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Angiotensin II and Anti Diuretic Hormone Exert Synergistic Effects on Thick Ascending Limb Transport in Spontaneously Hypertensive Rats

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Key Words
Ang II · Nitric oxide · NADPH oxidase · AVP

Abstract
Background: Sodium reabsorption is increased in the thick ascending limb (TAL) of Henle in several hypertensive models. In this segment, while transport is increased by ADH via cAMP, sodium reabsorption results from Ang II-induced superoxide (O_2^-) production. Surprisingly, it is unknown whether these mechanisms overlap in hypertension. We hypothesized that Ang II and ADH have accumulative effects on TAL's transport during hypertension. ***Methods:*** The effect of ADH/Ang II in TALs from spontaneously hypertensive rats (SHR) on oxygen consumption (QO_2), cAMP and O_2^- was measured. ***Results:*** Basal QO_2 was 113.3 ± 14.2 nmol O_2/min/mg protein. Addition of ADH (1 nM) increased QO_2 by 198%. In the presence of ADH, Ang II (1 nM) elicited a QO_2 transient response and then rose to 321.5 ± 28.3 ($p = 0.003$ vs. ADH). These accumulative effects could be due to nitric oxide synthase (NOS) uncoupling, lower Ang II ability to decrease cAMP or increased O_2^-. We first measured QO_2 using a NOS inhibitor. Pretreatment with L-NAME did not block the observed interaction ($p = 0.001$ Ang II vs. ADH). Also, Ang II blocked the ADH-stimulated cAMP accumulation in TAL of SHRs. In the presence of ADH, Ang II increased O_2^- production in TALs from SHR by 309% ($p = 0.015$ vs. basal). The O_2^- scavenger tempol blocked the Ang II effects on QO_2. In the presence of the NADPH oxidase inhibitor apocynin, the accumulative effects of ADH and Ang II were abolished. We conclude that (1) in SHR, Ang II has accumulative effects on ADH-stimulated transport; (2) this effect is mediated by AT1 receptors, and increased O_2^- production.

Introduction

The medullary thick ascending limb (TAL) of the loop of Henle reabsorbs 15–25% of the filtered NaCl. Increases in Na transport by this segment contribute to several models of hypertension. Unsurprisingly then, various hypertension models show increased Na transport in this segment [1, 2]. In agreement with this notion, diuretics that decrease TAL NaCl absorption are frequently used to reduce blood pressure [3]. Moreover, in many forms of hypertension, there is increased Na reabsorption in the distal nephron [1, 2, 4]. For instance, in spontaneously hypertensive rats (SHR), there is not only increased basal collecting duct water absorption

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but also increased basal ENaC apical expression in connecting tubules [5, 6]. Although it has been reported that SHRs have constitutive increased apical expression of the Na/K/2Cl co-transporter [7], synergic effects of hormones that regulate transport are yet to be evaluated in this model.

Independently of each other, Ang II and ADH are known to affect TAL regulation of Na transport. Indeed, Ang II and ADH increase transport in TAL. Acute increases of Ang II-induced O_2^- levels in the TAL enhance Na transport, whereas scavenging endogenous O_2^- decreases it [8]. Similarly, ADH increases cAMP levels enhancing Na transport [9] during dehydration or plasma volume contraction. Formerly, we demonstrated that Ang II and ADH interact under normotensive conditions, both by increasing Na reabsorption in this segment [10]. However, in hypertensive settings, the role of O_2^- and cAMP in mediating the effects of Ang II and ADH on vectorial transport in this nephron segment, remains undefined. Because exacerbation of Na retaining mechanisms are present during high blood pressure, we hypothesized that in hypertension, Ang II and ADH have summative effects on transport resulting in increased Na absorption in the TAL.

Methods

Animals

All studies were approved by the Institutional Animal Care and Use Committee of the J. Robert Cade Foundation and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. SHR rats weighing 200–250 g were fed ad libitum with normal chow diet for 7–10 days prior to the experiments and water.

Rat Medullary Thick Ascending Limb Suspensions

Medullary TAL suspensions were prepared as previously described [11, 12]. Both kidneys were perfused retrograde via the abdominal aorta with 40 ml of 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-buffered physiological saline containing (in mM): 130 NaCl, 4 KCl, 2.5 NaH_2PO_4, 1.2 $MgSO_4$, 2 calcium dilactate, 5.5 glucose, 6 D/L-alanine, 1 trisodium citrate, 10 HEPES and 100 U heparin. Both kidneys were excised and the inner stripe of the outer medulla was dissected from coronal slices. The tissue was minced (to approximately 1 mm^3 pieces) and incubated at 37°C for 30 min in HEPES-buffered physiological saline plus 0.1% collagenase type I while agitating the suspension and gassing it with 100% oxygen every 5 min. Then the tissue was centrifuged at 95 *g* for 2 min, resuspended in cold HEPES-buffered physiological saline and stirred on ice for 30 min. The resulting suspension was filtered using a 250-μm nylon mesh and centrifuged again for 2 min. The pellet was rinsed and resuspended in 1 ml cold HEPES-buffered physiological saline.

Measurement of Oxygen Consumption

Oxygen consumption was used to examine Ang II/ADH interaction. This technique allowed us to assess the effect of these hormones on both transport and cell metabolism. This is a suitable technique to measure transport because it is stoichiometrically related to Na transport: 35–50% of total oxygen consumption by the TAL is associated with NaCl reabsorption. To measure oxygen consumption, TALs were suspended in 0.1 ml of HEPES-buffered physiological saline, then warmed to 37°C and equilibrated with 100% oxygen. After that, the suspension was added to a closed chamber at 37°C and oxygen consumption recorded continuously using a Clark electrode. An initial constant slope was established for each experiment (3–5 min). Then, ADH was added and its effect was measured after a new stable slope was established for 6 min. After that, Ang II was added and its effects measured for at least an additional 6 min. Similar experiments were done reversing the order of exposure; that is, Ang II was added first and then ADH. In the protocols involving inhibitors, the drugs were present from the beginning of the experiment. All experiments were completed within 20 min. At the end of the experiment protein content was measured and total proteins were used to normalize the results as nmol O_2/min/mg of protein.

Measurement of O_2^- Production

O_2^- production was measured as described previously [10]. TAL suspensions were placed in glass tubes in HEPES-buffered physiological saline and N,N'-dimethyl-9,9′-biacridinium dinitrate (Lucigenin, Santa Cruz Biotechnology) at a final concentration of 5 μM. Then, tubules were incubated for 10 min at 37°C and placed in a luminometer. Following a 5-min steady baseline period, ADH was added and measurements were taken for 5 min. After that, Ang II was added and the emitted luminescence was recorded for an additional 10 min. Next, the O_2^- scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid (10 mM, tiron, Sigma) was added and the measurements were repeated. In the protocols involving inhibitors, the drugs were present from the beginning of the experiment. The difference in average luminescence between periods with and without tiron was used to calculate the luminescence produced by O_2^-.

Cyclic AMP Measurements

cAMP was measured as described previously [10, 13]. Aliquots of TALs suspensions were incubated in 95 μl of HEPES-buffered physiological saline containing 1 mM 3-isobutyl-1-methylxanthine at 37°C for 10 min before adding the different drugs. The reaction was stopped with methanol and cAMP was determined with an enzyme-immunoassay (Cyclic AMP EIA kit, Cayman). On the day of the assay, samples were centrifuged, the supernatant was transferred to another tube that was dried in a Savant dryer, and the pellet reconstituted in Na acetate buffer.

Determination of Protein Content

The total protein content was determined using Bradford's colorimetric method.

Statistics

Data are reported as mean ± SEM. Differences in means were analysed using analysis of variance with repeated measurements using Bonferroni as post-hoc test. p values <0.05 were considered significant for comparisons.

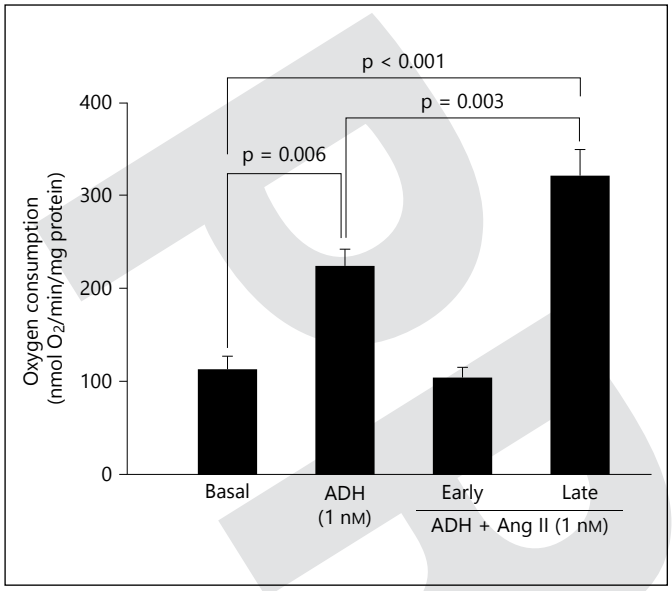

Fig. 1. Effects of ADH and Ang II on TAL oxygen consumption. (n = 5 in all the experiments). Compared to basal values, ADH increased oxygen consumption indicating increased transport. When both ADH and Ang II were added, no significant changes were observed early during the first 3 min. Then, at 6 min, oxygen consumption significantly increased not only when compared to basal values but also when compared to the ADH alone response.

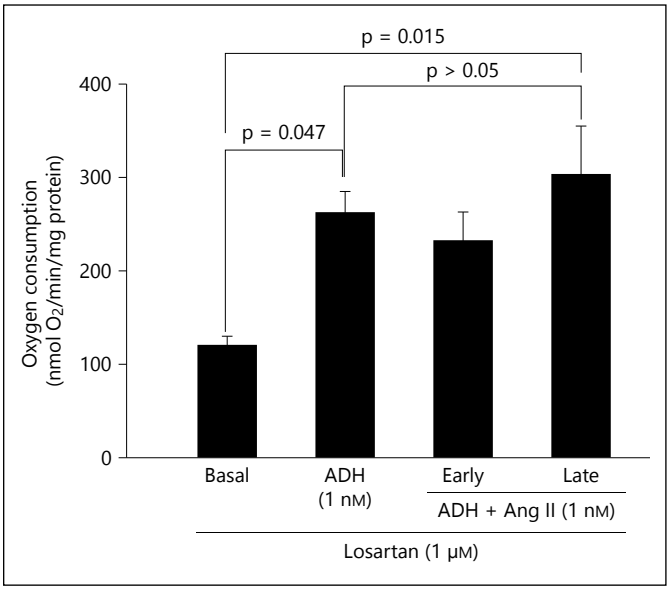

Fig. 2. Effects of ADH and Ang II on TAL oxygen consumption in the presence of the AT1 receptor inhibitor Losartan (n = 5). Losartan did not prevent the ADH-induced rise in oxygen consumption and the early response to combined ADH/Ang II but it did prevent the late response, suggesting this late rise in oxygen consumption is Ang II-related.

Results

To assess Na^+ transport in TAL, we measured oxygen consumption. This technique has been broadly used, as in this segment there is net luminal NaCl entrance into limb cells and therefore, vectorial NaCl transport. Indeed, 25–40% of total oxygen consumption in the TAL is related to Na transport. In fact, there is a stoichiometric link between oxygen consumption and Na transport [11, 12, 14]. Thus, we first measured the effect of ADH (1 nM) on basal oxygen consumption. The mean basal level for oxygen consumption was 113.3 ± 14.2 nmol O_2/min/mg protein (fig. 1). Then, after adding ADH, it increased to 224.1 ± 17.5 nmol O_2/min/mg protein ($p = 0.006$ vs. basal), a 198% increment. We then added Ang II (1 nM) in the presence of ADH and oxygen consumption decreased to 104.1 ± 11.6 nmol O_2/min/mg protein. After this initial inhibitory effect, oxygen consumption recovered and exceeded the ADH-stimulated oxygen consumption (from 104.1 ± 11.6 to 321.5 ± 28.3 nmol O_2/min/mg protein). This late effect remained at this level until the end of the experimental period (6 min; n = 5; $p = 0.003$ vs. ADH; fig. 1). In control experiments, vehicle of both ADH and Ang II had no effect on oxygen consumption.

Because AT1 receptor activation has been shown to increase transport in the TAL and to be involved in the Ang II/ADH-interaction in normotensive animals [10], we then tested whether the AT1 receptor was involved in the effects observed in our experiments and for this we used the AT1 receptor antagonist losartan. In the presence of losartan (1 μM), basal TAL oxygen consumption was 120.1 ± 10.9 nmol O_2/min/mg protein. Losartan had no effect on ADH-stimulated oxygen consumption that rose to 262.9 ± 23.3 nmol O_2/min/mg protein ($p = 0.047$ vs. basal). In contrast, in the presence of ADH, losartan prevented the Ang II-effects on oxygen consumption, remaining at 301.3 ± 52.9 nmol O_2/min/mg protein ($p > 0.05$ vs. ADH; fig. 2). This unchanged oxygen consumption remained at similar levels until the end of the experiment. These data indicate that AT1 receptors are involved in the cumulative effect of Ang II on ADH-stimulated transport.

To investigate this enhancing effect of Ang II on ADH-stimulated QO_2, we first evaluated the ability of Ang II to counteract the ADH-induced rise in intracellular cAMP in the SHR TAL. For this, we first measured cAMP accumulation. Basal cAMP was 65.4 ± 7.0 fmol/min/mg protein. After the addition of ADH, cAMP increased to 133.8 ± 14.3 fmol/min/mg protein ($p = 0.001$ vs. basal).

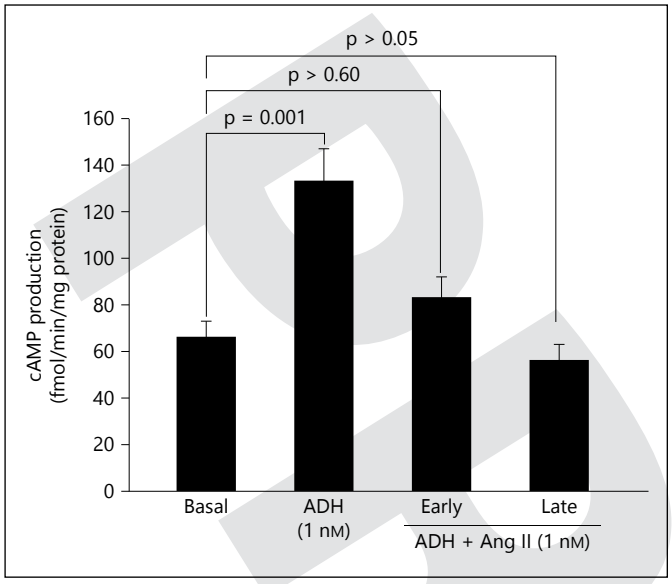

Fig. 3. Effect of Ang II on ADH-stimulated cAMP production in the TALs (n = 5). The ADH-induced rise in cAMP was blunted by Ang II, both during early and late experiments.

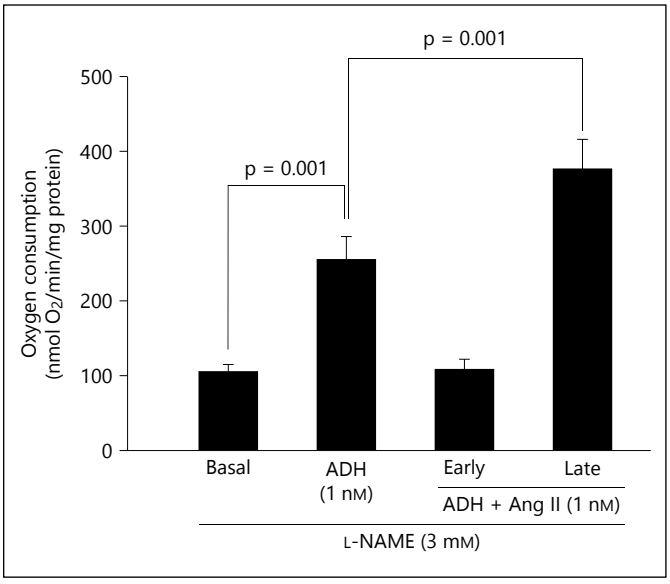

Fig. 4. Effects of ADH and Ang II on TAL oxygen consumption in the presence and absence of the NOS inhibitor L-NAME (n = 5). L-NAME neither prevented the ADH-induced rise in oxygen consumption nor the late response to combine of ADH + Ang II.

Ang II by itself yielded a cAMP level of 83.9 ± 9.2 fmol/min/mg protein ($p > 0.60$ vs. basal). Similarly, in the presence of Ang II, ADH resulted in a cAMP level of 56.6 ± 6.7 fmol/min/mg protein ($p > 0.05$ vs. basal; fig. 3). These data show that ADH-stimulated cAMP production is inhibited by Ang II in the TAL.

Because nitric oxide synthase (NOS) can produce O_2^- leading to enhanced sodium reabsorption by a mechanism known as NOS uncoupling [15] that has been shown to take place in kidney tissue in hypertension, we used the NOS inhibitor nitro-l-arginine methyl ester (L-NAME) to assess whether NOS uncoupling plays a role in the cumulative effects of Ang II on ADH-stimulated QO_2 in SHR. For this, we measured the Ang II capacity to increase ADH-stimulated QO_2 in the presence of L-NAME. Basal oxygen consumption was 105.1 ± 10.3 nmol O_2/min/mg protein in the presence of L-NAME (3 mM). After adding ADH, it increased to 255.7 ± 31.6 nmol O_2/min/mg protein ($n = 5$; $p = 0.001$ vs. basal). Then, after adding Ang II, it decreased first to 109.0 ± 14.7 nmol O_2/min/mg protein, but then it increased and superimposed the ADH-stimulated QO_2, once more until the end of the experiment (QO_2 376.5 ± 40.7 nmol O_2/min/mg protein, $p = 0.001$ vs. ADH; fig. 4).

To rule out other potential mechanisms that could rise O_2^- production – other than Ang II – thus increasing TAL transport [2, 3], we used the O_2^- scavenger tempol. In the presence of tempol, the mean basal oxygen consumption was 95.7 ± 6.6 nmol O_2/min/mg protein, increasing to 214.8 ± 7.5 nmol O_2/min/mg protein after adding ADH ($n = 5$; $p < 0.001$ vs. basal). Ang II decreased the mean oxygen consumption to 80.0 ± 5.9 nmol O_2/min/mg protein ($p < 0.001$ vs. ADH; fig. 5). After this, Ang II failed to stimulate QO_2 to levels like the ADH-stimulated QO_2. This inhibition was maintained until the end of the experiment ($p > 0.30$). Thus, tempol inhibited the cumulative Ang II effects on ADH-stimulated transport.

To confirm the role of O_2^- on the transient Ang II effects on ADH-stimulated transport, we measured O_2^- production in TALs after adding of ADH and Ang II. Mean basal TAL O_2^- was 262.9 ± 38.8 relative luminescence units (RLU)/mg protein. After adding ADH, TAL O_2^- levels remained unchanged at 294.5 ± 44.9 RLU/mg protein. However, addition of Ang II, TAL O_2^- amplified to 544.2 ± 70.9 RLU/mg protein during the first 3 min of the experiments ($p = 0.05$ vs. basal), while at 6 min of incubation with Ang II, TAL O_2^- production increased to 863.9 ± 207.0 RLU/mg protein ($p = 0.015$ vs. basal; fig. 6).

In brief, Ang II initially enhanced QO_2^- levels and this stimulated production was sustained during the late effects of Ang II on ADH-stimulated TAL transport. Because NADPH oxidase, one of the major sources of O_2^- in the TAL is activated by Ang II in Sprague Dawley rats, we

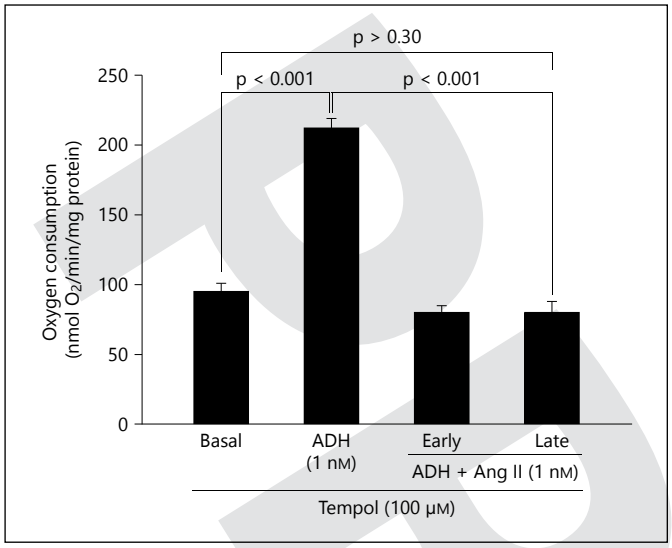

Fig. 5. ADH and Ang II effects on TAL oxygen consumption in the presence and absence of the O_2^- scavenger tempol (n = 5). The ADH-induced rise in oxygen consumption was unchanged by tempol. The late Ang II-induced rise in oxygen consumption was totally blunted by tempol.

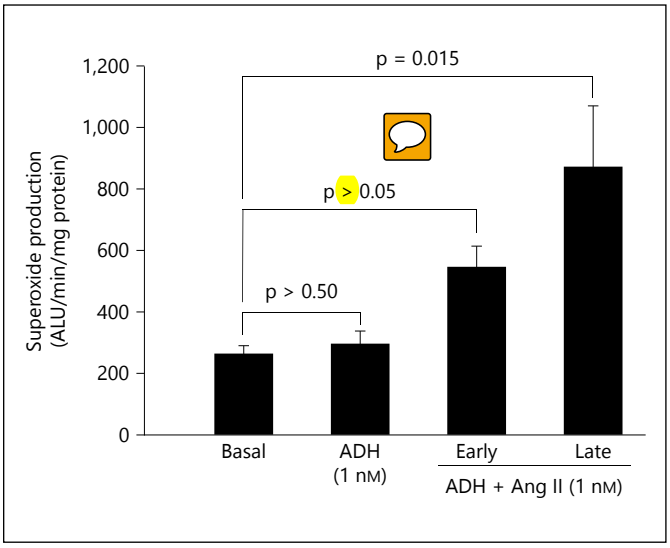

Fig. 6. Effect of ADH and Ang II on TAL O_2^- production (n = 5). ADH did not increase superoxide 'production', whereas Ang II caused an early significant increase in superoxide production. This was further increased during late assessment.

tested whether this was also the case in SHR rats. For this, we measured oxygen consumption in the presence of the NADPH oxidase inhibitor apocynin. In the presence of apocynin (30 μM), basal oxygen consumption was 111.5 ± 15.6 nmol O_2/min/mg protein, increasing to 187.9 ± 23.5 nmol O_2/min/mg protein after adding ADH (n = 5; $p < 0.04$ vs. basal; fig. 7). Ang II decreased oxygen consumption to 107.3 ± 16.4 nmol O_2/min/mg protein ($p < 0.03$ vs. ADH). This decrease was maintained until the end of the experiment.

Discussion

In the thick ascending loop of Henle, the abnormal modulation of signaling molecules may be responsible for increased NaCl reabsorption and therefore for the development of various forms of hypertension [4, 5]. Particularly, enhanced basal sodium reabsorption has been described in the TAL in hypertension [1, 4] and this could contribute to abnormal natriuresis. In addition, impaired responses to other factors such as endothelin-1 [6] and nitric oxide [4] have been described. Be that as it may, an abnormal interaction between the 2 major sodium transport regulators Angiotensin II and ADH are yet to be shown. To better understand the intrinsic mechanism by which sodium excretion is impaired in essential hyper-

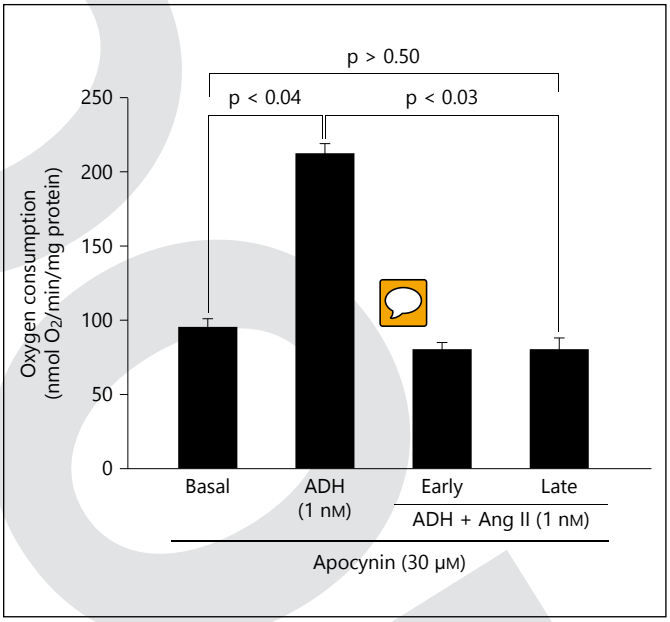

Fig. 7. Effects of ADH and Ang II on TAL oxygen consumption in the presence and absence of the NADPH oxidase inhibitor apocynin (n = 5). The ADH-induced rise in oxygen consumption was unchanged by apocynin. In contrast, the late Ang II-induced rise in oxygen consumption was blunted by the NADPH oxidase inhibitor, indicating that increased transport in this segment relates to increased O_2^- production.

tension, we studied the interaction of 2 antinatriuretic hormones, ADH and Ang II, both of which are involved in sodium balance and hypertension. First, we demonstrated that Ang II in normotensive rats decreases ADH-stimulated cAMP in the TAL. This effect should inhibit sodium reabsorption. However, after a transient fall in transport, Ang II stimulated O_2^- generation, and thereafter ADH-stimulated transport was recovered. Consequently, we hypothesized that in SHR animals, Ang II increases ADH-mediated transport and this mechanism is mediated by O_2^-.

The interaction between Ang II and ADH is well defined. Indeed, during water deficit, both ADH and Ang II releases rise in an attempt to generate positive sodium balance [7]. This is important because hypertension is a condition characterized by chronic positive sodium balance. To better evaluate these mechanisms, the effects of these 2 hormones on the TAL need to be defined. On these grounds, we studied the role of Ang II on ADH-stimulated transport in this nephron segment in SHR's rats, a model of enhanced renal O_2^- generation and essential hypertension. For this, we first tested the mechanisms involved in the Ang II/ADH interaction in this hypertension model (SHR) and its effects on Na transport-related oxygen consumption. Because of the increased medullary tissue fibrosis, isolation and perfusion of TALs from hypertensive animals are difficult to perform and are highly variable. Consequently, we chose to measure oxygen consumption.

To our knowledge, this is the first work reflecting the combined actions of these 2 transport inhibiting hormones at the TAL level in hypertension. Still, Ang II/ADH interactions have been shown in other tubular segments under normotensive conditions. The actions of these hormones include the modulation of aquaporin expression in collecting ducts [8] and Ang II-induced V2 receptor up-regulation in the medullary collecting duct [9]. These indicate that both compounds exert similar actions in other structures. In the present work, we focused on the joint Ang II/ADH effects on TAL in hypertension, as we had already observed similar interactions on oxygen consumption under normotensive conditions [10].

Our results showed that in the thick ascending loop of Henle from SHR, Ang II initially decreases ADH-stimulated QO_2, and this effect is associated with a decrease in intracellular cAMP, previously stimulated by ADH. After that, transport is over stimulated, and it even reaches higher levels than those induced by ADH alone. This effect is different from those we observed in the past with normotensive animals, in which Ang II did not exert any additional effects on the ADH-stimulated transport. In normotensive and hypertensive animals and during a short period of time, Ang II decreases ADH-stimulated QO_2. This effect seems to have similar mediators in normotensive and hypertensive populations, where a decrease in intracellular cAMP seems to be involved. However, the physiologic importance of this transient effect is still under study. However, it is probable that the enzyme kinetics of O_2^- generating machinery in hypertension might be also exacerbated after Ang II stimulation [1, 11].

Hypertension increases renal O_2^- production. Acute and chronic O_2^- treatment stimulates NaCl absorption in the TAL [1, 11]. Thus, we measured the effect of Ang II-dependent hypertension on O_2^- production in this segment. Although an increased reactive oxygen species have been shown in the kidney cortex [12] and medulla [3, 4, 10] in several models of hypertension, O_2^- production in the TAL has not been measured in SHRs. We found that O_2^- in medullary TALs was increased early in SHRs after stimulation with Ang II. However, this does not necessarily imply that this segment is the only nephron segment involved in the overproduction of O_2^- under this pathological condition.

We also found that in SHRs there is a stimulation of Na transport-related oxygen consumption in the TAL. In fact, abnormal Na handling by the TAL in this model has been shown before [13]. Be that as it may, there are no previous reports to our knowledge on the functional effects of the Ang II/ADH interactions on Na transport in any nephron segment in this hypertension model.

Although in a previous work we have tested the hormonal interaction between Ang II and ADH during normotensive conditions [13], it is considerable to compare the present results to those. We found in this work that in SHR, animals have superimposed hormonal effects; however, this was not observed before, even in normal blood pressure conditions. Although the mechanism of interaction remains the same, the additive or superimposed hormonal effects are observed only in hypertension. This could be due to the fact that V2 receptors and Ang II receptors might be upregulated [5] or because the genetic model we used has an enhanced tendency to increase transport [7], as has been shown by other investigators in other medullar segments of the nephron.

We found that in SHRs O_2^- production is increased in the TAL. These results expand other investigations showing that in SHRs there is increased production of reactive oxygen species in the kidney [14]. Our findings

are also supported by data showing that Ang II by itself can acutely stimulate O_2^- production in this segment. Indeed, significantly higher O_2^- levels were detected at the Ang II early period in SHRs compared to normotensive animals [15]. These findings point to and confirm that the O_2^- production mechanism is exacerbated in essential hypertension, hence supporting increased sodium retention.

Because the effects of Ang II on O_2^- production are mediated by AT1 receptors, we predicted that blockade of these receptors by losartan would decrease O_2^- levels, as seen when adding tempol. In this respect, the leading source of O_2^- in the loop of Henle is NADPH oxidase. Thus, we also anticipated the blockade of the synergic effects by apocynin in our experimental set up.

Increased O_2^- production raises transport in the TAL via the Na/K/2Cl co-transporter. This was studied by Sonalker et al. [7] who showed that Na/K/2Cl co-transporter expression is higher in outer medullas of hypertensive rats [9]. These data suggest a potential role for the Ang II/ADH interaction in the TAL in this model of hypertension, implying a concomitant enhancement of the Na/K/2Cl co-transporter membrane expression.

To evaluate this notion, we chose to use a single physiological dose of Ang II [16]. In addition, we used the maximal stimulating concentration of ADH in the physiological range [16]. This allowed us to define interaction and mediators between both hormones at that specific level in this nephron segment. Thus, we cannot avow the same net effect or mechanisms for different doses. Interpreting a dose-response for the 2 hormones could be flawed by other mechanisms and different involvement of other second messengers.

The present work shows that Na transport by the TAL in SHRs is increased mainly by Ang II-induced O_2^- production. Our results should help comprehending: (a) medullary Na transport in the pathogenesis of hypertension and (b) the task of combined increased Ang II and ADH secretion in the genesis of hypertension. It also justifies why at normal levels of intrarenal Ang II, most renal retention mechanisms are quickly activated resulting in extended periods of Na reabsorption. It also extends our knowledge on reabsorption mechanisms through the hypertensive course. Additionally, we believe that a more complete understanding of the signaling pathway involved in increased Na absorption may lead to the development and design of specific compounds, which could contribute to decrease Na retention during hypertension.

Perspective

We found that during essential hypertension the interaction and overlapping of the main sodium controller hormones are found to be abnormal. May be novel therapies could be directions not only to the normalization of Ang II levels or AT1 receptor blockade but also to normalize the interactions of Ang II with other hormones. That could bring a new era of modern diuretics.

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Disclosure Statement

None.

((Please insert a '**Statement of Ethics**'. If no statement is required the follow text may be used:
"This study did not require informed consent nor review/approval by the appropriate ethics committee."))

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