Augmentation of Aortic Ring Contractions by Angiotensin II Antisense Peptide

Patrick F. Dillon, Robert S. Root-Bernstein, Daniel D. Holsworth

Abstract—Previous biochemical experiments have revealed two antisense peptide antagonists to human angiotensin II (Ang II), one encoded in the cDNA in the antiparallel reading, the other in the parallel reading. Neither peptide’s ability to produce physiological antagonism has been demonstrated previously. Both peptides were tested for their ability to antagonize Ang II–induced contractions on rabbit aorta smooth muscle. Neither peptide had any direct contractile activity. The antiparallel Ang II peptide had physiological antagonism to Ang II contractions at a lower sensitivity than reported in biochemical studies, and its antagonist activity was partially blocked by Ang II antiserum, suggesting that it is not an antipeptide but an Ang II homologue. The parallel Ang II antipeptide also required high concentrations for physiological inhibition. Its contractile inhibition was not affected by Ang II antiserum and diminished the Ang II contraction at high micromolar concentrations, findings consistent with physicochemical data showing that it is an Ang II complement. The concentration of either peptide required to produce an antagonistic physiological effect was too high to predict any pharmacological usefulness. The parallel antipeptide, however, significantly increased the force of muscle contractions at high nanomolar concentrations, thus displaying a unique dual augmentation/antagonist activity. This antipeptide seems to have highly sequence-specific activity because other similar parallel antipeptides had no activity. The parallel antipeptide augmentation mimics the shift in the Ang II dose-response curve produced in hypertension studies of the slow pressor effect of Ang II and may be useful in deducing the currently unknown cause of the slow pressor effect. It may also have some uses in migraine studies. (Hypertension. 1998;31:854-860.)

Key Words: antisense elements ■ peptides ■ angiotensin II ■ muscle, smooth, vascular

These experiments were undertaken to test the mechanisms of action and physiological utility of Ang II antisense peptides. Antisense peptides are the logical analogues of antisense oligonucleotides. Just as the noncoding strand of DNA can be used either directly to generate an antisense DNA sequence or indirectly to generate antisense mRNA that can bind to and inactivate the coding strand of DNA or its respective mRNA, some investigators believe that it is possible to use the coding strand of DNA or its respective mRNA to generate antisense peptides that are assumed to bind directly to and inactivate the sense-generated peptide. Thus, four different antisense approaches exist. All have been applied to Ang II regulation. Antisense DNA sequences have been genetically inserted into cells to regulate Ang II receptor expression.1

Antisense RNA has been exogenously introduced to limit angiotensinogen expression.2,3 Assuming that antisense DNA encodes a protein complementary to Ang II, Ruiz-Opazo et al4 have used the antisense approach to isolate a novel dual receptor for Ang II and vasopressin. Finally, investigators have synthesized antisense peptide sequences based on two different reading frames of Ang II mRNA (parallel and antiparallel; see later section) resulting in Ang II antagonist activity.5-13 This study focuses on Ang II antisense peptide activity.14,15

Two fundamentally different approaches to antisense peptide design have been used to generate both Ang II antipeptides and antisense peptides against other peptide targets. One, first suggested theoretically by Mekler,16 was reinvented later by Biro17 and Blalock and Smith18 and experimentally was pioneered by Blalock and colleagues.19-20 It uses the RNA sequence that would be generated by the noncoding (-) or complementary strand of the DNA encoding Ang II if it were read in the normal or antiparallel reading frame (5′→3′). The resulting antisense peptide for human Ang II has the sequence NH2-GLU-GLY-VAL-TYR-VAL-HIS-PRO-VAL-COOH. This sequence shall be referred to as “Blalock peptide,” or “Bl peptide” for short. The second approach to antisense design was initiated both theoretically and experimentally by Root-Bernstein21-24 It also uses the (−) strand DNA sequence but reads it in the parallel or backward reading frame (3′→5′). The resulting antisense peptide for human Ang II is NH2-LEU-ALA-HIS-MET-TYR-VAL-GLY-LYS-COOH. In previous publications,8,9 this peptide was referred to as RB1 peptide. An inverted version of this peptide—NH2-LYS-GLY-VAL-TYR-MET-HIS-ALA-LEU-COOH, referred to as RB6—has significantly greater activity than RB1.8,9 Both approaches are often used, sometimes interchangeably, by those generating so-called antisense or “complementary” peptides.
Selected Abbreviations and Acronyms

Ang II = angiotensin II
BI = Blalock design
PSS = physiological salt solution
RB = Root-Bernstein design

(reviewed in References 14, 25, and 26). Among the unresolved issues of the field are whether these two approaches are interchangeable and whether the resulting peptides actually represent “complements” or antipeptides that bind directly to their target peptides. This assumption has been seriously questioned. Despite claims of low nanomolar activity, several groups independently have demonstrated, using nuclear magnetic resonance spectroscopy, soft ionization electrospray mass spectrometry, and binding of radiolabeled Ang II to immobilized peptides, that there is no detectable binding between BI peptide and Ang II even at millimolar concentrations. Moreover, DeGasparo et al and Guillenette et al have directly challenged the finding that the form of BI peptide generated from rat mRNA has anti–Ang II activity, whereas Soffer et al, Moore et al, Weist et al, and Jackson et al have concluded that BI peptide acts not as an Ang II antipeptide but as an Ang II receptor antagonist. Moreover, BI peptide is more than 80% similar to Ang II (Table 1). This sequence similarity would tend to indicate that BI peptide is not complementary to Ang II but acts by binding to the receptor as a simple sequence–variant antagonist.

RB6 peptide has much lower activity as an antagonist to Ang II using the synaptic membrane assay ($K_p$ = $5 \times 10^{-5}$), but direct evidence of antisense peptide binding to Ang II exists for this peptide from all of the techniques mentioned above. The binding constant obtained from nuclear magnetic resonance studies of RB6 peptide binding to Ang II in aqueous solution is somewhat lower, but it is consistent with measured antagonist activity. We note that this micromolar binding is more in line than nanomolar affinity with what would be expected from peptide interactions in aqueous solution.

We have compared our results with published data on the inhibition of Ang II contractions with the nonpeptide agent losartan, which reduces contraction of the rabbit aorta and has been found to lower blood pressure after infusion into rats. Although this antagonism is not directly related to antisense peptides, it provides a benchmark for functionality. Moreover, losartan is known to antagonize Ang II activity due to allosteric binding to the Ang II receptor, a model that should merit further consideration in analyzing Ang II–antisense peptide activity.

Methods

Solutions

PSS contained the following (in millimolar concentrations): NaCl 116; KCl 5.4; NaHCO$_3$ 19; NaH$_2$PO$_4$, 1.1; CaCl$_2$, 2.5; MgSO$_4$, 1.2; EDTA 0.01; and glucose 11. PSS was aerated with 95% O$_2$/5% CO$_2$ to maintain pH 7.4 and warmed to 37°C before addition to tissue baths. Isomolar high K$^+$-PSS was made by reducing the NaCl concentration to 46 mmol/L and increasing KCl to 75.4 mmol/L. Ang II and Ang II antipeptides (BI human configuration) were obtained from BaChem Bioscience. Ang II antipeptide (RB6 configuration) and variants (RB3, RB4, RB5, RB7) were manufactured at the Michigan State University Peptide Synthesis Facility (Department of Biochemistry). Additional variants lacking the glycine residue (see sequences in Table 1) were synthesized by Daniel Holsworth at Houghten Pharmaceuticals, Inc (San Diego, Calif). Rabbit anti-Ang II antiserum was obtained from Peninsula Laboratories. All pharmaceuticals were refrigerated until serially diluted in PSS on the day of the experiment.

Tissue Procedures

All rabbits used were kept in university-approved facilities before experimental use. All proper procedures were followed. Adult New Zealand White rabbits of either sex were relaxed with 55 mg/kg ketamine administered intramuscularly. After 15 minutes, the rabbits were anesthetized with 50 mg/kg Nembutal administered intraperitoneally. When the rabbits were unresponsive to toe pinch, the abdomen was opened and the abdominal aorta exposed. The aorta was teased from the vena cava and clamped at both the rostral and caudal ends. The aorta was removed using surgical scissors and placed in 4°C PSS. The aortic clamps were removed to induce death.

The tissues were prepared for mechanical measurements using the procedures previously established for carotid strips, with modifications as described below for the use of tissue rings. The aorta was debindered of excess connective tissue, flushed of any remaining blood, and placed in fresh PSS. Aortic rings of 3 mm were cut using a single-edge razor blade, and the rings were placed in fresh PSS. The scissor-cut ends were not used. A pair of stainless steel loops with a flat, central straight section was passed through the lumen of each aortic ring. Upper and lower loops were secured to Plexiglas–stainless steel clamps with stainless steel screws. The lower clamp was attached to a micrometer (Newport Corp) for length adjustment. The upper clamp was connected to a 50-g force transducer (Kulite Semiconductor) with a gold chain. The force transducers were interfaced with an eight-channel Gould Instruments signal conditioner and recorder.

The rings were immersed in 20-mL or 25-mL aerated, jacketed tissue baths (Harvard Apparatus) and maintained at 37°C with a Haake (Karlsruhe) circulator. After mounting, each ring was stretched to 5 g and allowed to stress-relax for 2 hours before activation. If stress-relaxation reached 0 g, the ring was restretched to 2 g and allowed to stress-relax until the passive force was stable. The rings had a stretched linear length of 3 to 4 mm.

The tissues were activated with either K$^+$ or Ang II. Modifications of the Ang II contractions were made with different combinations and
of the maximum force that vascular smooth muscle can generate using hypercalcemic K+ or histamine solutions.36

Fig 2 shows the relative force of Ang II cumulative dose-response curves in control solutions or in the presence of 10 μmol/L Bl or 10 μmol/L RB6 peptides. As expected, the Bl peptide caused a rightward shift of the dose-response curve, with the relative force at 3, 10, and 30 nmol/L Ang II being significantly lower in the presence of Bl peptide. Unexpectedly, Ang II contractions in the presence of RB6 peptide were shifted to the left, with significantly higher relative force generated at 1 and 3 nmol/L Ang II. At these concentrations, it can make this tissue more sensitive to Ang II.

Fig 3 shows the same data as Fig 2, but here the force is measured relative to the tissue weight. In the case of the RB6 peptide–generated leftward shift of the Ang II curve, this presentation demonstrated that the increased relative contractile strength seen in Fig 2 is not due to a reduction in the maximum force generated by Ang II in the presence of RB6 peptide. The tissue weight–normalized force at 1 and 3 nmol/L Ang II is higher in the presence of 10 μmol/L RB6 peptide. The Bl peptide still inhibits the normalized force at 3, 10, and 30 nmol/L Ang II.

Results

Fig 1 shows the dose–response curves for control contractions of Ang II using individual 4-minute contractions or cumulative contractions without relaxation between the different doses. In these experiments, only at 1 nmol/L did the individual contraction show a significantly higher relative force than the cumulative force. In either case, 10 nmol/L Ang II produced a maximum Ang II contraction. For 29 rings, 10 nmol/L Ang II produced 0.85±0.06 g/mg force. Ang II produced 0.62±0.03 (n=8) of the K+ contractions. This force is =50%

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Inhibition of 10 nmol/L Ang II (AII) contractions of rabbit aortic rings by 0.07 mg/mL Ang II antiserum (AS) alone or in combination with either 100 μmol/L RB6 peptide or 3 μmol/L Bl peptide. The measurements are mean±SE (n=4) and are relative to 10 nmol/L Ang II contractions on the same rings. *Significantly different force (P<.02) from the RB6-alone inhibition. Antiserum+Bl is not different from Bl alone. These results are striking. Ang II antiserum antagonizes Ang II activity by direct binding to it. Blalock has asserted that Bl peptide also antagonizes Ang II activity by binding to it. The effect of Ang II antiserum plus Bl peptide on Ang II should therefore be additive. It is not. The observed result is 0.59 log units less than is predicted from the individual effects of the Ang II antiserum and Bl peptide. We conclude that the Ang II antiserum must recognize Bl peptide as an antigen, interfering with its activity as well as the activity of Ang II. Bl peptide must therefore be an Ang II homologue antagonist rather than an Ang II antipeptide. RB6, however, behaves like an antipeptide. The effects of RB6 plus Ang II antiserum on Ang II activity are additive and differ from the value predicted from their independent activities by only 0.16 log units. Thus RB6 is not recognized significantly by Ang II antiserum as an antigen and is not behaving as an Ang II homologue antagonist.

The intriguing augmentation of Ang II contractions by RB6 peptide was investigated further. In Fig 4, different concentrations of RB6 peptide were added to 1 nmol/L contractions of Ang II. There was a significant increase in Ang II force at every RB6 peptide concentration from 0.1 μmol/L to 10 μmol/L. Additions of RB6 peptide alone over this range did not produce any contractions at any concentration. The reversal of the curve at 1 μmol/L RB6 peptide indicates two competing processes, one augmenting Ang II contractions competing with another that inhibits its contraction.

Fig 5 shows the inhibition of individual 10 nmol/L Ang II contractions by increasing concentrations of Bl peptide. For each concentration, the peptide was added to the solution before it was prewarmed before addition to the tissue bath. Half-maximal inhibition occurs at approximately 2 μmol/L Bl peptide. Addition of Bl peptide alone from 0.1 μmol/L to 10 μmol/L in half-log increments did not produce any measurable force at any concentration.

Inhibition of Ang II contractions with RB6 peptide is demonstrated in Fig 6. Addition of 100 μmol/L RB6 peptide to 10 nmol/L Ang II solutions produced 0.49±0.05 (n=4) of the Ang II-only force. No variant of RB6 produced a similar reduction in the force generated by Ang II contractions (Table 2). Because of the uncertainty of the mechanism(s) of the antipeptide inhibition, Ang II contractions were made in the presence of Ang II antiserum, Ang II antipeptide, or both. Using the Fig 1 individual dose-response curve to approximate the decrease in Ang II using antiserum and/or RB6 peptide in individual contractions, the combination of antiserum and RB6 reduces the Ang II force by the expected amount if both antiserum and RB6 bind directly to Ang II. The variation from the predicted force is 0.16 log units or a factor of 1.45. The combination force is significantly less than with the RB6 alone. The antiserum+RB6 contractions were compared with Ang II-alone contractions, which followed. Potential tissue damage by RB6 was measured by comparing K⁺ contractions at the beginning and end of the experiment. The postcontrol terminal K⁺ contractions after 10 μmol/L RB6 were 1.03±0.04 (n=4) of the initial K⁺ contractions. Terminal K⁺ contractions after 30 μmol/L RB6 were 1.01±0.04 (n=4) of the initial K⁺ contractions.
contractions. Neither pair was significantly different. The augmentation of Ang II contractions by RB6 occurred at 10 μmol/L. RB6 and less (Fig 5), making damage to the tissue by RB6 unlikely.

The Bl peptide does not give the same result (Fig 6). The combined Ang II inhibition of antiserum and Bl not only does not produce the expected further decrease in Ang II force, but an Ang II force that is not significantly different than the Bl peptide alone, and the mean is higher. The predicted mean change is in the wrong direction and varies by 0.59 log units or a factor of 38.9. This result is not consistent with Bl peptide as well as antiserum binding Ang II, but it is consistent with both Ang II and Bl binding directly to antiserum.

Various modifications of RB6 peptide listed in Table 1 were all inactive when tested at concentrations 10,000 times higher than the Ang II available to the muscle tissue, as were a number of these sequences lacking the glycine residue in the sequence (data not shown). While it is possible that one of these peptides was tested just at the cusp between its antagonistic and augmenting activities, it is extremely unlikely that this is the case for all of the variant peptides; even if this were the case, the antagonist activity of such a peptide would be in the millimolar range and thus of no significance. These data are consistent with RB6 activity being highly sequence specific.

Conclusions

The physiological results of these experiments indicate that Blalock peptide and some other antisense peptides of Ang II have no clinical usefulness in inhibiting Ang II contractions. The concentrations required are too high. On the other hand, an unexpected nanomolar augmentation effect of both investigative and clinical potential was discovered for RB6 peptide.

The most interesting and surprising result concerning RB6 peptide activity was that at high nanomolar and low micromolar concentrations it significantly increased the force of Ang II–induced contractions in a dose-dependent manner (Figs 2 through 4), whereas Bl peptide at the same concentrations decreased the force of such contractions. RB6 peptide, in other words, has an augmenting effect on Ang II at low concentrations and an antagonistic effect at higher concentrations. This is, as far as we are aware, the first report of a compound that has dual augmentation/antagonist effects on smooth muscle and the first demonstration of a tissue activation effect of an antisense peptide. We note, however, that a similar force increase in smooth muscle contraction, known as the slow pressor effect, occurs naturally as a result of chronic low-dose Ang II exposure. The mechanism by which this slow pressor effect is induced is unknown. RB6 peptide may provide clues to this mechanism and a novel and much faster way to explore the phenomenon.

Clearly, two opposing types of activity must be present to develop the inverting curve observed in Fig 4. Several possibilities exist to explain the augmentation results. RB6 alone did not induce contractions. This makes it unlikely that the increase in force was due to either the opening of calcium channels or the release of intracellular calcium stores by RB6. A direct effect on the complex smooth muscle contractile mechanism, either the latch mechanism or a thin filament process, has not been demonstrated for any hydrophilic agent, and it is unlikely that RB6 has such an effect. It is unlikely that RB6 causes upregulation of receptor affinity or number because the effect is transient on a time scale of a few minutes. Therefore, RB6 most likely has its augmentation effect by facilitating the effectiveness of Ang II activation, resulting in an increase in intracellular calcium and thus increased force. Among the possibilities that further research must distinguish are (1) RB6 peptide may bind to Ang II, placing it in a preferred conformation for binding to its receptor; (2) RB6 peptide may work allosterically at the receptor itself to facilitate Ang II activity; (3) RB6 peptide may prevent cellular sequestration or degradation of the Ang II receptor; and (4) RB6 peptide may poorly activate a synergistic receptor system in smooth muscle (a possibility made particularly interesting due to the unexplained nature of the slow pressor effect). In each of these cases, it is assumed that as the concentration of RB6 peptide increases, its antagonistic activity to Ang II overwhelms that of its contractile augmentation activity. The direct effect of losartan on the Ang II receptor indicates the receptor’s sensitivity to functional conformational change. The augmentation of the response by such a mechanism (possibility 2 above) increases the scope of the potential manipulation of this receptor. RB6 peptide may therefore open up new drug development opportunities.

Whatever the mechanism of RB6 activity, the nature of its dual augmentation/antagonistic activity raises an interesting possible application of this or related compounds to migraine treatment. There are several theories of migraine production, and no definitive mechanism has been determined as yet. In one of those theories, migraines are thought to be characterized by an initial decrease in cerebral vascular perfusion accompanied by focal neurological deficits, followed by a phase in which there is an increase in cerebral blood flow accompanied by headache. A quickly degrading peptide given in high concentrations in the decreased-blood-flow, neurological-deficit phase would relax arterial blood vessels, increasing blood flow. The lower concentrations of peptide available at a later time would augment vascular contraction during the increased-blood-flow headache phase.

The negative results obtained with sequence variants of RB6 are also interesting (see Tables 1 and 2). RB7, which is the reverse of the RB6 sequence, was previously shown to have some anti-Ang II activity in physicochemical tests and an Ang II–receptor assay but had no measurable activity on smooth muscle. Adding amino acids to the N and C termini of RB6 destroyed its activity (RB5) and had no effect on the lack of activity displayed by RB7 (RB3 and RB4). We conclude that both sequence and end effects are critical components of the activity we are reporting here. We also note that sequences lacking the glycine residue in the sequence also destroyed peptide activity (data not shown). Thus, not only is the activity displayed by RB6 unique, it is also extremely sensitive to any alterations in the peptide.

Our results are also very important because they clarify a long-standing debate over the activity and mechanisms of Ang II antisense peptides. Elton et al have previously reported that Bl peptide antagonizes Ang II activity with a Ki of 58×10−9 mol/L, but assumed in their calculation is that all of the observed activity was due to direct binding of Blalock peptide to Ang II. In light of the failure of any physicochemical technique to validate this assumption, their calculation is
suscept, 5, 8, 9, 11–13, 27, 30 Direct measurement of activity gives a functional $K_d$ on the order of $2 \times 10^{-7}$ mol/L, some 200 times lower than previously reported (Fig 5). Such low sensitivity in vascular smooth muscle contractions obviates its usefulness. Also, in contrast to the 1 order of magnitude shift produced by Bl peptide (Fig 1), losartan shifts the Ang II dose-response curve of the rabbit aorta by 2.5 orders of magnitude. 31, 32 Mid-micromolar activity in conjunction with the lack of evidence for direct binding to Ang II and the strong similarity between Bl peptide and Ang II all suggest that Bl peptide is a sequence-variant antagonist of the Ang II receptor. To test this possibility, the effect of Ang II antisemur on Bl peptide activity was assayed. If Bl peptide bound directly to Ang II and not at all to the Ang II receptor, then one would expect that the effects of Ang II antisemur would be additive with those of Bl peptide. Both would bind up some proportion of Ang II, so that Ang II–induced muscle contractions would be less than if only the antisemur or only the Bl peptide were present. In fact, the effect of Ang II antisemur in the presence of Bl peptide was not additive (Fig 6). The degree of muscle contraction shows that, if anything, more Ang II was functionally available. This result is only possible if the Ang II antisemur bound up a significant proportion of Bl peptide, proving that Bl peptide and Ang II are antigenically similar. In light of the fact that Ang II antisemur can block Bl peptide activity, that Bl peptide is sequentially very similar to Ang II, and that DeGasparo et al 29 have found that antibodies against Bl peptide do not bind to the Ang II receptor, it is likely that antibody to Bl peptide binds directly to Ang II because of molecular mimicry between Ang II and Bl peptide.

We note that the same basic problems observed with Bl peptide in this study exist for a very wide range of peptides derived by the Mekler-Biro-Blalock approach. 14, 25, 26 Calculated binding constants often assume direct binding of the antipeptide to its peptide in the absence of physicochemical evidence or despite direct evidence to the contrary. 43–48 In some cases, 45, 49 the Root-Bernstein approach yields active antipeptide when the Mekler-Biro-Blalock approach does not. Reported binding constants vary from nanomolar values to no measurable binding by different research group, technique used, and the particular antipeptide being studied. 14, 25, 26

These experiments also negate any functional antipseptide-binding contractile-inhibition effect of Ang II antipeptides generated in the parallel reading frame. Although we were unable to complete a full activity curve for Root-Bernstein peptide (this procedure would have required approximately a gram of peptide to reach the high concentrations necessary), the data available suggest that the previously reported $K_d$ of $\approx 5 \times 10^{-7}$ mol/L (Reference 13) is within the right range. We calculate $\approx 80 \times 10^{-7}$ mol/L for the inhibition constant from the muscle contraction data. The augmentation effect will alter the apparent inhibition constant measurement because a higher concentration will be needed to overcome the augmentation effect. Adding Ang II antisemur to RB6 peptide in the presence of Ang II resulted in an additive decrease in Ang II activity, as would be expected if each acted directly on Ang II without interaction between the antisemur and RB6 peptide (Fig 6). Thus, our results are consistent with physicochemical data indicating that RB6 peptide is a true Ang II complement or antipeptide, not a receptor antagonist, 12, 13 but not at a physiologically useful concentration.

To summarize, our data demonstrate that although neither parallel- nor antiparallel-generated Ang II antipeptides have sufficient sensitivity to be pharmacologically useful in inhibiting Ang II contractions, the parallel antipeptide RB6 at high nanomolar to low micromolar concentrations exhibits unique previously unreported properties in augmenting Ang II contractions that may have great clinical and investigative potential. Elucidating the mechanism of the augmentation effects of RB6 may lead to a better understanding of the slow pressor effect of chronic Ang II exposure and of migraine treatment. The dual activity of RB6 on Ang II contractions could create a novel paradigm for peptide therapies if other antipeptide systems show similar dual activities.

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