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LETTER TO THE EDITOR

ANGIOTENSIN II INHIBITS ADH-STIMULATED cAMP. ROLE ON O_2^- AND TRANSPORT-RELATED OXYGEN CONSUMPTION IN THE LOOP OF HENLE

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Dehydration and acute reductions of blood pressure increases ADH and Ang II levels. These hormones increase transport along the distal nephron. In the thick ascending limb (TAL) ADH increases transport via cAMP, while Ang II acts via superoxide (O_2^-). However, the mechanism of interaction of these hormones in this segment remains unclear. The aim of this study was to explore ADH/Ang II interactions on TAL transport. For this, we measured the effects of ADH/Ang II, added sequentially to TAL suspensions from Wistar rats, on oxygen consumption (QO_2) -as a transport index-, cAMP and O_2^-. Basal QO_2 was 112±5 nmol O_2/min/mg protein. Addition of ADH (1nM) increased QO_2 by 227%. In the presence of ADH, Ang II (1nM) elicited a QO_2 transient response. During an initial 3.1±0.7 minutes after adding Ang II, QO_2 decreased 58% (*p*<0.03 initial vs. ADH) and then rose by 188% (*p*<0.03 late *vs* initial Ang II). We found that Losartan blocked the initial effects of Ang II and the latter blocked ADH and forskolin-stimulated cAMP. The NOS inhibitor L-NAME or the AT2 receptor antagonist PD123319 showed no effect on transported related oxygen consumption. Then, we assessed the late period after adding Ang II. The O_2^- scavenger tempol blocked the late Ang II effects on QO_2, while Ang II increased O_2^- production during this period. We conclude that 1) Ang II has a transient effect on ADH-stimulated transport; 2) this effect is mediated by AT1 receptors; 3) the initial period is mediated by decreased cAMP and 4) the late period is mediated by O_2^-.

PROOF

By actively reabsorbing 15-25% of the filtered NaCl, the medullary thick ascending limb contributes to build up the osmotic gradient needed to drive the counter-current multiplication mechanism that is responsible for water absorption at the collecting ducts. In this process, several hormones interact to create the osmotic gradient (1-4). The combined effects of these hormones may play a significant role in several pathophysiological conditions. For instance, both Angiotensin II (Ang II) and antidiuretic hormone (ADH) may interact in response to dehydration (5, 6) or acute blood pressure reduction (7).

A key concept is that ADH not only promotes water permeability in the collecting tubules, but also promotes active NaCl transport across the thick ascending limb via increased cAMP (8-10). This mechanism increases interstitial osmolality and thus

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water reabsorption in the collecting duct (9, 11). Consequently, ADH's action on the medullary thick ascending limb is important for water conservation under conditions of water deprivation.

Unlike ADH, acute exposure to Ang II has been shown to produce inhibition (12) or stimulation (13) of NaCl transport, depending on the experimental conditions. On the other hand, like ADH, chronic exposure to Ang II enhances NaCl reabsorption in the thick ascending limb (14) thereby increasing blood pressure (15). In this segment, most of the Ang II effects are due to increased superoxide (O_2^-) production, a potent antidiuretic autacoid (14, 16). Thus, it could be reasoned that ADH and Ang II interact to preserve body volume. For instance during dehydration, both ADH and Ang II should trigger Na retention mechanisms in the distal nephron. Because the combined effects of Ang II and ADH are poorly defined, we aimed to investigate their interaction as well as the role played by mediators involved in thick ascending limb transport-related oxygen consumption.

MATERIALS AND 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. Male Wistar rats weighing 150 to 200 g were fed normal chow diet for 7 to 10 days prior to the experiments.

Rat medullary thick ascending limb suspensions

Medullary thick ascending limb suspensions were prepared as described previously (14, 17-22). 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 100U heparin. Both kidneys were excised and the inner stripe of the outer medulla was dissected from coronal slices. The tissue was minced (to approximately 1mm^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 x g for 2 min, re-suspended 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 re-suspended in 1 ml cold HEPES-buffered physiological saline.

Measurement of oxygen consumption

To examine the interactions of Ang II and ADH on tubular transport we used oxygen consumption. This technique allowed us to assess these hormones effects on both transport and cell metabolism. Oxygen consumption is a suitable technique to measure transport because it is stoichiometrically related to Na transport: 35-50% of total oxygen consumption by the thick ascending limb is associated with NaCl transport (14, 20). To measure oxygen consumption, thick ascending limbs 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. At the beginning of the experiment maximal concentration of dissolved oxygen was determined and constantly monitored, showing a decrease in a time dependent manner due to thick ascending limb consumption (20). An initial constant slope was established for each experiment (3 to 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; 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 (14, 23, 24). Thick ascending limb 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. Difference in average luminescence between periods with and without tiron was

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used to calculate the luminescence produced by O_2^-.

Cyclic AMP measurements

cAMP was measured as described previously (25). Aliquots of thick ascending limbs 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 after 30 min of incubation 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

Total protein content was determined using Bradford's colorimetric method.

Statistics

Data are reported as mean±SEM. Differences in means were analysed using ANOVA with repeated measurements using Bonferoni as *post-hoc* test. P values <0.05 were considered significant for comparisons.

RESULTS

Ang II and ADH increase transport in the thick ascending limb by independent mechanisms (9, 13). We first measured the effect of ADH (1nM) on basal oxygen consumption. Figure I shows that basal oxygen consumption was 112±5 nmol O_2/min/mg protein. After adding ADH, it increased to 255±29 nmol O_2/min/mg protein ($p<0.03$ *vs* basal), a 127% increment. We then added Ang II (1nM) in the presence of ADH, oxygen consumption decreased to 148±15 nmol O_2/min/mg protein ($p<0.03$ *vs* ADH). This Ang II-induced initial fall was sustained up to 3.1±0.7 min. Then, oxygen consumption increased to 279±17 nmol O_2/min/mg protein. This late effect remained at this level until the end of the experimental period (6 min) (n = 5; $p<0.03$ ADH + Ang II initial *vs* ADH + Ang II late; Fig. 1A).

In reverse experiments, we tested the effects of adding first Ang II and then ADH. Under these conditions, basal oxygen consumption was 118±16 nmol O_2/min/mg protein. After adding Ang II, it increased to 139±22 nmol O_2/min/mg protein (n = 6; N.S *vs* basal), an 18% increment that lasted 6 min, we immediately added ADH (1 nM) in the presence of Ang II and oxygen consumption increased further to 271±35 nmol O_2/min/mg protein ($p<0.05$ Ang II + ADH *vs* Ang II) remaining at this level until the end of the experiment (Fig. 1B). These reverse studies lacked the biphasic effects seen in the previous experiments in which we had applied the sequence: ADH first and then Ang II. In control experiments, vehicle of both ADH and Ang II had no effect on oxygen consumption. These data indicate that in the thick ascending limb there is a sequential and coordinated signaling between ADH and Ang II.

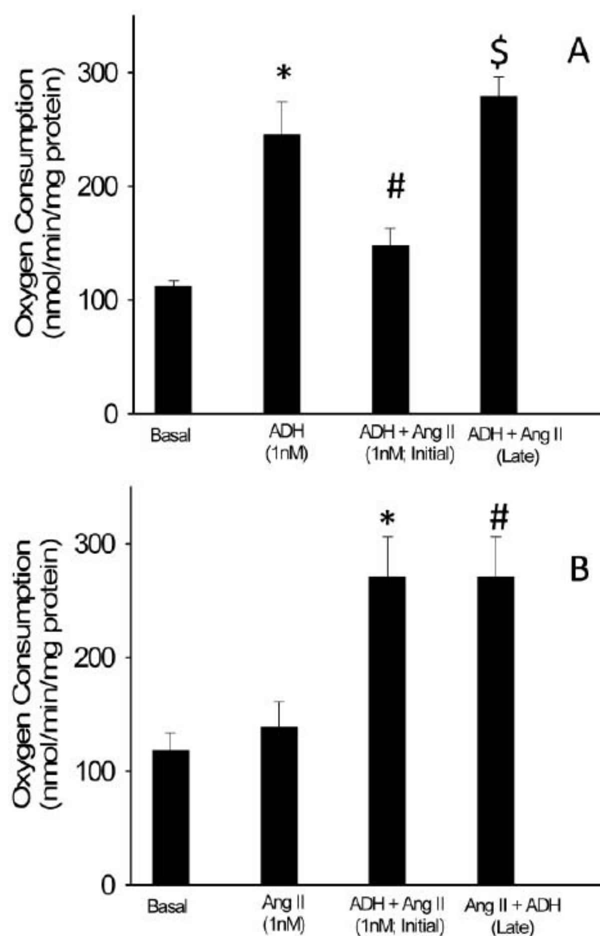

Fig. 1. ***A****) Effects of ADH (first) and Ang II (second) on thick ascending limb oxygen consumption (n = 5 in all the experiments). * = p<0.03 vs. basal; # = p<0.03 vs ADH; $ = ; p<0.03 ADH + Ang II initial vs ADH + Ang II late.* ***B****) Effect of Ang II (first) and ADH (second) on thick ascending limb oxygen consumption (n = 6 in all the experiments). * = p<0.05 Ang II + ADH vs Ang II; # = p<0.05 Ang II + ADH vs Ang II.*

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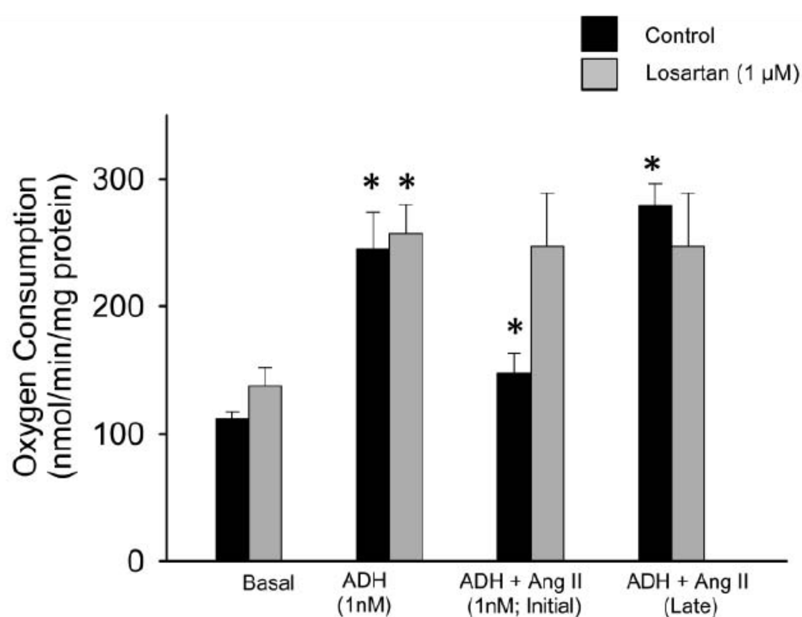

Fig. 2. *A) Effects of ADH (first) and Ang II (second) on thick ascending limb oxygen consumption in the presence and absence of the AT1 receptor inhibitor Losartan (n = 5). * = p<0.03 vs basal.*

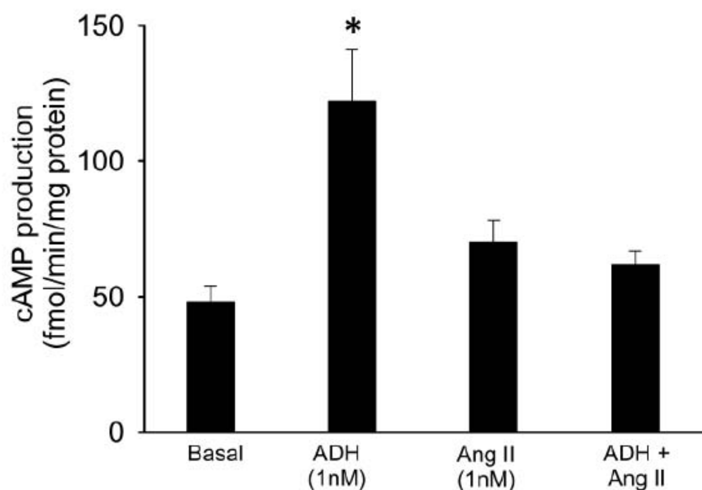

Fig. 3. *Effect of Ang II on ADH-stimulated cAMP production in the thick ascending limbs (n = 5). * = p<0.01 vs basal.*

We next evaluated the signaling involved in the biphasic time-dependent effect. AT1 and AT2 receptors are expressed in the thick ascending limb. To test whether the transient effect of Ang II on ADH-stimulated oxygen consumption was due to differential receptor activation, we first used the AT1 receptor antagonist losartan. In the presence of losartan (1 μM), basal thick ascending limb oxygen consumption was 138±14 nmol O_2/min/mg protein. Losartan had no effect on ADH-stimulated oxygen consumption that rose to 257±23 nmol O_2/min/mg protein (*p* 0.03 *vs* basal). In contrast, in the presence of ADH, losartan prevented the *initial* Ang II-induced fall in oxygen consumption, remaining

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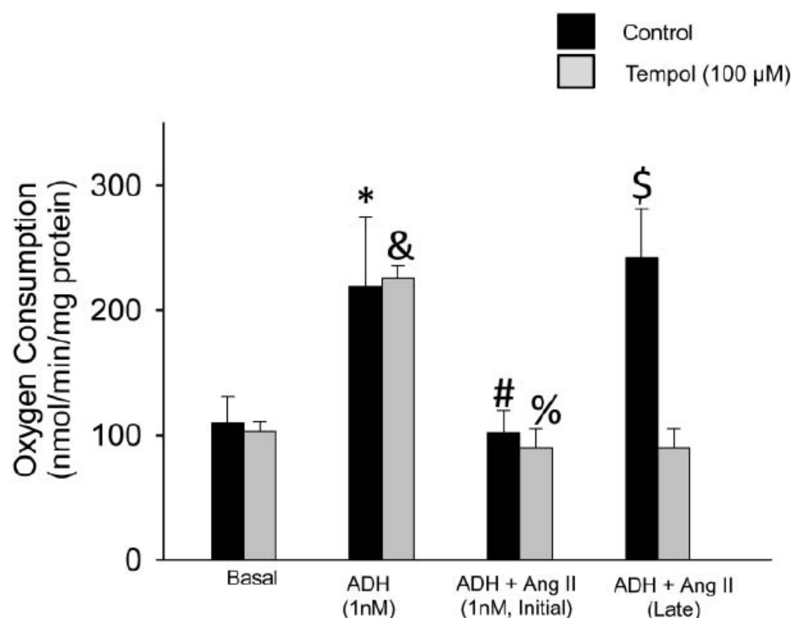

Fig. 4. *ADH and Ang II effects on thick ascending limb oxygen consumption in the presence and absence of the O_2^- scavenger Tempol (n = 5). In Controls (black bars): * = p<0.05 vs basal; # = p<0.02 ADH vs ADH + initial Ang II; & = p<0.04 ADH + Ang II initial vs ADH + Ang II late. In the presence of Tempol (grey bars): & = p<0.03 ADH vs. basal; % = p<0.02 ADH vs ADH + initial Ang II.*

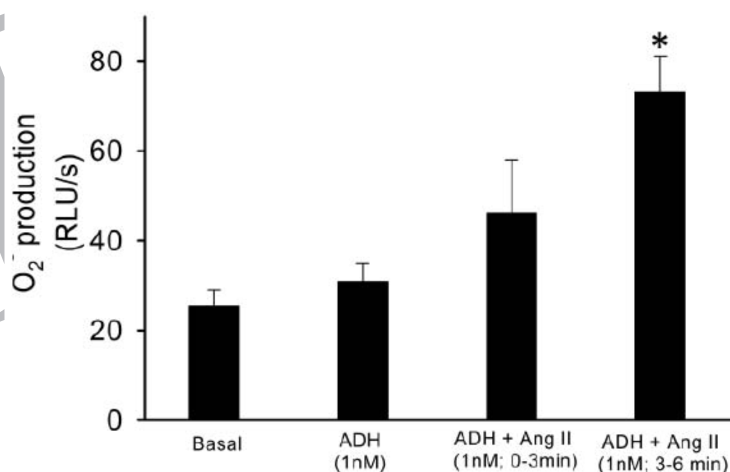

Fig. 5. *Effect of ADH and Ang II on thick ascending limb superoxide production (n = 5). * = p<0.05 vs basal.*

at 247±42 nmol O_2/min/mg protein. (*p* = N.S; ADH *vs* ADH + Ang II initial; Fig. 2). This unchanged oxygen consumption remained at similar levels until the end of the experiment.

To explore the role of AT2 receptors we used the AT2 antagonist PD123319. In the presence of PD123319 (1 μM), basal thick ascending limb oxygen consumption was 118±9 nmol O_2/min/mg protein. After adding ADH, thick ascending limb oxygen consumption increased to 248±17 nmol O_2/min/mg protein (*n* = 5; *p*<0.03 *vs* basal). Addition of Ang II in the presence of ADH, initially decreased oxygen consumption to 180±18 nmol O_2/min/mg protein (*p*<0.05 ADH *vs* ADH + Ang II initial), before rising

to 335±37 nmol O_2/min/mg protein until the end of the experiment (p<0.04 ADH + Ang II initial *vs* ADH + Ang II late). Taken together these data indicate that AT1 receptors and not AT2 receptors mediate the transient effect of Ang II on ADH-stimulated transport-related oxygen consumption.

Nitric oxide (NO) inhibits transport in the thick ascending limb. To assess whether NO plays a role during the initial period of Ang II-induced inhibition, we measured the effects of the NOS inhibitor, L-NAME. In control experiments, basal oxygen consumption was 102±4 nmol O_2/min/mg protein and increased after adding ADH to 256±17 nmol O_2/min/mg protein (n = 5; p<0.03 ADH *vs*. basal). Then, we added Ang II and oxygen consumption fell initially to 102±5 nmol O_2/min/mg protein (p<0.03 ADH *vs* ADH + Ang II initial), after approximately 3 min it increased again until the end of the experiment to 219±20 nmol O_2/min/mg protein (p<0.02 ADH + initial *vs* ADH + Ang II late). In the presence of L-NAME (2 mM), basal oxygen consumption was 96±2 nmol O_2/min/mg protein, it increased after adding ADH to 215±17 nmol O_2/min/mg protein (n = 5; p<0.02 *vs*. basal) and finally after adding Ang II, it decreased first to 80±2 nmol O_2/min/mg protein (p<0.02 ADH *vs* ADH + Ang II initial), and increased again until the end of the experiment to 228±22 nmol O_2/min/mg protein, (p<0.02 ADH + initial Ang II *vs* ADH + Ang II late). The changes in oxygen consumption in the presence of L-NAME were analogous to the changes in its absence. These data indicate that NO is unlikely to be involved in the initial inhibition of Ang II on ADH-stimulated thick ascending limb transport-related oxygen consumption.

The initial Ang II-induced transport-related oxygen consumption effect could also be due to reduced cAMP levels. Indeed, in the thick ascending limb, cAMP mediates the ADH stimulatory effect on transport (26), while Ang II decreases cAMP levels in other nephron segments (27). To test the role of cAMP in our experiments we measured cAMP accumulation. Basal cAMP was 48±6 fmol/min/mg protein. After addition of ADH, cAMP increased to 122±19 fmol/min/mg protein (p<0.01 *vs* basal). Ang II by itself yield a cAMP level of 70±8 fmol/min/mg protein (p = N.S. *vs* basal). Similarly, in the presence of Ang II, ADH resulted in a cAMP level of 62±5 fmol/min/mg protein (p = N.S *vs* basal). These data indicate that ADH-stimulated cAMP production is inhibited by Ang II in the thick ascending limb (Fig. 3). To further evaluate the role of Ang II on cAMP in the thick ascending limbs, we used forskolin to stimulate cAMP in thick ascending limb suspensions. Basal cAMP was 102±9 fmol/min/mg protein. After incubation with forskolin (1μM), cAMP increased to 218±25 fmol/min/mg protein (p<0.04 *vs* basal). Next, we added Ang II. Early, during a 3 minute period, Ang II inhibited cAMP production to 106±6 fmol/min/mg protein (p = N.S *vs* basal) and this inhibition persisted until the end of the experimental period (98±13 fmol/min/mg protein; p = N.S *vs* basal). Taken together, these data indicate that cAMP is involved in the inhibitory effects of Ang II, during the initial effects of Ang II on ADH -stimulated thick ascending limb transport-related oxygen consumption.

We then studied potential mediators of the late Ang II-induced effects on ADH-stimulated transport-related oxygen consumption. Ang II enhances transport in the thick ascending limb (14) by increasing O_2^- production (28). Thus, to assess a potential O_2^- role on thick ascending limb transport-related oxygen consumption, we used the O_2^- scavenger tempol. In the absence of tempol (100 μM), basal oxygen consumption was 109±21 nmol O_2/min/mg protein. After adding ADH, thick ascending limb oxygen consumption increased to 219±56 nmol O_2/min/mg protein (n = 5; p<0.05 *vs* basal). Addition of Ang II initially decreased oxygen consumption to 102±18 nmol O_2/min/mg protein (p<0.02 ADH *vs* ADH + initial Ang II), and then increased to 242±39 nmol O_2/min/mg protein until the end of the experiment (p<0.04 ADH + Ang II initial *vs* ADH + Ang II late). In contrast, in the presence of tempol, basal oxygen consumption was 103±8 nmol O_2/min/mg protein, increasing to 226±10 nmol O_2/min/mg protein after adding ADH (n = 5; p<0.03 ADH *vs* basal). Ang II decreased oxygen consumption to 91±15 nmol O_2/min/mg protein (p<0.02 ADH *vs* ADH + initial Ang II), and this decrease was maintained until the end of the experiment (Fig. 4). Thus, tempol inhibited the late Ang II effects on ADH-stimulated transport-related oxygen consumption, suggesting that O_2^- mediates these actions in the thick ascending limb.

PROOF

To confirm the role of O_2^- on the transient Ang II effects on ADH-stimulated transport-related oxygen consumption, we measured O_2^- production in thick ascending limbs after addition of ADH and Ang II. Basal thick ascending limb O_2^- was 25.5±3.5 Relative Luminescence Units (RLU)/mg protein. After adding ADH, thick ascending limb O_2^- levels remained unchanged at 30.9±4.0 RLU/mg protein. After adding Ang II thick ascending limb O_2^- was 46.2±12.8 RLU/mg protein during the first 3 min of the experiments (p = N.S *vs* basal). However, at 6 minutes of incubation with Ang II, thick ascending limb O_2^- production increased to 73.2±8.7 RLU/mg protein (p=<0.05 *vs* basal; Fig. 5). Taken together, these data indicate that O_2^- is involved in the stimulatory effects of Ang II, during the late effects of Ang II on ADH -stimulated thick ascending limb transport-related oxygen consumption.

DISCUSSION

The thick ascending limb reabsorbs 15% to 25% of the filtered NaCl, and by these means regulates the cortico-medullary osmotic gradient that controls water absorption in the collecting duct. In this segment, abnormal modulation of signaling molecules is responsible for increased NaCl reabsorption and very likely for the development of various forms of hypertension (14, 29). Water deficit increases ADH release and through hypovolemia may increase Ang II release (5).

Both ADH and Ang II may interact in regulating the thick ascending limb transport mechanisms. On these grounds, we studied the role of Ang II on ADH stimulated transport-related oxygen consumption in this nephron segment. We found that Ang II causes a time-dependent biphasic effect on ADH stimulated transport-related oxygen consumption but it appears that ADH does not produce such similar effect on Ang II- stimulated transport-related oxygen consumption. These data suggest that Ang II at first interferes with ADH-induced reabsorption and then, reverses these initial inhibitory actions mainly through Ang II-induced O_2^- release. For these reasons we then focused our study on studying the transient phenomena.

Ang II / ADH interactions have been shown in other tubular segments. For instance, Wand et al. suggested that Ang II, regulates aquaporin expression in collecting ducts via the AT1 receptor and by these means modulates urinary concentration *in vivo* in ADH treated animals (30). This view is also supported by studies showing that Ang II induces V2 receptor up-regulation in the medullary collecting duct (31). To our knowledge, even though Ang II / ADH interactions have been consistently shown in other segments, the thick ascending limb has not been independently evaluated in this regard.

Transport-related oxygen consumption inhibition in the thick ascending limb could be mediated by NO or cAMP. On the one hand, Herrera et al have reported acute Ang II-induced NO synthesis in thick ascending limb via AT2 receptor activation (32). We measured thick ascending limb oxygen consumption in the presence of the NOS inhibitor L-NAME and found no differences compared to control. Moreover, the initial Ang II inhibition was not altered by the AT2 antagonist. These results add more evidence to support the hypothesis that NO does not mediate the inhibitory effects of Ang II on ADH-stimulated oxygen consumption.

On the other hand, the initial Ang II inhibitory effect on ADH stimulated transport- related oxygen consumption associated with decreased cAMP production in thick ascending limbs suspensions, suggesting that Ang II prevents ADH transport-related oxygen consumption stimulation by cAMP inhibition. To further explore the role of cAMP in our experiments, we stimulated thick ascending limbs adenylate cyclase by using forskolin. We found that Ang II decreased forskolin-stimulated cAMP. In fact the Ang II-induced initial inhibition of oxygen consumption was also reversed by the AT1 receptor antagonist Losartan, a finding consistent with the current notion that AT1 receptors can inhibit cAMP accumulation through Gi protein coupled receptors (33). Indeed, in proximal renal epithelia, Ang II activates Gi protein, blocking by these means adenylate cyclase and thus cAMP production (27).

After transport-related oxygen consumption inhibition of Ang II a late recovery period was observed. This late increase in transport-related oxygen consumption was blunted by the O_2^- scavenger tempol suggesting that O_2^- mediates the late effect. These results are in line with our experiments showing that Ang II increases thick

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ascending limb O_2^-. In this regard, we assume that Ang II activates NADPH oxidase, as this enzyme is the main source of O_2^- in the thick ascending limb, where intracellular Ca^{2+} or diacylglycerol activate PKCα, an enzyme involved in the assembling of the NADPH oxidase complex (23, 28)

Our findings show that Ang II, in previously ADH stimulated thick ascending limb, triggers two different signaling cascades with separate transport purposes: an early inhibition and a delayed stimulation. These effects appear not to be present in experiments done in reverse sequence (ADH added after exposing the thick ascending limb to Ang II).

In addition, our study shows that the AT1 receptor mediates the Ang II initial and late effects. However, these seemingly puzzling opposite effects of Ang II on Na transport have been previously described in medullary loop of Henle. Moreover, Ang II is known to generate natriuretic or antinatriuretic responses depending on the dose (12, 13).

For this study, we chose to use single dose of Ang II that do not exceed the physiological levels (34). In addition, we used the maximal stimulating concentration of ADH in the physiological range (35). 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 of the two hormones could be flawed by other mechanisms and different involvement of other second messengers.

We found that Ang II increased O_2^- levels, but not ADH. This is at variance with reports on other tissues where ADH has shown to increase O_2^- by various mechanisms. For instance, in pathologic conditions, ADH stimulates O_2^- release in vascular cells via Endothelin-1 (36). This was not the case in our study, perhaps related to differences in cell type, phenotype and cellular function.

A limitation of our study lays on the fact that our experiments were conducted in the absence of flow, avoiding by these means a potential mechanical effect that could activate NADPH oxidase, as it was shown in the past. NADPH oxidase induced O_2^- production by Ang II during no-flow *vs* flow remains undefined. Although our model does not allow to assess the influence of flow changes on O_2^- production; it has been reported that Ang II has the ability to increase O_2^- levels even during flow conditions (23).

An obvious question would be why the renal medulla needs two different mechanisms for a single hormone at different times. We found opposite results of Ang II during a short period of time. In the conditions presented in this paper Ang II by first inhibiting transport-related oxygen consumption, operates as a volume conservative hormone. Evolutionary Ang II seemed to play a crucial role in blood pressure control and extracellular volume and Na conservation, since losses of volume at the origin of the species were critical for survival plus Na was scarce (37, 38). Later on in time, with the Na abundance and the development of human society, the volume conservation actions of Ang II were no longer required and possibly forced to adapt to reverse its original effects. Apparently our observations could show a transition phase of the Ang II actions. Still, whether a failure in the balance or adaptation of this mechanism causes pathological phenotypes remains unknown.

In summary, our study shows that ADH-stimulated transport-related oxygen consumption by the thick ascending limb can be modulated by Ang II through two different and consecutive signaling pathways involving cAMP and O_2^-. The latter mediates a recovery mechanism from the initial inhibitory transport-related effects of Ang II.

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