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ORIGINAL ARTICLE

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Novel high molecular weight albumin-conjugated angiotensin II activates β-arrestin and G-protein pathways

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Abstract

Purpose To study the ability of a novel bovine serum albumin-angiotensin II (BSA-Ang II) conjugate to effect responses of the AT_1 angiotensin II receptor subtype mediated by the G-protein-coupled and the beta-arrestin pathways.

Methods Angiotensin II (Ang II) was conjugated with bovine serum albumin and compared with Ang II for competition binding to AT_1 receptors, to stimulate aldosterone release from adrenocortical cells, to promote beta-arrestin binding to AT_1 receptors, to promote calcium mobilization, and stimulate drinking of water and saline by rats.

Results The BSA-Ang II conjugate was less potent competing for AT_1R binding, but was equally efficacious at stimulating aldosterone release from H295R adrenocortical cells. Both BSA-Ang II and Ang II stimulated calcium mobilization and beta-arrestin binding to AT_1 receptors. BSA-Ang II and Ang II stimulated water appetite equivalently but BSA-Ang II stimulated saline appetite more than Ang II. Both BSA-Ang II and Ang II were considerably more potent at causing calcium mobilization than β-arrestin binding.

Conclusions Addition of a high molecular weight molecule to Ang II reduced its AT_1 receptor binding affinity, but did not significantly alter stimulation of aldosterone release or water consumption. The BSA-Ang II conjugate caused a greater saline appetite than Ang II suggesting that it may be a more efficacious agonist of this beta-arrestin-mediated response than Ang II. The higher potency calcium signaling response suggests that the G-protein-coupled responses predominate at physiological concentrations of Ang II, while the beta-arrestin response requires pathophysiological or pharmacological concentrations of Ang II to occur.

Keywords AT1 receptor · Biased agonism · Bovine serum albumin-conjugated angiotensin II · Calcium mobilization · Aldosterone release · Salt appetite

Abbreviations

Ang II	angiotensin II;
ARBs	angiotensin receptor blockers;
AT_1R	angiotensin II receptor subtype 1;
AT_2R	angiotensin II receptor subtype 2;
BSA-Ang II	Ang II conjugated to bovine albumin;
G-protein	guanine nucleotide binding protein;
GPCR	G-protein-coupled receptors;
ICV	intracerebroventricular;
MAPK	mitogen-activated protein kinase;
SI Ang II	Sarcosine[1] Isoleucine[8] angiotensin II;
SII Ang II	Sarcosine[1] Isoleucine[4] Isoleucine[8] angiotensin II;

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SMCC succinimydyl 4-[N-maleimidomethyl1]cyclohexane-1-carboxylate.

Introduction

The renin-angiotensin system (RAS) is a potent physiological system that regulates water homeostasis, electrolyte balance, blood pressure, and blood volume by several different mechanisms [1]. Angiotensin II (Ang II) indirectly regulates blood volume through activation of adrenocortical Ang II type 1 receptors (AT_1R), stimulating release of aldosterone [2], to increase sodium reabsorption in the kidney [3]. AT_1R is also present in other tissues such as liver, kidney, vascular smooth muscle, endothelium, and brain. Ang II activation of brain AT_1R, elicits a dipsogenic response [4–6], salt appetite [7, 8] and vasopressin (antidiuretic hormone) release. AT_1R stimulation in the kidney also directly promotes sodium reabsorption from the kidney [9]. AT_1R stimulation of vascular smooth muscle increases vascular resistance which increases blood pressure [10]. Hence, it is vital to elucidate the precise mechanisms of AT_1R activation and function.

The AT_1R is a member of Class A guanine nucleotide binding protein (G protein)-coupled receptor (GPCR) family characterized by seven-transmembrane spanning domains, with intracellular domains that interact with G proteins, kinases, arrestins, and phosphatases. Ang II-mediated activation of $G_{\alpha(q)}$ and $G_{\alpha(i)}$ proteins via the AT1R is well-documented [11]. In contrast, the β-arrestin signaling pathway, first observed more than 20 years ago [12] revealed that β-arrestin was not only involved in receptor internalization, but also activated a G-protein independent transduction signal. Subsequent work [13] identified the AT_1R as a GPCR that also signaled via a G-protein-independent signaling pathway involving ß-arrestin-1 and 2 [14]. β-arrestin has distinct MAPK (mitogen-activated protein kinase) pathways that promote cell survival, cytoskeletal rearrangement and other cellular actions [15, 16]. Developing a drug that selectively activates AT_1R β-arrestin signaling, reported to have cardioprotective effects [17], while simultaneously opposing its G-protein signaling, has been an area of focus in pharmacological research. Interestingly, recent works have shown that despite β-arrestin and G-protein pathways being separate, they do not always cause opposing responses. Overexpression of ß-arrestin increases aldosterone secretion, while muting of the G-protein pathway reduced Ang II stimulated aldosterone secretion in H295R cells [18–20].

Conformation of the ligand that binds to AT_1R can dictate its signaling, internalization, and phosphorylation [21]. An Ang II-derived peptide, such as sarcosine^1, isoleucine^4, isoleucine^8 (SII)-Ang II, that lacks the position-8 aromatic ring cannot activate the G-protein pathway, but can cause receptor internalization and is thereby β-arrestin-biased. This information provided new insights into the pharmacology of AT_1R leading to development of other high affinity, pathway selective ligands. These ligands, when tested in rats showed improved cardiac performance which was more beneficial than the effects of the sartan class of AT_1R antagonists, the angiotensin receptor blockers (ARBs). Such biased agonists were developed to provide greater benefits to cardiac hypertrophy patients [16]. However, the prototype biased agonist, sarcosine^1, D-alanine^8 Ang II (TRV027) showed no clinical advantage over conventional ARBs [22]. The mean arterial pressure reduction by TRV027 was similar to the unbiased ARB, telmisartan [22]. Although TRV027 initially showed promise in human studies, the clinical trials discontinued because TRV027 did not meet their criteria for treating heart failure patients [23].

Biased agonism of the AT_1R is reported to differentially stimulate consummatory responses. Downstream stimulation of PLC/PKC/IP_3 has been shown to elicit water intake and SII biased agonism via β-arrestin-mediated activation of saline intake [24, 25]. PEP7, a seven amino acid peptide encoded by the AT1 receptor gene, upstream of the AT1R coding region, is a novel biased ligand that selectively inhibits Ang II-mediated Erk1/2 activation, presumably by interfering with the ß-arrestin signaling pathway [26]. In addition, PEP7 inhibited Ang II-induced saline consumption in rats without affecting water consumption. As a result, PEP7 or a PEP7 mimetic might be used to control hypertension via sodium intake modulation [25]. In this study, we aimed to determine the binding characteristics and functionality of a novel BSA-conjugated Ang II (BSA-Ang II) ligand, developed to be a less internalizable agonist by steric hindrance [27], thereby inhibiting ß-arrestin signaling by the internalized AT_1R. We compared this BSA-Ang II conjugate with Ang II to determine its ability to bind to the AT_1R, and stimulate calcium mobilization, ß-arrestin binding to the AT_1R, aldosterone release, thirst, and salt appetite.

Methods

Preparation of BSA-Ang II conjugate

Cys^0-angiotensin II was conjugated to bovine serum albumin (BSA) via SMCC (succinimydyl 4-[N-maleimidomethyl1]cyclohexane-1-carboxylate) according to the manufacturer's instructions. SMCC (ThermoFisher Scientific; Cat. #22360) is a heterobifunctional, non-cleavable crosslinker consisting of a maleimide reactive group on one end and an NHS-ester reactive group at the opposite end, separated by a 8.3 angstrom cyclohexane

spacer arm. The SMCC was first attached to BSA using a molar excess of the linker and any remaining free linker was subsequently removed via dialysis. The cys-AngII peptide was then later reacted with the BSA-SMCC, again using a molar excess of peptide and any remaining free peptide removed via dialysis. Purity of the final conjugate was determined via SDS-PAGE and an average molecular weight determined from the gel. It was determined that there were five angiotensin II molecules bound to each bovine albumin molecule. For comparison purposes the molecular weight of the BSA-Ang II conjugate was determined to be 72 kDa. The initial concentration of the BSA-Ang II conjugate was 6.5 mg/ml which was calculated to be 90 μM BSA-Ang II conjugate and 450 μM Ang II. For comparison with Ang II, the 450 μM concentration of Ang II was used.

Cell culture and aldosterone release assays

H295R cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM: F12 medium, containing 15% cosmic calf serum (CCS Hyclone SH3008703) and 1% penicillin/streptomycin (Gibco 15140122). For treatment, cells were trypsinized and plated into 24-well plates at 50,000 cells per well. On the following day, cells had 500 μM APA-inhibitor pre-incubation for 1 h to inactivate proteases in serum-free DMEM: F12. Cells were then treated with BSA-Ang II and regular Ang II at concentrations ranging from 1 to 100 nM and 20 mM KCl for 3 h stimulation. Culture medium was collected after a 3-h incubation period and frozen until analyzed by ELISA (Aldosterone EIA Kit, ALPCO diagnostics, Salem NH, USA). Data were in concentrations of pg/ml in medium then normalized to untreated control.

Radioligand binding: saturation and competition assays

H295R cells and rat livers, collected fresh or frozen immediately following euthanasia were used for radioligand binding assays. On the day of the assay they were thawed, homogenized in water, and then washed by centrifugation (20,000 × *g* for 20 min at 4 °C). The supernatant was discarded and the membrane pellet was resuspended in assay buffer (150 mM NaCl, 5 mM EDTA, 0.1 mM Bacitracin, 50 mM Na_2HPO_4), re-centrifuged as before, followed by a final resuspension of the membrane pellet in assay buffer at 20 mg initial wet weight/ml. The membranes were incubated with six concentrations of ^{125}I-sarcosine^1, isoleucine^8 angiotensin II (^{125}I-SI Ang II) ranging from 0.25 to 3 nM in the presence or absence of 3 μM Ang II for 60 min at ~22 °C. Membrane-bound radioligand was separated from unbound radioligand using a Brandel M-24R cell harvester and GF/B filters. Filter bound radioligand was assayed in a gamma counter. Specific, angiotensin receptor binding, was calculated by subtracting non-specific binding, in the presence of 3 μM Ang II, from total binding, in the absence of Ang II. For experiments in H295R cells measuring AT_1R subtype, PD123319, 10 μM, was added to all tubes to saturate all the AT_2R. For experiments in H295R cells measuring AT_2R subtype, losartan, 10 μM, was added to all tubes to saturate all the AT_1R.

Binding data were analyzed with Graphpad PRISM software using a one-site specific binding equation. B_{max} values were in units of fmol/mg initial wet weight, or fmol/mg protein, in liver or H295R cells, respectively. AT_1R or AT_2R expression in cells were determined in the presence of 10 μM PD123319 or losartan, respectively. Protein concentration in the H295R cell saturation assays was determined by bicinchononic acid (BCA) assay (Pierce, Illinois).

Competition binding assays were performed with 0.2 nM ^{125}I-SI-Ang II with competing ligand concentrations of BSA-Ang II ranging from 10 nM to 10 μM and Ang II ranging from 3 nM to 100 μM. Graphpad PRISM software was used to determine IC_{50} values using a one-site competition binding equation.

In vivo experiments

Animals

Male Sprague Dawley rats were purchased from Envigo Laboratories (Indianapolis, IN). Rats were maintained in standard rodent plastic shoebox cages until at least 1 week before behavioral testing, at which time they were singly housed in hanging wire-mesh stainless steel cages affixed with an electrically isolated metal plate with 3.2 mm openings through which rats need to lick to reach a spout on the other side. Rats had ad libitum access to food (Teklad 2018; Envigo Laboratories) tap water, and were also given a second bottle containing 1.5% saline for at least a week before the start of experiments, and throughout the entire experiment. The temperature- and humidity-controlled colony rooms were maintained on a 12:12 h light-dark cycle. All experiments occurred during the early portion of the light phase. All experimental protocols were approved by the University at Buffalo Institutional Animal Care and Use Committee, and the handling and care of the animals was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Surgery

Rats were implanted with intracerebroventricular (ICV) cannulae as described previously [25, 28]. Surgical anesthesia was induced by ketamine (70 mg/kg) and xylazine

(5 mg/kg) and post-surgical care included injection of carprofen (5 mg/kg) and a bolus injection of isotonic saline (5 ml). Accurate placement of cannula was verified by measuring the drinking response to a single injection of Ang II (10 ng) and rats that did not drink at least 6 ml in 20 min after the injection were excluded from further testing.

Experimental design and intake measures

Rats were habituated to the injection procedure by regular handling and mock injections. A repeated measures design was used in which each rat received an injection of vehicle (2 µl tris-buffered saline; TBS), 10 ng Ang II, 130 ng BSA-Ang II (9.4 ng Ang II equivalents), or 650 ng BSA-Ang II (47 ng Ang II equivalents). This dose of AngII was chosen because it produces a submaximal response [29]), thereby allowing us to test for increases or decreases in intake without concern of a floor or ceiling effect. At least 2 days were allowed between injections, and rats included in the results section received all four injections. Injection order was controlled for as best as possible given the sample size.

On each experiment day, rats were injected and returned to their cages with pre-weighed bottles (one containing water, the other containing 1.5% saline). Licks at each spout were counted by a contact lickometer (Psychology Electronics Shop, University of Pennsylvania, Philadelphia, PA) with custom software running in a MATLAB environment. The software used a 1 kHz sampling rate to avoid missing any spout contacts, and non-tongue contact with the spout was minimized because of the electrically isolated metal plate separating the inside of the cage from the spouts. Licking was measured for the duration of the test, after which bottle rates were recorded.

DiscoveRx assays

DiscoveRx Corporation was contracted to perform two assays: β arrestin binding to AT1Rs and calcium mobilization. The assays were performed in the same cell line to ensure that receptor expression level was consistent between each type of test. These studies are utilized to delineate activation of ß-arrestin pathway or G-protein pathway. All assays were done according to DiscoveRx protocols. The GPCR arrestin binding assay was performed with cell lines that stably express inactive complementary portions of ß-galactosidase linked to ß-arrestin and the GPCR. When ß-arrestin binds to the GPCR the two fragments of ß-galactosidase are joined, activating the enzyme which then acts upon its substrate producing a fluorophor whose light emission is then measured. The calcium mobilization assay used PathHunter® cell lines or other cell lines stably expressing G_q-coupled GPCRs uses a calcium-sensitive dye that is loaded into the cells. GPCR activation by an agonist causes the release of calcium from intracellular stores which increases dye fluorescence that is measured in real-time.

Statistical analyses

Determinations of B_{max} and K_d for receptor binding were made using Prism (Graphpad software) one-site specific binding model. IC_{50} values were determined using Prism one-site log (IC_{50}) competition model. Comparisons of IC_{50} values for BSA-Ang II conjugate and Ang II were made using a paired *t*-test. Concentration-response curves were obtained using Prism monophasic log (agonist) vs response model with variable Hill slope. Resulting EC_{50} and maximal response % were compiled and analyzed by two-way ANOVA, with Sidak's post hoc multiple comparisons test. Aldosterone secretion levels were compared using two-way ANOVA. Values given are mean ± SEM.

Analysis of drinking behavior was conducted on volumes consumed over the test, and on licking in discrete 10-min bins. Licks for water or saline in 10-min bins following ICV Ang II or BSA-Ang II conjugate administration were also analyzed using separate 2 factor ANOVAs with Drug and Time as within subjects factors. For all statistical tests, $p < 0.05$ was considered statistically significant, and Newman Keul's post hoc tests were used to further probe significant results in the ANOVAs.

Results

BSA-Ang II and Ang II have significantly different IC_{50}

The half-maximal inhibitory concentration (IC_{50}), is a measure of how well a substance is able to displace another ligand, in this case, ^{125}I-SI Ang II, at a given receptor, in this case, AT_1R. Rat liver tissue has abundant AT1R [30]. Using 0.2 nM ^{125}I-SI-Ang II, as the AT_1R radioligand and BSA-Ang II and Ang II at concentrations ranging from 1 nM to 100 µM, the IC_{50} values for BSA-Ang II and Ang II was determined to be 52.8 ± 17 and 12.4 ± 3 nM, respectively which is approximately a fourfold difference (Fig. 1). The difference in potency was significant by paired Student's *t*-test ($p < 0.05$).

H295R cells express AT1R but not AT2R

Bird et al. [31, 32] showed that H295R cells primarily express AT_1R mRNA and protein. We documented the expression of AT_1R in H295R cells with a B_{max} of 156 ± 62 fmol AT1R/mg protein, and a K_D of 0.72 ± 0.14 nM using ^{125}I-SI Ang II in the presence of 10 µM PD123319

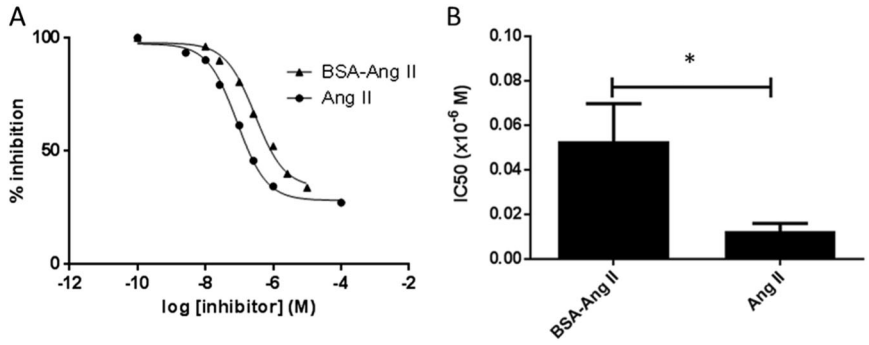

Fig. 1 Competition for ^{125}I-SI Ang II binding to AT1R in rat liver by Ang II and BSA-Ang II. **a** Representative competition curves for Ang II and BSA-Ang II binding to ^{125}I-SI Ang II binding sites in rat liver membrane suspensions. **b** IC_{50} values were 12.4 and 52.8 nM, for Ang II and BSA-Ang II, respectively, summarized for seven independent assays (mean ± SEM). Paired *t*-test, $*p < 0.05$

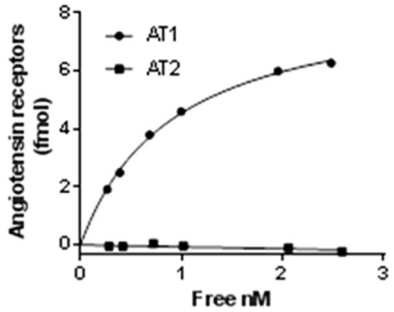

Fig. 2 Representative saturation binding curve for ^{125}I-SI Ang II binding to AT1R (in presence of 10uM PD123319) or AT2R (in presence of 10 µM Losartan) in H295R cells. ^{125}I-SI Ang II concentration range 0.25–2.5 nM. Non-specific binding was determined in the presence of 3 µM Ang II. Specific receptor binding was derived by subtracting "total" binding (in the absence of 3 µM) from non-specific binding. The average B_{max} values AT1R for $n = 3$ assays was 156 ± 62 fmol/mg protein with $K_D = 0.72 \pm 0.14$ nM. AT2R binding was not detectable

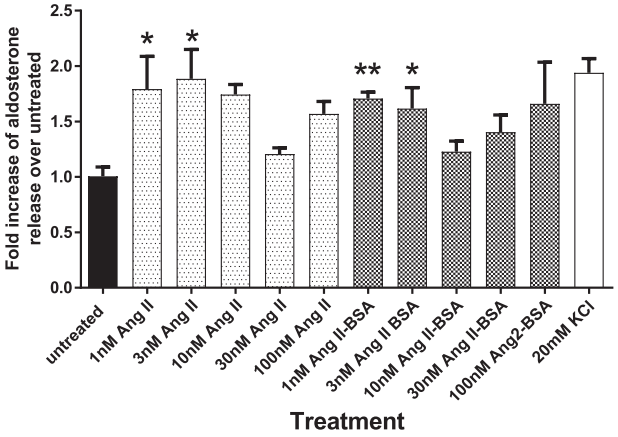

Fig. 3 Ang II and BSA-Ang II concentration-response relationships for aldosterone secretion from H295R cells measured by EIA (mean ± SEM, $n = 4$). Normalized untreated value was 22.3 ± 1.9 pg/ml. One-way ANOVA of the concentration-response curves for Ang II ($F_{3,15} = 3.87$, $p = 0.038$) and BSA-Ang II ($F_{3,14} = 0.007$) up to 10 nM revealed that only the 1 and 3 nM concentrations of each agonist significantly increased aldosterone release. Two-way ANOVA of revealed no significant difference between the abilities of Ang II and BSA-Ang II to stimulate aldosterone release at all doses tested. $*p < 0.05$ compared with control, $**p < 0.01$ compared to control

(Fig. 2). AT_2R (Angiotensin II receptor type 2) binding of ^{125}I-SI Ang II in the presence of 10 µM losartan was not detectable in the H295R cells (Fig. 2). AT_2R is expressed in rat adrenal but occurs predominantly in the medulla [33].

Ang II and BSA-Ang II are equipotent in stimulating aldosterone secretion in H295R cells at physiological concentrations

After verifying AT_1R binding in the H295R cells, a concentration-response assessment of aldosterone secretion responses to Ang II and BSA-Ang II was run. The concentration-response curves demonstrated that both ligands stimulated aldosterone secretion in an equivalent manner. The two lowest concentrations of Ang II and BSA-Ang II caused near maximal aldosterone release compared with 20 mM KCl (Fig. 3), suggesting that the cells were highly sensitive to both agonists. There was no significant difference in the aldosterone secretion responses to Ang II and BSA-Ang II which would suggest that internalization of receptors does not affect aldosterone secretion in this adrenal cortical cell model.

G-protein mediated calcium mobilization versus ß-arrestin binding to AT_1R by Ang II and BSA-Ang II

Assessment of calcium mobilization (a G-protein mediated response to angiotensinergic stimulation of the AT) and the binding of ß-arrestin to the AT_1R using a split enzyme activation protocol (DiscoveRx, California) revealed that both Ang II and the BSA-Ang II conjugate were significantly more potent at stimulating intracellular calcium mobilization than

they were at causing ß-arrestin to bind to the AT_1R (Fig. 4, Table 1). The EC_{50}s for Ang II and BSA-Ang II to stimulate calcium mobilization were 172 and 179 pM, respectively, whereas the EC_{50}s for Ang II and BSA-Ang II to stimulate ß-arrestin binding were 2.56 and 4.53 nM, respectively. Ang II was significantly more potent than the BSA-Ang II conjugate to promote ß-arrestin binding, although the maximal ß-arrestin binding to AT_1R was significantly greater with BSA-Ang II (Fig. 4, Table 1). In contrast, both agonists were statistically equipotent at stimulating calcium mobilization, but the maximal response to Ang II was significantly greater than that of the BSA-Ang II conjugate, suggesting that the BSA-Ang II conjugate is a partial agonist of the G-protein mediated response (Fig. 4, Table 1).

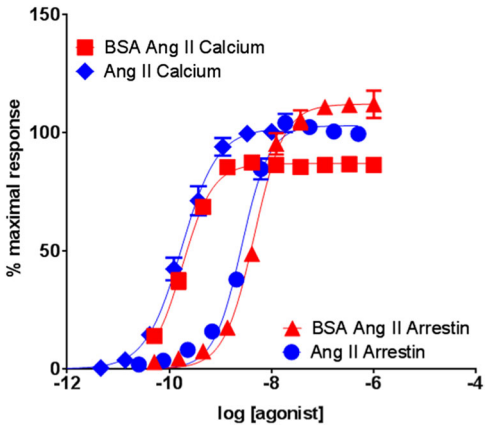

Fig. 4 DiscoveRx Calcium vs Arrestin signaling with BSA-Ang II or Ang II ligands. Arrestin signaling is measured by enzyme-catalyzed fluorescence when arrestin binds GPCR. Calcium signaling is measured by calcium-sensitive dye. Data presented (from two replicates) were fitted on log (agonist) vs (response-variable slope) four parameters. Resulting EC_{50} and maximal response % were compiled and analyzed by two-way ANOVA, Sidak's multiple comparisons. Maximal stimulation % showed significant differences between Ang II and BSA-Ang II for arrestin ***$p < 0.0003$ and calcium pathways ****$p < 0.0001$. In addition, log (EC_{50}) was significantly different between Ang II and BSA-Ang II in arrestin pathway ****$p < 0.0001$

Also of note, the Hill slopes of both agonists for both responses were significantly >1 (Table 1). While this is normally interpreted as indicating positive cooperativity, it could also be indicative of spare receptors, i.e., the calcium response may only require activation of a small proportion of the AT1R to provide a maximal response, which would increase the slope of the concentration–response curve. The steep dose–response curve for ß-arrestin binding could indicate a threshold effect, such that only with a high proportion of receptor occupancy would there be measurable ß-arrestin binding, which would then rapidly increase to attain a maximum level of interaction with the near fully occupied receptor population.

BSA-Ang II and Ang II induced a similar water appetite but greater saline appetite when administered ICV to rats

Rats with stable cannulae throughout the entire experiment ($n = 8$) were given injections of 10 ng Ang II, 130 ng (9.4 ng Ang II equivalent) BSA-Ang II, and 650 ng (47 ng Ang II equivalent) BSA-Ang II. Measures of drinking behavior after these injections are shown in Figs 5 and 6. Measurement of total water and saline intake ($n = 8$) revealed no difference in the amount of water consumed in response to 10 ng Ang II, 9.4 ng of Ang II in the BSA Ang II conjugate or 47 ng of Ang II in the BSA Ang II conjugate, $F_{2,14} = 1.34$ (Fig. 5 panel a). However, there was a significant difference in saline intake $F_{2,14} = 5.01$, $p = 0.02$. Post hoc tests indicated that the 47 ng dose of BSA Ang II conjugate response was greater than the 10 ng Ang II dose (Fig. 5 panel b).

To further probe the drinking behavior, we analyzed lick data in discrete 10 min bins. One rat was excluded from this analysis because lick data were unavailable. Analysis of

Table 1 DiscoveRx™ assay results comparing EC_{50}, maximum response and Hill slope for Ang II and BSA-Ang II, for ß-arrestin binding and calcium signaling responses

	Log (EC_{50}) Mean ± SEM		Maximum response Mean ± SEM		Hill slope Mean ± SEM	
	ß-arrestin binding	Calcium signaling	ß-arrestin binding	Calcium signaling	ß-arrestin binding	Calcium signaling
Ang II	−8.59 ± 0.03[a]	−9.76 ± 0.03[b]	103 ± 2.0	102 ± 2.1[c]	1.60 ± 0.15	1.23 ± 0.10
BSA-Ang II	−8.34 ± 0.03	−9.75 ± 0.02[b]	112 ± 1.8[d]	86.9 ± 0.7	1.47 ± 0.13	1.45 ± 0.09

Two-way ANOVA of EC_{50} values were significantly different between response ($F_{1,116} = 2098$, $p < 0.0001$), Ang II versus BSA-Ang II ($F_{1,116} = 22.0$, $p < 0.0001$) and interaction ($F_{1,116} = 16.7$, $p < 0.0001$). Two-way ANOVA of maximum response values was significantly different between response ($F_{1,116} = 65.6$, $p < 0.0001$), but was not significantly different for Ang II versus BSA-Ang II ($F_{1,116} = 3.14$, $p > 0.05$), with a significant interaction ($F_{1,116} = 54.2$, $p < 0.0001$). Hill slopes were all significantly different from 1.0, $p < 0.02$, but did not differ otherwise

[a]Significantly more potent than BSA-AngII, $p < 0.05$

[b]Significantly more potent than the ß-arrestin binding response $p < 0.05$

[c]Significantly greater than BSA-Ang II, $p < 0.05$

[d]Significantly greater than Ang II, $p < 0.05$

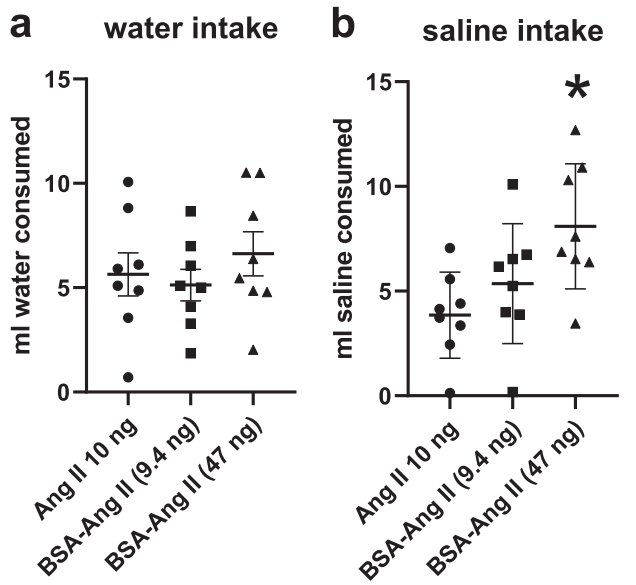

Fig. 5 Cumulative water and saline intake in response to BSA-Ang II conjugate and Ang II administered ICV. The total amount of water consumed (**a**) and saline consumed (**b**) in response to Ang II (10 ng) and BSA Ang II conjugate (9.4 and 47 ng) over a 60 min period post-treatment is shown. $n = 8$, $^*p < 0.05$ versus 10 ng Ang II. Bars indicate mean ± SEM

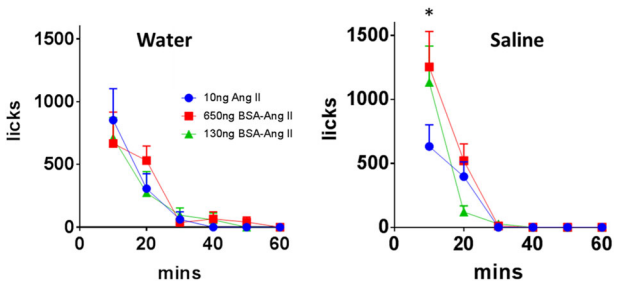

Fig. 6 Lick responses to BSA-Ang II conjugate and Ang II. The number of licks for water or saline in response to the administration of Ang II and two doses of BSA-Ang II conjugate are shown in 10-min bins over time. There were no significant differences in licks for water (left panel) caused by Ang II versus the two doses of BSA-Ang II. There were significantly more saline licks (right panel) with both doses of BSA-Ang II conjugate compared with Ang II in the first 10-min bin ($p < 0.05$). $n = 7$, $^*p < 0.05$ greater and 10 ng Ang II response. Bars represent mean ± SEM

licking for water revealed a main effect of Time ($F_{5,30} = 14.7$, $p < 0.001$), but did not detect any effect of drug on licks for water ($F_{2,12} = 0.19$, $p = 0.83$) or a significant time × drug interaction ($F_{10,60} = 0.55$, $p = 0.85$). Analysis of licking for saline also revealed a main effect of time ($F_{5,30} = 35.7$, $p < 0.001$), without a main effect of drug ($F_{2,12} = 3.23$, $p = 0.08$). However, in contrast to the analysis of licks for water, there was a significant time × drug interaction on licks for saline ($F_{10,60} = 2.02$, $p = 0.047$). Post hoc tests probing the interaction revealed that treatment with either dose of the BSA-Ang II conjugate produced more licks for saline in the first 10 min than was observed after treatment with 10 ng Ang II ($p < 0.05$, Fig. 6).

Discussion

In this study, we designed a high molecular weight Ang II conjugate; based on the hypothesis that it would reduce receptor internalization and thereby β-arrestin signaling [27, 34]. Based upon the duration of the responses to Ang II of different molecular weight conjugates of Ang II determined to be reflective of the rate of receptor internalization: ~3.5-fold increase in duration half-time at 45 kDa and 8.7-fold increase in duration of half-time at 527 kDa [27], we estimate that the 72 kDa BSA-Ang II conjugate bound AT_1R would have a fourfold extended duration of response and thus be internalized at 1/4th the rate of Ang II bound AT_1R. We tested this ligand in vitro, and in vivo. The pharmacological characteristic of this novel ligand was first defined by competition for the AT_1R-binding site in rat liver tissue. Competition binding assays revealed the BSA-Ang II had a ~4-fold lower affinity compared with Ang II, for competing with ^{125}I-SI Ang II for the AT1R binding site. Possible explanations for this difference are that only one, or possibly two, of the BSA-conjugated Ang II molecules are able to access AT_1R simultaneously in tissue membrane preparations, or that the BSA molecule may affect binding by steric hindrance, thereby decreasing the conjugated Ang II's ability to interact with AT1R, compared with the individual Ang II molecule.

After defining how BSA-Ang II interacts with the AT_1R, we assessed the physiological effects of BSA-Ang II in H295R adrenal cortical cells to observe BSA-Ang II effects on aldosterone secretion. Bird et al. [35] characterized H295R cells as a suitable model to observe the Ang II stimulated aldosterone response. In our characterization of these cells, we confirmed these cells express abundant AT1R and no detectable AT2R (Fig. 2). This characteristic of adrenal cortical cells expressing high AT1R expression is consistent with a previous study [33]. H295R cells respond to Ang II stimulation by increasing aldosterone secretion. In Fig. 3, BSA-Ang II and Ang II at 1 and 3 nM stimulated aldosterone release equivalently at near maximal rates as defined by responsivity to 20 mM KCl. Overall, Ang II and BSA-Ang II did not display any major difference in stimulating aldosterone secretion.

The intracellular signaling assays that delineated ß-arrestin binding to AT1R and G-protein mediated calcium mobilization revealed interesting perspectives and minor divergences with respect to how each of the Ang II ligands activate intracellular signaling pathways. BSA-conjugated Ang II was essentially equipotent with Ang II to stimulate calcium mobilization, although it was ~15% less

efficacious, suggesting it is only a partial agonist for the calcium mobilization response. However, the equivalent aldosterone secretion and water drinking responses mediated by both agonists could suggest the presence of spare AT_1R for the stimulation of aldosterone and thirst by Ang II.

BSA-conjugated Ang II was slightly, but significantly less potent (1.8 times) than Ang II at promoting ß-arrestin binding to the AT_1R, however, it was 9% more efficacious than Ang II (Fig. 4, Table 1). The decrease in potency of the BSA-conjugated Ang II to promote ß-arrestin binding would suggest that it should be less efficacious at stimulating saline intake since the salt appetite response to Ang II binding to the AT_1R is mediated by the ß-arrestin MAPK pathway [24–26]. However, the greater maximum ß-arrestin binding would be consistent with a greater saline appetite response.

The ß-arrestin binding to the AT_1R is dependent upon phosphorylation of the AT_1R by G-protein-coupled receptor kinases (GRKs) [36]. One possible explanation for the altered affinity and maximum binding of ß-arrestin to the AT_1R could be that the conformation of the GRK phosphorylated BSA-Ang II conjugate bound AT_1R is less optimal for ß-arrestin binding than Ang II bound AT_1R, but that a greater proportion of AT_1R is phosphorylated in response to BSA-Ang II conjugate binding leading to a greater number of AT_1R that are available for ß-arrestin binding.

While MAPK activation can occur via both G-protein and ß-arrestin-stimulated pathways [37, 38], the activation of MAPK by ß-arrestin appears to be the primary pathway for stimulation of salt appetite [25, 26]. Biased ligands such as sarcosine[1], isoleucine[4], Isoleucine[8] (SII) which selectively activate MAPK pathways while simultaneously inhibiting G-protein-mediated pathways [24], increase NaCl intake in male rats, while inhibitors of the G-protein linked activation of MAPK do not inhibit NaCl intake [25].

If there are differences in agonist bound AT_1R–ß-arrestin binding, then it is possible that different agonists of the AT1R might have different abilities to promote ß-arrestin-mediated signaling by the AT_1R. Thus, the higher amount of ß-arrestin binding to BSA-Ang II bound AT_1R may be mediating the increased saline appetite caused by the agonist compared with Ang II bound AT_1R. By virtue of this increased ß-arrestin binding to the BSA-conjugated Ang II bound AT_1R, BSA-Ang II could be a biased agonist in favor of the ß-arrestin pathway, for which it has increased efficacy measured as licks for a saline solution, compared with Ang II bound AT_1R. However, recent studies by Eichel and colleagues [39, 40] suggest that activation of ß-arrestin by GPCRs can be sustained via transfer of the activated ß-arrestin from the receptor to the endosome independently of the G-protein-coupled receptor

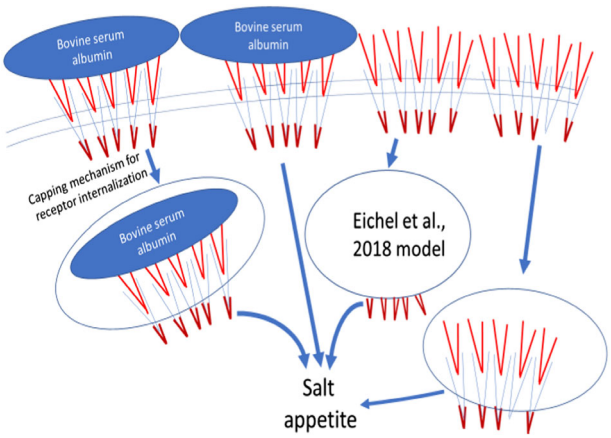

Fig. 7 Proposed mechanisms for stimulation of salt appetite by agonist stimulation of brain AT_1 receptors. Four proposed mechanisms for stimulation of salt appetite by either BSA-conjugated Ang II molecules or single Ang II molecules. From left to right, the capping mechanism whereby a group of receptors is occupied concurrently by the BSA-conjugated Ang II molecules, forming a cap which is internalized in an endosome with ß-arrestin and which subsequently activates an intracellular cascade of phosphorylation events eventually causing salt appetite. The second mechanism as proposed from this study is that the BSA-conjugated Ang II molecules bind to a cohort of AT_1 receptors, all of which bind ß-arrestin, do not internalize, but are still able to initiate the intracellular cascade of phosphorylation events eventually causing salt appetite. The third mechanism is that proposed by Eichel et al., 2018 in which ß-arrestin becomes activated by binding to the AT_1R, dissociates from the AT_1R and binds to an endosome which is internalized leading to initiation of the intracellular cascade of phosphorylation events eventually causing salt appetite. The last mechanism proposed in this study is that not all of the individual Ang II-bound AT_1R's bind to ß-arrestin, such that fewer ß-arrestin molecules are internalized with the endosome, leading to a diminished intracellular cascade of phosphorylation events, causing a smaller salt appetite than is seen with the BSA-conjugated Ang II molecules. Ang II molecules are portrayed as long red V shapes, AT_1 receptors are portrayed as long blue V shapes and ß-arrestin is portrayed as short rust colored V shapes. The curved parallel lines indicate the plasma membrane of an AT_1R-containing cell. The white ovals with blue outline represent endosomes. For purposes of clarity, AT_1R phosphorylation and the intracellular kinase cascade of phosphorylation events are not included in this diagram

(see depiction in Fig. 7), which is not consistent with our observations of increased saline consumption which occurs via the ß-arrestin MAPKinase pathway. Rather, the saline drinking response to the BSA-conjugated Ang II during the first 10-min bin following ICV administration, would suggest that internalization of the ß-arrestin bound AT_1 receptor may not be required for the ß-arrestin-mediated saline appetite response, as also depicted in Fig. 7.

Alternatively, another possible explanation for similar potency in activating ß-arrestin signaling with our ligands is through binding and internalization via a capping effect [41, 42]. Antibodies, which are larger than the BSA-Ang II ligand, have been shown to bind more than one receptor, forming a patch that is subsequently internalized by cells. When this "cap" is internalized, it may then activate

ß-arrestin signaling as it is traditionally understood [16]. Concurrent BSA-Ang II binding to multiple receptors could thus form a cap for internalization of this receptor-ligand complex (as depicted in Fig. 7), yielding the higher ß-arrestin maximal response for the saline drinking response as opposed to a single-uncapped Ang II-AT_1R response.

While MAPK activation occurs via both G-protein and ß-arrestin-stimulated pathways [37, 38], the activation of MAPK by ß-arrestin appears to be the primary pathway for stimulation of salt appetite [25, 26]. Biased ligands such as sarcosine[1], isoleucine[4], Isoleucine[8] (SII) which selectively activate MAPK pathways while simultaneously inhibiting G-protein-mediated pathways [24], increase NaCl intake in male rats, while inhibitors of the G-protein linked activation of MAPK do not inhibit NaCl intake [25].

We provided new insight to this puzzle by tying together higher ß-arrestin binding maximal response observed with BSA-Ang II conjugate binding to AT1R, and higher saline intake observed in BSA-Ang II-treated rats. Consistent with a role of ß-arrestin/MAPK in saline, but not water, intake, we found that water intake was not different after Ang II or BSA-conjugated Ang II. However, contrary to the anticipated reduction in saline intake with the BSA-Ang II conjugate, saline appetite as indicated by licks was significantly greater after treatment with BSA-Ang II compared with Ang II. Analysis of volumes of water and saline consumed gave similar results although only the 47 ng dose of BSA-Ang II conjugate caused significantly greater saline intake than that of 10 ng Ang II. We used a dose of Ang II (10 ng) that would produce a submaximal response, thereby allowing us to observe increases or decreases in intake without concern of a floor or ceiling effect. Moreover, it is important to note that after treatment with Ang II, males consume more water than saline, particularly with the 1.5% concentration used here [25, 29] and the greater water licking data in response to 10 ng Ang II in this study confirmed that observation. However, after treatment with the BSA-Ang II conjugate, saline licking was approximately twice as much as water licking (Fig. 6 panels a and b). Similar patterns of water to saline intake ratios were seen when cumulative water and saline intake in response to Ang II or BSA-Ang II were compared (Fig. 5 panels a and b). This may indicate that ß-arrestin signaling by the AT1R may not require receptor internalization, and that the enhanced binding of ß-arrestin to the BSA-Ang II conjugate agonist-occupied AT1R may selectively enhance ß-arrestin-mediated signaling. A diagram of the four putative mechanisms for stimulation of salt appetite by the BSA-Ang II conjugate and Ang II binding to AT_1R discussed herein is shown in Fig. 7.

Some additional observations that can be made from this study are that G-protein signaling is most likely considerably greater than ß-arrestin signaling under physiological conditions, wherein Ang II levels will be in the picomolar range. The EC_{50} of both Ang II and BSA-Ang II for calcium signaling was in the picomolar range, whereas the EC_{50} of both ligands for ß-arrestin binding was in the nanomolar range, which is more representative of a pathophysiological or pharmacological concentration of Ang II. Most studies of receptor internalization use supraphysiological concentrations of agonist ligand to achieve a measurable response and may overestimate the significance of this response relative to the G-protein-coupled response. The lack of advantage of biased agonists such as TRV-027 over ARBs to antagonize classical AT_1 receptor mediated responses could indicate that higher concentrations of biased agonists are required to cause receptor internalization than is required for them to compete for Ang II binding to AT_1Rs.

Another notable observation from this study is the near equal physiological potency of BSA-conjugated Ang II with Ang II in view of the fact that the BSA-Ang II molecules were tethered together in groups of five on a BSA backbone, whereas the Ang II molecules exist individually. This comparison is even more pronounced for the saline appetite study in which slightly less than the concentration of Ang II from the total Ang II molecules in the BSA-Ang II conjugate gave a greater salt appetite response than the individual Ang II molecules. Indeed, a pilot study showed that a subset of four rats that received an injection of 100 ng of Ang II, in addition to the treatment conditions described above, still consumed less saline and had fewer licks for saline than they did in response to either the 9.4 or the 47 ng doses of the BSA Ang II conjugate (data not shown). This suggests that more than one, and perhaps all five of the Ang II molecules tethered to the BSA can bind simultaneously to Ang II receptors that are in close apposition to each other, possibly as multimers, and that this simultaneous binding produces a stronger response than the individual binding of Ang II to AT_1Rs. Alternatively, reduced internalization of AT_1R leading to a more sustained response to the BSA-Ang II conjugate (second scenario from the left in Fig. 7) in which beta-arrestin signaling occurs without receptor internalization, with the receptors remaining on the surface, still responsive to agonist activation.

A potential limitation to this study is the report that ß-arrestin signaling can increase calcium mobilization, however, the potency for both Ang II and BSA-Ang II to increase ß-arrestin binding was more than 10-fold less than for the calcium mobilization response. Accordingly, the contribution of ß-arrestin signaling to the calcium response would be minimal at best.

Another limitation of this study is that it did not directly measure AT_1R internalization, so the extent of receptor internalization that may have occurred in response to the BSA-Ang II conjugate cannot be definitively determined.

In summation, we have created a novel Ang II molecule that displays both similarities and differences from Ang II in its interactions with AT_1Rs. The binding affinity of this BSA-Ang II conjugate for AT_1R is slightly less than that for Ang II, but its ability to stimulate aldosterone release and water intake is equipotent with Ang II. It has a similar potency to stimulate calcium mobilization mediated by AT_1R but only as a partial agonist. In contrast to Ang II, BSA-Ang II is less potent at stimulating ß-arrestin binding to the AT_1R, but has greater efficacy for promoting ß-arrestin binding to the AT1R. This increased efficacy may explain the greater ability of BSA-Ang II to stimulate saline intake compared with Ang II. BSA-conjugated Ang II may be a useful ligand in investigating partial agonism of angiotensin receptors or studies of salt appetite and management in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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