement of insulin-regulated aminopeptidase and/or aminopeptidase N in the angiotensin IV-induced effect on dopamine release in the striatum of the rat. *Brain Res* 2007; 1131: 97–105.
15. Demaegdt H *et al*. Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [^{125}I]Ang IV binding and catalytic activity. *Biochem Pharmacol* 2004; 68: 885–892.
16. Handa RK *et al*. Characterization and function of the bovine kidney epithelial angiotensin receptor subtype 4 using angiotensin IV and divalinal angiotensin IV as receptor ligands. *J Pharmacol Exp Ther* 1999; 291: 1242–1249.
17. Wright JW, Harding JW. The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning and memory. *Prog Neurobiol* 2004; 72: 263–293.
18. Golding BJ *et al*. The role of IRAP/ApN in a murine model of cognition. *Eur J Pharmacol* 2010; 641: 154–159.