



Update on angiotensin II: new endocrine connections between the brain, adrenal glands and the cardiovascular system

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Abstract

In the brain, angiotensinergic pathways play a major role in chronic regulation of cardiovascular and electrolyte homeostasis. Increases in plasma angiotensin II (Ang II), aldosterone, [Na⁺] and cytokines can directly activate these pathways. Chronically, these stimuli also activate a slow neuromodulatory pathway involving local aldosterone, mineralocorticoid receptors (MRs), epithelial sodium channels and endogenous ouabain (EO). This pathway increases AT₁R and NADPH oxidase subunits and maintains/further increases the activity of angiotensinergic pathways. These brain pathways not only increase the setpoint of sympathetic activity *per se*, but also enhance its effectiveness by increasing plasma EO and EO-dependent reprogramming of arterial and cardiac function. Blockade of any step in this slow pathway or of AT₁R prevents Ang II-, aldosterone- or salt and renal injury-induced forms of hypertension. MR/AT₁R activation in the CNS also contributes to the activation of sympathetic activity, the circulatory and cardiac RAAS and increase in circulating cytokines in HF post MI. Chronic central infusion of an aldosterone synthase inhibitor, MR blocker or AT₁R blocker prevents a major part of the structural remodeling of the heart and the decrease in LV function post MI, indicating that MR activation in the CNS post MI depends on aldosterone, locally produced in the CNS. Thus, Ang II, aldosterone and EO are not simply circulating hormones that act on the CNS but rather they are also paracrine neurohormones, locally produced in the CNS, that exert powerful effects in key CNS pathways involved in the long-term control of sympathetic and neuro-endocrine function and cardiovascular homeostasis.

Key Words

- ▶ angiotensin II
- ▶ aldosterone
- ▶ endogenous ouabain
- ▶ neurohormones
- ▶ CNS regulation

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Introduction

The classical view of the role of the central nervous system (CNS) in the regulation of the cardiovascular homeostasis focuses on the critical importance of the CNS in the acute, short-term control. In this paradigm, a variety of reflexes send afferent information to the brain and control sympathetic nerve traffic to arteries and veins, heart and kidneys. According to the Guytonian view

of the circulation, these reflexes reset and the CNS and sympathetic activity have only limited involvement in the chronic regulation of the cardiovascular homeostasis and are not involved in the setpoint of the blood pressure (BP). In the last two decades, it has become apparent that this concept is too narrow. Endocrine afferent and efferent connections to and from the brain can play a



powerful role in the long-term control of the circulation in, for example, heart failure and hypertension. In this brief review, we address only one aspect of these extensive endocrine interactions, i.e. the important new concept that angiotensin II (Ang II), aldosterone and endogenous ouabain (EO) are not simply circulating hormones that act on the CNS but rather that they are also paracrine neurohormones, locally produced in the CNS, that exert powerful effects in key CNS pathways involved in the long-term control of sympathetic and neuroendocrine function and the regulation of cardiovascular homeostasis. We will first provide an update on CNS angiotensinergic pathways, and then review the actions of plasma Ang II, aldosterone and EO mediated through these pathways. The next section reviews studies on the role of brain Ang II in the regulation of plasma aldosterone and EO, and effects of EO on arteries and the heart. We conclude with a review of recent studies on blockade of the CNS angiotensinergic pathways as a therapeutic strategy for treatment of hypertension and heart failure.

CNS pathways contributing to long-term regulation of the circulation

Daily living depends critically on the acute millisecond-to-millisecond regulation by the CNS of sympathetic and parasympathetic nerve traffic to the heart and arteries. After release into the synaptic cleft, neurotransmitters such as glutamate or GABA bind to ligand-gated ionotropic receptors, which change their conformation and open their ion channels in less than a millisecond. The resulting ion currents can initiate neuronal membrane depolarization and generate high-speed action potentials along the axon, as well as subsequent repolarization. The effectiveness of this fast synaptic transmission can be influenced by a variety of mechanisms. For example, the quantity of neurotransmitter release from the presynaptic terminal in response to an action potential can be modulated. Also, the responsiveness of the post-synaptic neuron to a given amount of neurotransmitter can be altered by changing the number of receptors and/or the activity of intracellular signaling pathways (1). Such pre- and post-synaptic changes may blunt or enhance autonomic and cardiovascular responses. In general, however, autonomic activity will return quickly to its setpoint due to buffering mechanisms such as arterial baroreflex resetting. These glutamate-/GABA-ergic autonomic pathways are primarily intended for the acute regulation of autonomic tone.

Nonetheless, overcoming this resetting by chronic electrical stimulation of the carotid baroreceptors causes persistent decreases in sympathetic activity and BP (2). The resulting enhanced GABA-ergic inhibition lowers the setpoint for sympathetic tone but may also inhibit responsiveness to acute stresses.

In contrast, slow-acting CNS pathways involve signal transduction over seconds to minutes. In the case of angiotensinergic pathways, Ang II released into the synaptic cleft binds to angiotensin type 1 receptors (AT₁R); this stimulates a G-protein signaling pathway, activates NADPH oxidases and increases intracellular reactive oxygen species (ROS) (3, 4). The increased ROS, by altering gene expression and other mechanisms, leads to the activation of presympathetic neurons that project to the intermediolateral cell column in the spinal cord (5, 6). Angiotensinergic pathways originate from neurons in circumventricular organs (CVOs) in the forebrain such as the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT). These CVOs are located outside the blood–brain barrier (BBB), and project to the paraventricular nucleus (PVN) in the hypothalamus and to the rostral ventrolateral medulla (RVLM) in the brainstem. Ang II-containing neuronal cell bodies and axonal end-terminals are present in these major cardiovascular regulatory nuclei and AT₁R are found on both pre- and post-synaptic terminals in these nuclei. Besides direct neuronal actions, Ang II/AT₁R can also activate microglia to generate proinflammatory cytokines, which in nuclei such as the PVN contribute to ROS production and downstream sympatho-excitatory and pressor responses (7, 8).

Active renin is almost undetectable in the brain. Recently, it has become apparent that instead, prorenin binds to the membrane prorenin receptor (PRR) and plays a major role in the local production of Ang II both in physiological and pathophysiological conditions such as hypertension (9, 10). Upon release into the synaptic cleft, Ang II (Ang 1–8) could directly bind to AT₁R, but in the brain Ang III (Ang 2–8) appears to act as the primary AT₁R agonist (11, 12). High aminopeptidase A (APA) activity is present in hypothalamic and brainstem nuclei (13, 14), and Ang III, generated from Ang II by APA, may act as the main AT₁R agonist of the brain RAS (15).

The above outlined angiotensinergic signaling pathways initiate sympatho-excitatory, neurohormonal and pressor responses to a variety of stimuli. These mechanisms, alone, are apparently not sufficient for persistent activation. Chronic s.c. infusion of Ang II

increases the expression of immediate early genes (IEGs) in the SFO only for the first few days, but the increase is progressive and persistent in the supraoptic nucleus (SON) and magno- and parvocellular subdivisions of the PVN (16, 17). Similarly, ROS is markedly increased in the SFO at 7 days but no longer after 2 weeks, whereas ROS activation persists in the PVN and RVLM (4). These findings are consistent with the concept that Ang II/III induced AT₁R/ROS signaling is the primary driver for the increase in neuronal activity in the SFO/PVN/RVLM pathways, but that another, more slowly acting, signaling mechanism is also being activated that amplifies and maintains downstream responses. Several lines of evidence indicate that a slow-acting neuromodulatory pathway involving aldosterone, mineralocorticoid receptors (MR), epithelial sodium channels (ENaC), EO and its receptor, the α_2 Na⁺ pump, plays a critical role in this regard. All components of this pathway are present in hypothalamic nuclei such as the SFO, PVN and SON (5). These nuclei co-express MR (18, 19) and 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) (18, 20, 21, 22) or 11 β -HSD type 1 (23), which both can confer aldosterone specificity to MRs (23). A variety of groups have demonstrated the presence in the hypothalamus of an ouabain-like factor. Aldosterone via MR increases the release of this factor from magnocellular neurons of the PVN and SON (24) for local action (25) or via the pituitary (26) release into the circulation. This brain factor appears to be identical to plasma EO (27, 28). In the brain, the ouabain-sensitive α_3 -isoform of Na⁺, K⁺-ATPase is highly expressed in neurons, and the ouabain-sensitive α_2 -isoform is the isoform in glia (29). Unexpectedly, in mice with an ouabain-resistant α_2 -isoform ($\alpha_2^{R/R}$), pressor responses to central infusion of ouabain or sodium (acting by release of EO) are largely absent (30, 31). This finding suggests that ouabain or EO may act mainly on glia rather than neurons in the CNS.

Aldosterone, acting through this slow neuromodulatory pathway, increases mRNA and protein expression of ACE, AT₁R and NADPH oxidase subunits (25) and elevates intracellular superoxide levels and Ca²⁺ influx (32, 33). Aldosterone can, thereby, enhance Ang II release and AT₁R signaling in, for example, the SFO (34) and the PVN (25). Consistent with this concept, central infusion of aldosterone or ouabain causes a gradual increase in sympathetic nerve activity (SNA), BP and heart rate that can be prevented by central infusion of an AT₁R blocker (32, 35). Finally, and most importantly, central infusion of Ang II at a low rate (not effective when infused

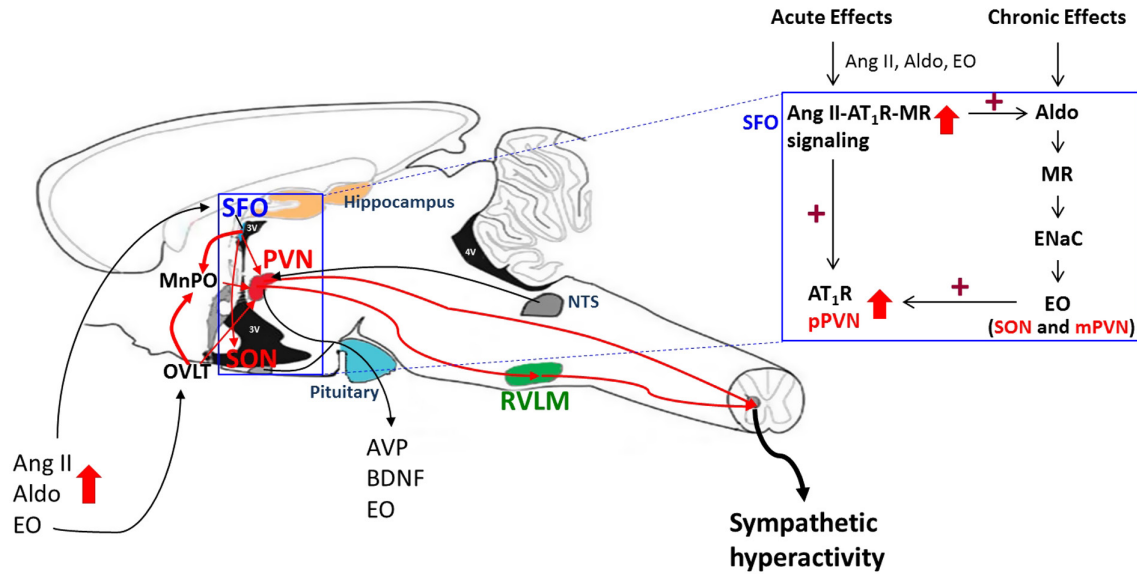
peripherally) increases hypothalamic aldosterone 2- to 3-fold; this increase can be prevented by central infusion of an aldosterone synthase (AS) inhibitor. Moreover, when combined with central infusion of an AS inhibitor or MR blocker, central Ang II only causes a transient increase in BP rather than persistent hypertension (36). Together, these findings suggest that aldosterone, produced locally in the CNS, acts on MR in, for example, the SON or PVN, and via this neuromodulatory mechanism causes persistent activation of angiotensinergic sympatho-humoral pathways.

CNS actions of circulating Ang II, aldosterone and EO

The CNS actions of Ang II, aldosterone and EO are often overlooked and/or not integrated with the peripheral actions. The relative role of these different actions is still being debated and may depend on the disease model or the extent of the increase in plasma levels. In a recent 'state-of-the-art' article (37), we reviewed the accumulating evidence that, at modest increases in plasma levels, the CNS actions can play a critical role, with peripheral actions of the same hormones acting as important amplifiers. Newer studies have provided additional support for these CNS actions (Fig. 1).

Plasma Ang II

Chronic small increases in plasma Ang II by s.c. infusion gradually increase BP by up to 60 mmHg (17), associated with an increase in neuronal AT₁R and IEG expression, and in ROS in hypothalamic and brainstem nuclei (4, 17). Knockdown of AT₁R specifically in the SFO prevents the increase in AT₁R in the PVN and of ROS in the PVN and RVLM. Knockdown of AT₁R or of NADPH oxidase in the SFO also prevents circulating Ang II-induced hypertension in rats or mice (4, 38). Activation of angiotensinergic projections from the SFO to the PVN appears critical in this regard, because knockdown of AT₁R in the PVN also prevents the Ang II induced hypertension (39), and infusion of an AT₁R-blocker in the PVN reverses the hypertension (40). These findings indicate that circulating Ang II increases AT₁R/ROS in the SFO, followed by AT₁R/ROS activation in the PVN, presumably by locally produced Ang II. Activation of this angiotensinergic pathway appears essential for the circulating Ang II-induced hypertension.

**Figure 1**

Acute and chronic effects of plasma Ang II, aldosterone and EO on brain angiotensinergic pathways. Circulating Ang II, aldosterone or EO can activate Ang II/AT₁R/MR signaling in circumventricular organs such as the SFO and OVLT, which are located outside of the BBB, followed by activation of angiotensinergic projections to the PVN, SON and RVLM (red lines). Prolonged stimulation of AT₁R/MR in the SFO also activates a slow neuromodulatory pathway in the hypothalamus (box at upper right) that involves locally produced aldosterone, which binds MR in, for example, SON and PVN and, via ENaC increases hypothalamic EO. Ouabain or EO acutely inhibits α_2 Na⁺ pumps and, chronically, also activates an α_2 Na⁺ pump-associated protein kinase cascade that increases the expression of ACE, AT₁R and NADPH oxidase subunits. As a consequence, chronic Ang II/AT₁R/MR signaling is maintained/enhanced. Effective sympathetic tone is increased by enhanced secretion of EO (see Figs 2 and 3). See text for references.

As observed for central infusion of Ang II (see above), persistent activation of AT₁R/MR in the CNS by plasma Ang II also appears to depend on the stimulation of the aldosterone-MR-EO neuromodulatory pathway. Elevation of plasma Ang II by s.c. infusion increases both the expression of aldosterone synthase (AS) (22) and aldosterone levels (17) in the hypothalamus. Central infusion of an AS inhibitor prevents this increase in hypothalamic aldosterone without affecting the Ang II-induced increase in plasma aldosterone (17); this implies that plasma Ang II increases local production of aldosterone. Central infusion of an AS inhibitor or MR blocker also markedly attenuates the IEG activation in both the magnocellular (neurosecretory) and parvocellular (presympathetic) parts of the PVN (17). Thus, persistent PVN activation depends on aldosterone/MR signaling. Further supporting this concept, central AS/MR blockade fully prevents the gradual ~20 mmHg increase in BP induced by a low s.c. Ang II infusion rate, that does not cause a detectable increase in plasma Ang II levels. On the other hand, a higher rate of infusion, that raises plasma Ang II ~3-fold, increases BP by 15–20 mmHg within 1–2 days, and then gradually, by another 40–45 mmHg, over the next 1–2 weeks. Central AS/MR blockade does not affect the initial increase in BP, but does block the

subsequent further increase (17). Central MR blockade also normalizes the BP and normalizes the enhanced BP decrease in response to AT₁R blockade in the PVN of rats with established s.c. Ang II-induced hypertension (40). These patterns of change in the hypothalamus/brainstem and in BP in response to central or circulating Ang II indicate that central aldosterone-MR signaling plays a critical role in the persistent activation of CNS angiotensinergic pathways and hypertension.

Recent studies have addressed where in the CNS these MR are located. Knockdown of either MR or AT₁R in the SFO, by intra-SFO infusion of AAV-MR- or AAV-AT₁R siRNA, similarly prevents Ang II-induced increases in AT₁R expression in the SFO, in ROS in the PVN and RVLM and most of the hypertension (4). Knockdown of either MR or AT₁R in the PVN also prevents the s.c. Ang II-induced hypertension (39). These findings suggest that MR-AT₁R cross-talk in the SFO represents a critical first step, and (in the PVN) an essential downstream step. Both steps are apparently required to maintain increased activity in the angiotensinergic pathways and for long-term control of cardiovascular homeostasis by plasma Ang II. An increase in plasma aldosterone may contribute to the MR activation in the SFO (22), whereas locally produced aldosterone (possibly in magnocellular neurons

in the SON and/or PVN) may, by volume transmission, be responsible for the MR activation in the PVN (17).

The above reviewed findings all indicate that activation of hypothalamic MR/AT₁R signaling is essential for Ang II-induced hypertension, at least at small/modest increases in plasma Ang II. However, when comparing BP responses to central vs peripheral infusion of Ang II, it appears that these CNS actions *per se* are not sufficient for the progressive increase in BP caused by plasma Ang II. Central infusion of Ang II at a rate of 2.5 ng/min (well below peripheral rates) increases BP by ~20 mmHg within a day, but in contrast to s.c. Ang II, does not further increase BP over the subsequent days despite activating the same CNS mechanisms (36). This different pattern of BP responses suggests that the peripheral actions of plasma Ang II play an essential amplifier role for the CNS actions. The higher the plasma [Ang II], the more these peripheral actions on their own may be sufficient to cause severe hypertension. Indeed, in mice, the rapid development of s.c. Ang II-induced severe hypertension (+50–60 mmHg within 1–2 days) appears to depend solely on renal AT₁R activation (41).

Further confounding the traditional view of the pre-eminence of the kidneys are studies on Ang II-high salt hypertension. A high salt diet markedly enhances the hypertensive response to chronic s.c. infusion of Ang II. For example, in rats on a 'regular' salt diet (0.4% NaCl), chronic s.c. infusion of Ang II at the low rate of 150 ng/kg/min increases BP by 15–20 mmHg after 8–10 days, but in rats on 2.0% high salt diet, this dose increases BP by ~50 mmHg (42, 43). In the classical paradigm, one might expect that this is a typical kidney-dependent form of severe hypertension. In fact, however, CNS mechanisms play a critical role, and as in the case for Ang II alone, MR/ENaC/AT₁R signaling (43, 44) and the PVN (45, 46) are involved.

Plasma aldosterone

The well-known renal actions of aldosterone are important for the maintenance of sodium homeostasis and are often considered to be pivotal for aldosterone's role in hypertension or heart failure. In rodents on regular salt intake, s.c. infusion of aldosterone at low rates, that increase plasma levels 2–3 fold, does not, alone, elevate BP (22), but when combined with high salt intake gradually raises BP by 20–30 mmHg over 2–3 weeks (22, 47). Studies in adrenalectomized rats showed hypothalamic

aldosterone levels parallel plasma aldosterone levels (48). In contrast, in intact rats, circulating aldosterone poorly penetrates most brain areas protected by the BBB, and chronic infusion of aldosterone at rates that increase plasma aldosterone 5- to 8-fold cause only a minimal increase in hypothalamic aldosterone (17). Substantial competition by corticosterone for both uptake and binding to MR in the cell (49) may explain this different penetration pattern. However, circulating aldosterone can readily access neurons in brain nuclei outside the BBB, such as the SFO, which express both MR and ENaC (19). Aldosterone can enhance the Ang II-induced increase in intracellular Ca²⁺ in SFO neurons (33), and thereby activate downstream angiotensinergic pathways. Indeed, lesioning of the SFO prevents 60%–70% of the hypertension induced by aldosterone salt (22), as does central infusion of an AT₁R blocker (47). Not surprisingly then, central infusion of an MR blocker is similarly effective (22, 47), but, unexpectedly, central infusion of an AS inhibitor is as effective (22). This suggests that circulating aldosterone, like Ang II, activates central angiotensinergic pathways involving local production of aldosterone. These pathways appear to project from the SFO to the PVN since knockdown of either AT₁R or MR in the PVN also prevents most of the hypertension (22). Altogether, it appears that a modest increase in plasma aldosterone when combined with an increase in plasma [Na⁺] (induced by, e.g., 0.9% saline drinking water), activates the aldosterone/MR/ENaC/EO neuromodulatory pathway via MR/AT₁R signaling in the SFO. This is critical for persistent Ang II/AT₁R signaling in the PVN and, therefore, the hypertension.

The preceding studies employed direct central blockades to assess for CNS actions of plasma aldosterone. In humans and dogs, sympathetic activity often serves as an index for central actions of plasma aldosterone. Humans with primary hyperaldosteronism (PA) caused by an aldosterone-producing adenoma or adrenal hyperplasia have high plasma aldosterone levels and elevated BP. One study (50) reported that muscle sympathetic nerve activity (MSNA) was increased by about 30% in a group of US patients with PA. Both MSNA and BP normalized after removal of the adenoma. In contrast, Japanese patients with PA exhibited a significantly reduced MSNA, and MSNA increased to normal after the removal of the adenoma (51). A variety of factors (50) may contribute to these opposite findings, including duration of aldosterone excess, extent of cardiac and renal fibrosis and damage by

the hypertension and aldosterone *per se*, and the level of salt intake. In a recent study in dogs (52), i.v. infusion of aldosterone and saline increased plasma aldosterone by 15-fold, plasma Na⁺ by 3 mM and plasma vasopressin by 2-fold and caused a marked drinking response. BP increased ~20 mmHg associated with a minor decrease in plasma norepinephrine (NE). Prolonged baroreflex activation similarly lowered plasma NE and heart rate before and after the induction of aldosterone-salt hypertension, but the BP fall was less: -7 ± 1 vs -16 ± 2 mmHg in control conditions. The authors concluded that sympatho-excitation does not contribute to the hypertension induced by mineralocorticoid excess. However, even if their conclusion is correct, this would not exclude central actions of plasma aldosterone on neuroendocrine outputs involved in cardiovascular regulation. Indeed, chronic central infusion of aldosterone in dogs increased TPR and elevated BP by ~20 mmHg, but in contrast to studies in rats (32), did not increase plasma NE or heart rate (53). The authors suggested that release of a Na⁺, K⁺ ATPase inhibitor from the hypothalamus could contribute to the increase in TPR and BP.

In summary, although CNS actions of aldosterone are well established, their contribution to the chronic cardiovascular effects of circulating aldosterone is still being debated. Species differences, modest vs large increases in plasma aldosterone and the level of salt intake may all be relevant factors in this regard.

Plasma EO

In 1991, a substance ‘indistinguishable from ouabain’ was identified by mass spectroscopy (MS) as an endogenous cardiostonic steroid (CTS) in human plasma (54). This discovery was validated by MS and NMR identification of EO in bovine, rodent and human plasma and tissue samples by laboratories on 3 continents (26, 27, 55, 56, 57). A few investigators reported that they were unable to detect EO in human plasma (58), but none of these reports detailed how they attempted to separate EO by column chromatography prior to MS. A recent commentary provides a more in-depth review of this topic (28).

The adrenal glands are highly enriched with EO in humans, rodents and cattle (54, 56), and plasma EO is extremely low in adrenalectomized rats (54) and patients with adrenal insufficiency (59). Also, primary cultured bovine and human adrenocortical cells exhibit net synthesis of EO (60) and, in awake dogs, the adrenal venous EO concentration is 4- to 5-fold higher than that

in the arterial blood (61). Thus, the adrenals are likely the primary source of circulating EO under normal conditions. In addition, there is evidence for local biosynthesis in the brain (see above). The extent to which brain EO contributes to the plasma levels is yet to be determined (26). The full EO biosynthetic pathway has not yet been elucidated. Nevertheless, EO, like aldosterone, is derived from progesterone but the distal steps are not yet known (62, 63). A related CTS, marinobufagenin (MBG), also has been linked to rodent hypertension (64, 65). MBG binds preferentially to rodent α_1 Na⁺ pumps (65, 66), whereas EO favors the α_2 and α_3 isoforms.

Chronic increases in plasma EO or plant-derived ouabain activate multiple intracellular signaling pathways involved in cardiac and arterial function and remodeling (67, 68). CNS actions appear to play an important role as well. Several groups have shown that in rats chronic s.c. or i.v. infusion of ouabain causes a gradual, dose-dependent increase in BP, TPR and heart rate up to 40 mmHg and 100 bpm (69, 70). Ouabain appears to initiate these CNS actions through the forebrain CVOs (71). Both the hypertension and the tachycardia can be prevented by central infusion of antibody Fab fragments raised against ouabain and can be reversed by acute ganglionic blockade. This suggests that central effects of ouabain increasing sympathetic tone play an essential role (70). Moreover, central infusion of an AT₁R blocker prevents the sympatho-excitatory and pressor responses to both central and s.c. infusion of ouabain (35, 72), indicating that these responses to ouabain are mediated by AT₁R-dependent activation of angiotensinergic pathways in the CNS. Since the actions of ouabain appear to be glia dependent, and components of the RAS are also present in glia (73), this interaction may occur in glia rather than neurons, i.e. ouabain may increase Ang II release by glia cells, acting on AT₁R in glia and/or neurons.

In conclusion, there is now compelling evidence that the CNS actions of plasma Ang II, aldosterone and EO play an important role in their cardiovascular effects. All of these effects depend on the activation of angiotensinergic pathways.

Brain Ang II and neuroendocrine control of the circulation

There is extensive literature on the regulation of the secretion of a number of hormones by CNS angiotensinergic pathways. In this section, we will

focus on recent studies on the chronic effects of brain Ang II on plasma aldosterone and EO. Central infusion of Ang II at low rates (2.5–12.5 ng/min), that are not effective when infused peripherally, markedly increases plasma levels of aldosterone and corticosterone as well as EO (26, 36). s.c. infusion of Ang II at the rate of 150 ng/kg/min does not cause detectable increases in plasma Ang II or the levels of these 3 steroids. When combined with high (2%) salt diet (HS), however, this dose of Ang II causes severe hypertension associated with 2- to 3-fold increases in the plasma levels of all 3 steroids (74). Concomitant central infusion of an AS inhibitor or MR blocker prevents most of these increases (26, 36, 74), indicating that central aldosterone-MR signaling is needed for these actions of central Ang II or peripheral Ang II-HS. ACTH release could be the link between the brain pathways and increased secretion of the 3 steroids by the adrenal cortex (60). However, ACTH tends to elevate plasma aldosterone only transiently and therefore seems unlikely to be the intermediary for chronic CNS Ang II actions (75). Alternative mechanisms include enhanced sympathetic drive to the adrenals (76), increases in plasma vasopressin (77) or direct adrenocortical effects of EO itself that stimulate aldosterone secretion (78). Low nanomolar concentrations of ouabain acutely stimulate basal and Ang II-evoked aldosterone secretion from bovine zona glomerulosa cells in primary culture. This stimulatory mechanism depends upon the ability of ouabain to raise intracellular Ca^{2+} , augment the release of renin from the cells and, in the presence of angiotensinogen, to form

locally high levels of Ang II (78). Moreover, *in vivo*, the chronic elevation of plasma ouabain into the range of 1.5–2.5 nM produced by s.c. infusion increases the mass of the adrenal glands, especially the zona glomerulosa layer, and elevates plasma aldosterone and corticosterone (79, 80) i.e., the same classical products of the rat adrenal zona glomerulosa layer that are augmented by brain Ang II. In addition, chronic active immunological neutralization of EO lowers plasma aldosterone (~30%) in otherwise normal rats on a low salt intake (81). When taken together, these results are consistent with the following interpretations: (1) The brain, via circulating EO, can provide a physiologically relevant stimulus to zona glomerulosa function; (2) The elevation of brain Ang II augments the CNS production of EO, raises circulating EO and thereby stimulates the adrenal secretions of aldosterone and corticosterone. Collectively, the rodent work summarized in Fig. 2 implicates EO as the humoral intermediary that may link brain Ang II with augmented adrenal secretion of corticosteroids (Fig. 2).

The most evident readily observable consequences of chronically elevated brain Ang II in the rat can therefore be summarized as hypertension with co-elevated circulating EO and aldosterone. The obvious question concerns the broader clinical significance of these experimental observations. Are the latter humoral features present among some patients with hypertension? In a recent study involving 590 patients with never-treated mild-to-moderate essential hypertension, co-elevated circulating levels of EO and aldosterone were found in approximately

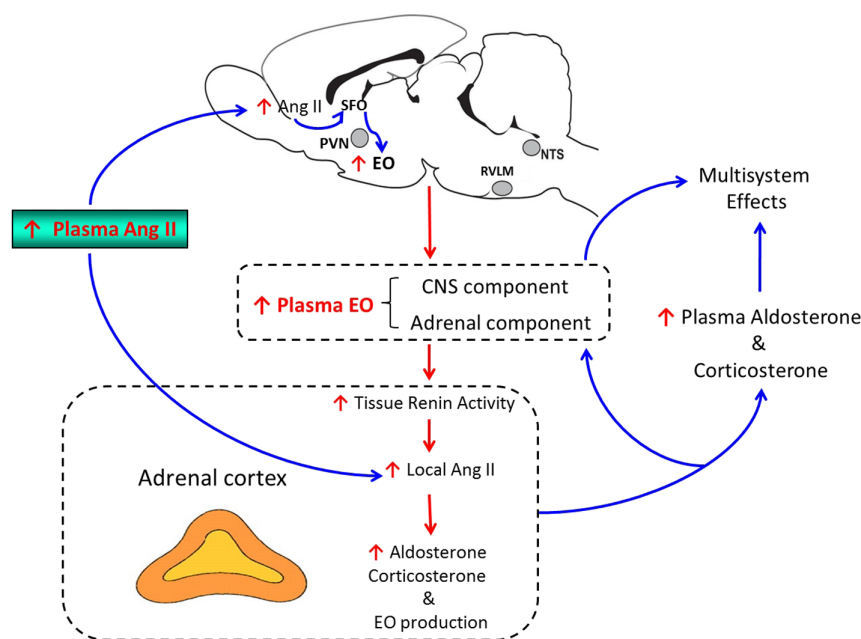


Figure 2

Proposed role of EO as a link between the effects of brain Ang II and adrenocortical activity. In response to the prolonged elevation of brain Ang II arising from either local or peripheral sources, a CNS aldosterone-MR-ENaC pathway (see Fig. 1) stimulates the production and secretion of CNS EO into the circulation. The elevated plasma EO, reflecting a (yet to be determined) variable mixture arising from CNS and adrenal sources, raises intracellular sodium and, via sodium-calcium exchange, augments Ca^{2+} signaling, stimulates intra-adrenal renin activity and enhances the local generation of Ang II. The combination of these actions leads to the enlargement of the zona glomerulosa layer and augmented adrenal production of aldosterone, corticosterone and EO. The stimulatory effects of Ang II on EO secretion from the CNS as well as intra-adrenal effects of EO involve AT_1R -mediated signaling. See text for references.

one-third of the patients (82). In addition, it has long been known that co-elevated levels of EO and aldosterone occur in ~50% of patients with aldosterone-producing adrenal adenomas but, intriguingly, not among patients with hyperaldosteronism due to bilateral hyperplasia (83, 84, 85). Among the patients with unilateral adenomas, removal of the diseased gland normalized EO, aldosterone and BP (84). This suggests that, in these patients, the elevated EO originates primarily from the adrenal glands and that the co-elevation of EO and aldosterone are sufficient to drive the hypertension. Moreover, it may be recalled that circulating EO and aldosterone both have actions that activate brain Ang II pathways and that the central blockade of either of these agents prevents hypertension. This implies that the CNS is positioned as a critical component between adrenocortical hyperfunction and the elevated arterial tone and BP even in patients with adenoma producing hyperaldosteronism. Further, as EO is a stimulus to aldosterone secretion, the question arises as to whether in some patients, their zona glomerular adenomas might primarily hypersecrete EO and thereby evoke a secondary form of aldosterone excess (86). Irrespective of the primacy of aldosterone in hyperaldosteronism, the impact of elevated plasma levels of EO, aldosterone and corticosterone extends beyond their effects on BP; these steroids and their receptors have been implicated in cardiac growth and fibrosis, podocyte damage, memory and mood (86, 87, 88, 89, 90, 91). Thus, among those individuals in whom EO and aldosterone are inappropriately co-elevated, there may be a higher incidence of adverse cardiac, renal and CNS sequelae. Further mechanistic studies are clearly needed to elucidate the specific pathways through which the brain regulates and is influenced by the plasma levels of these important steroids.

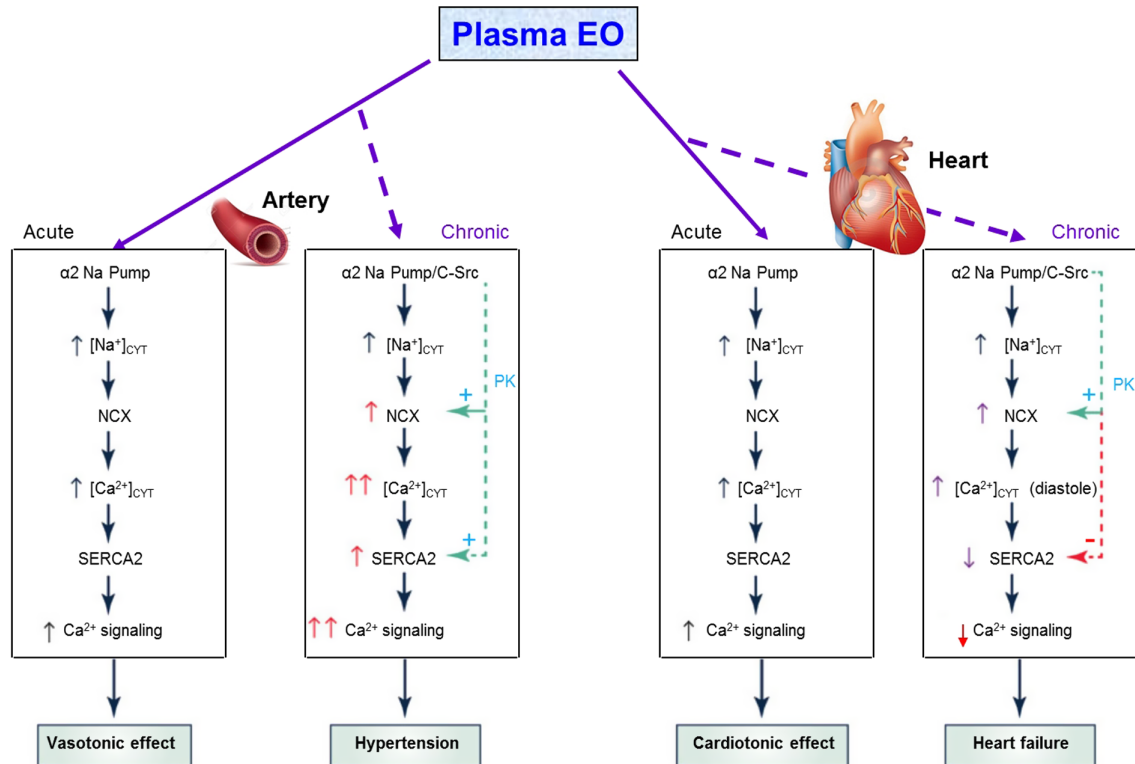
Direct effects of EO on the heart and arteries

EO inhibits ouabain-sensitive cardiac Na⁺ pumps and raises local intracellular Na⁺ ([Na⁺]_i), thereby promoting net Ca²⁺ gain via NCX and enhancing cardiac Ca²⁺ signaling and contraction: the well-documented ‘positive inotropic effect’ (68). If sustained, this can foster cardiac hypertrophy (68). Similar mechanisms are responsible for the analogous vasotonic effect of EO in arteries (68, 92). This action may contribute (along with enhanced

sympathetic drive) to the elevated total peripheral vascular resistance that is a hallmark of hypertension (93) (Fig. 3).

In addition to these Na⁺ transport-dependent effects, sustained EO binding to Na⁺ pumps has Na⁺ transport-independent, chronic tissue-specific actions that involve protein kinase (PK) cascade activation. This leads to changes in the expression (reprogramming) of Ca²⁺ transporters and other proteins (94, 95, 96, 97). In arteries, for example, EO-induced upregulation of Na/Ca exchanger-1 (NCX1), sarcoplasmic reticulum Ca²⁺ pump-2 (SERCA2) and several Ca²⁺-permeable channels further promotes Ca²⁺ entry, vasoconstriction and hypertension (68, 94). In the heart, however, EO-induced PK activation upregulates NCX1, but downregulates SERCA2 expression; this fosters Ca²⁺ extrusion and attenuates Ca²⁺ signaling and contraction, thereby impairing systolic function (68). Note that the driving force on cardiac NCX1 favors Ca²⁺ efflux during diastole whereas, in arteries with tone, the myocyte plasma membrane is partially depolarized and the driving force on NCX1 favors Ca²⁺ influx (68). Both the chronic arterial and cardiac effects involve EO/ouabain-triggered activation of PK cascades (98, 99, 100, 101, 102, 103). These can be stimulated by α₁ Na⁺ pumps when micromolar concentrations of CTS are used (101). However, in rodents nanomolar ouabain concentrations can activate these signaling pathways (95, 103). This implies that α₂ and α₃ pumps are the functionally relevant receptors *in vivo* for EO and is consistent with circulating levels of EO that range from ~0.2 to 1 nM.

Data from genetically engineered mouse models strongly support the view that the EO-Na⁺ pump endocrine system plays a pivotal role in cardiovascular function (68, 104). Normally ouabain-sensitive α₂ Na⁺ pumps (the EO receptors in cardiac muscle and vascular smooth muscle) can be made ouabain resistant without altering transport activity by mutating just two amino acids (105, 106). This attenuates or delays pressure overload-induced cardiac hypertrophy (107) and prevents several forms of hypertension (105, 106, 107, 108, 109). Importantly, immuno-neutralization of EO has the same effects (107, 108). Furthermore, cardiac-specific knockout or overexpression of α₂ pumps also delays or attenuates pressure overload-induced hypertrophy (110, 111). Clearly, the EO-α₂ Na⁺ pump endocrine system, and not just myocyte stretch (112), contributes to the hypertrophy.

**Figure 3**

Acute and chronic effects of plasma EO on cardiac and arterial function. The elevated plasma EO acutely inhibits α_2 Na⁺ pumps in both the heart and arteries, and the rise in intracellular Na⁺ rapidly induces Na⁺/Ca²⁺ exchanger (NCX)-mediated Ca²⁺ gain, and cardiotoxic and vasotonic effects. Prolonged plasma EO elevation also activates an α_2 Na⁺ pump-associated protein kinase cascade (e.g. C-Src-mediated) that increases cardiomyocyte and arterial smooth muscle cell NCX expression, and arterial sarcoplasmic reticulum (SR) Ca²⁺ pump (SERCA2) expression. In arteries with tone, NCX normally favors Ca²⁺ entry and helps to sustain cytosolic Ca²⁺ ($[Ca^{2+}]_{cyt}$) above contraction threshold. The EO-induced NCX and SERCA2 upregulation enhance Ca²⁺ signaling and SNA to increase vascular tone and elevate BP. In the heart, NCX promotes Ca²⁺ extrusion during diastole, but prolonged α_2 pump inhibition by EO reduces the Na⁺ gradient driving force so that $[Na^+]_{cyt}$ and diastolic $[Ca^{2+}]_{cyt}$ are both elevated; consequently, cardiac relaxation is slow and/or incomplete. Also, cardiac SERCA2 expression is usually reduced in heart failure (perhaps due to the high EO), as are SR Ca²⁺ stores and Ca²⁺ transients, and systolic function is impaired. See text for references. Reproduced, with permission, from Blaustein M, Chen L, Hamlyn J, Leenen FHH, Lingrel J, Wier W & Zhang J, Pivotal role of α_2 Na⁺ pumps and their high affinity ouabain binding site in cardiovascular health and disease, *Journal of Physiology*, 2016, 594, 6079–6103. Copyright 2016 The Physiological Society.

Therapeutic implications of brain angiotensinergic pathways for cardiovascular disease

In addition to Ang II and aldosterone, plasma/CSF $[Na^+]$ and cytokine levels increase and activate brain angiotensinergic pathways in a variety of cardiovascular diseases. One may therefore anticipate that their CNS actions play a role in cardiovascular pathophysiology. Knockdown of for example MR or AT₁R in specific brain nuclei or central infusions of blockers at low rates, that are ineffective peripherally, can be used to assess the overall role of CNS angiotensinergic signaling for the activation of peripheral mechanisms contributing to cardiovascular disease. This concept has so far mainly been employed to test for the role of CNS-activated peripheral mechanisms that contribute to hypertension or heart failure in animal models.

Hypertension

Using these approaches, many groups have shown that central AT₁R signaling plays a pivotal role in both the development and maintenance of hypertension in a number of models including hypertension induced by renal ‘injury’/dysfunction (reviewed in (37)). For example, central blockade of the aldosterone/MR/ENaC-EO neuromodulatory pathway can prevent or reverse the hypertension in many models (37), demonstrating that these CNS pathways are a valid potential therapeutic target. The key question is whether peripheral (for example s.c. or oral) dosing can cause central blockade, not only in brain nuclei outside the BBB, such as the SFO, but also in nuclei inside the BBB such as the PVN. Important factors in this regard are the lipophilicity of the specific drug (or active metabolite), drug dose and duration of dosing

(i.e., acute studies are not useful). Indeed, in rats, AT₁R blockers, when administered in high enough doses, can cause marked and sustained AT₁R blockade, not only in nuclei outside the BBB but also in important regulatory nuclei inside the barrier such as the PVN (113, 114). For example, in Dahl salt-sensitive (S) rats, central treatment with an AT₁R blocker prevents salt-induced hypertension, and systemic treatment is similarly effective if the dose is high enough to cause sufficient central blockade (114, 115). These findings suggest that the degree of central and not peripheral AT₁R blockade determines the extent of the antihypertensive effect in the Dahl model.

Studies in humans with hypertension on possible CNS effects of AT₁R blockers evaluated the effects of conventional, ‘clinically recommended’ doses, e.g. losartan 50mg/day (116) or irbesartan 150mg/day (117). Neither study showed a decrease in the elevated resting sympathetic tone, assessed by MSNA or whole-body norepinephrine spillover. According to Krum and coworkers (116) ‘reversal of sympathetic activation in hypertension cannot be expected with renin-angiotensin antagonism’. In contrast, Raheja and coworkers (117) considered the possibility that central AT₁R may not have been inhibited, and that the results might have been different if a higher dose of irbesartan had been used. This concept has not yet been tested in patients with hypertension.

APA inhibitors are presently being tested in clinical studies as a new class of antihypertensive drugs to specifically target the brain RAS (118). RB150/QGC001 is a systemically active prodrug that crosses the BBB and is cleaved into 2 active EC33 molecules by brain reductases. Centrally administered EC33 or systemically administered RB150 similarly inhibit brain APA activity and lower BP in several experimental hypertension models (119, 120). The antihypertensive effectiveness of RB150 is presently being evaluated in patients with hypertension. The potential differences with standard systemic RAS blockade, for example, on renal function under different clinical conditions will need to be determined.

Experimental studies have shown that systemic treatment with an MR blocker at low-to-regular doses appears to be able to cause sufficient central MR blockade to lower sympathetic hyperactivity (121). In humans with hypertension, the sympathetic effects of MR blockers are distinctly different from those caused by chlorthalidone or hydrochlorothiazide. Treatment with chlorthalidone and spironolactone similarly lowered BP, but only chlorthalidone increased MSNA (122). In older hypertensives, treatment with hydrochlorothiazide

or spironolactone similarly lowered BP, but only spironolactone lowered plasma norepinephrine (123). The increase in MSNA by chlorthalidone was fully prevented by concomitant treatment with spironolactone at 25mg/day (117). These findings suggest that the modest increase in plasma aldosterone by chlorthalidone appears sufficient to activate CNS MRs, which may mediate the sympathetic hyperactivity induced by chlorthalidone. Moreover, low doses of the MR blocker appear sufficient suggesting that the relevant MR population is readily accessible to spironolactone or its active metabolites.

Heart failure

In animal studies, central MR or AT₁R blockade by central infusions of blockers or knockdown of MR or AT₁R in specific nuclei such as the PVN inhibits the activation of several peripheral mechanisms, including sympathetic hyperactivity, that contribute to progressive cardiac dysfunction (121, 124, 125, 126, 127). Systemic (oral or s.c.) treatment with MR or AT₁R blockers also can inhibit the activation of the peripheral mechanisms (121, 126, 127). Central treatment with spironolactone at low doses and oral treatment with much higher doses have comparable beneficial effects on cardiac remodeling as well as dysfunction suggesting that CNS MR blockade by chronic oral treatment with spironolactone plays an important role (126). On the other hand, although central and systemic treatment with the AT₁R blocker losartan similarly prevent sympathetic hyperactivity and improve cardiac fibrosis and LVEDP, only central treatment improves LV systolic function, whereas s.c. or oral losartan treatment further lowers LVPSP and dP/dt_{max} (127, 128). Thus, peripheral AT₁R blockade appears to exert an adverse effect on cardiac systolic function that overrides the positive effects of central AT₁R blockade. The mechanisms contributing to this apparent adverse effect have not yet been elucidated, but may involve unopposed, enhanced cardiac AT₂R activation (129) or blockade of Ang II-AT₁R signaling in myocytes, thereby decreasing Ca²⁺-influx and myocardial contractility (130). If cardiac AT₁R blockade does exert adverse effects on cardiac systolic function in heart failure, then a new therapeutic approach is needed. For example, orally active APA inhibitors that specifically inhibit the activation of brain angiotensinergic pathways (34), may lead to more optimal and specific approaches to the prevention/management of heart failure after MI. In patients with heart failure such an approach may lower/normalize CNS-dependent activation of peripheral mechanisms contributing to heart failure progression.

Sympathetic hyperactivity could be prevented/reversed without interfering with the acute millisecond-to-millisecond sympathetic regulation of cardiovascular homeostasis and without causing nonspecific sympatho-inhibition or nonspecific central sympatholysis, which may adversely affect outcome in patients with heart failure (131). Similarly, centrally induced decreases in plasma Ang II, aldosterone and EO would leave acute regulation intact and therefore may minimize adverse effects such as hypotension, hyperkalemia and renal dysfunction. Further preclinical and clinical studies are needed to assess whether orally active APA inhibitors are similarly effective in lowering SNA, but with less adverse effects and perhaps better cardiac outcomes.

Conclusions

The renal, cardiac and vascular actions of the endocrine, circulating RAAS play a major role in the short-term and chronic maintenance of the cardiovascular and electrolyte homeostasis. As a result, AT₁R or MR blockade may lead to hypotension, renal dysfunction or hyperkalemia in, e.g., dehydration or low salt intake, or diseases such as heart failure or renal disease.

Plasma Ang II, aldosterone as well as EO (primarily via CVOs such as the SFO) also activate slow-acting CNS angiotensinergic pathways involved in cardiovascular regulation, e.g., by determining the setpoint of sympathetic activity without affecting its acute, second-to-second role. AT₁R-ROS signaling is the primary driver for neuronal activation in these pathways. Nevertheless, persistent activation depends on parallel gradual activation of a hypothalamic aldosterone/MR/ENaC/EO pathway, which increases the expression of key AT₁R signaling components. These brain pathways appear to not only increase the setpoint of SNA *per se*, but also to enhance its effectiveness by increasing plasma EO and EO-dependent reprogramming of arterial and cardiac function. In contrast to peripheral blockades, blockade of central AT₁R-ROS signaling normalizes the activity of the peripheral pathways and maintains physiological regulation and function.

The general outline of these concepts has been established, but important aspects of the regulation of these central and peripheral modulatory pathways still need to be established. The therapeutic potential of inhibiting these pathways to normalize rather than block (components of) the sympathetic nervous system or the

circulating RAAS is apparent, and results from preclinical studies are promising. However, new therapeutic drug strategies will be needed as well as comparative clinical studies for efficacy and adverse effects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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