Endothelin Upregulates the Expression of Vasopressin V_2 mRNA in the Inner Medullary Collecting Duct of the Rat

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Recent studies in our laboratory have demonstrated that bosentan, a mixed endothelin ET_A/ET_B receptor antagonist, prevented the upregulation of the arginine vasopressin (AVP) V_2 receptor in the inner medullary collecting duct (IMCD) of cardiomyopathic hamsters. These results suggested that endothelin-1 (ET-1) is involved in the upregulation of AVP V_2 receptors. Studies were performed to detect the effect of ET-1 on the expression of AVP V_2 receptors and the ET receptor mediating these effects within the IMCD of the rat. Rat IMCD tissue was isolated and incubated with the following: ET-1, or ET-1 in combination with ET_A and ET_B receptor antagonists BQ-123 and BQ-788, respectively, and sarafotoxin c (S6c), an ET_B receptor–specific agonist. Tissue samples were then analyzed using quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blotting. ET-1 treatment resulted in increased V_2 mRNA from a control level of 186.8 ± 15.0 amol/μg total RNA to 430.7 ± 49.0 amol/μg total RNA ($P < .003$). ET-1/ET_A treatment resulted in no significant decrease in V_2 mRNA expression 335.0 ± 38.0 amol/μg total RNA. Whereas ET-1/ET_B, and ET-1/ET_B/ET_A treatment resulted in V_2 mRNA approaching control 256.0 ± 15.0 amol/μg total RNA, and 215.6 ± 42.3 amol/μg total RNA. However, ET-3 treatment produced no significant changes in V_2 receptor mRNA expression. Sarafotoxin treatment corroborated both the ET-1 and ET receptor antagonist data, demonstrating striking significant increases in V_2 receptor mRNA and protein expression. S6c treatment increased V_2 mRNA expression from a control level of 199 ± 17.3 amol/μg total RNA to 284.3 ± 42.1 amol/μg total RNA ($P < 05$). Western blotting revealed that changes in V_2 mRNA expression in the various treatment conditions were similar to changes in protein expression. Overall, these data indicate that in the IMCD ET-1 increases AVP V_2 receptor expression and these changes are mediated by the ET_B receptor.

VASOPRESSIN'S ROLE in the control of water reabsorption in the inner medullary collecting duct (IMCD) is central to our understanding of the control of water reabsorption. The V_2 receptor is the primary AVP receptor expressed by the IMCD.[1] V_2 receptor stimulation results in the activation of a protein kinase A (PKA) pathway; leading to increased aquaporin-2 (AQP-2) synthesis, and insertion into the apical membrane of the principal cells.[2] Rat left coronary artery ligation congestive heart failure models have demonstrated up regulated AQP-2 water channel expression. Further, it has also been shown that these changes in expression could be attenuated by a V_2 receptor antagonist treatment and were correlated to V_2 receptor expression.[3-5] Intuitively, factors that affect V_2 expression would be expected to play significant roles in the control of water homeostasis, and provide insight into disease pathology.

The role of endothelin-1 (ET-1) has also been investigated in congestive heart failure. Endothelin levels are elevated and effective as a prognostic indicator in congestive heart failure.[6-8] ET-1's role is of particular interest given that the kidney's sensitivity to ET-1 is greater than any other tissue of the body and its high level of expression within the IMCD.[9,10] Increased levels of ET-1 have been shown to reduce glomerular filtration rate, renal plasma flow, natriuresis, and diuresis, and to increase medullary blood flow.[11-13] Except for the ET_B-mediated changes in the medullary blood flow, these hemodynamic effects have been shown to be mediated by ET_A receptors.[13,14] As in the medullary capillaries, ET_B mediation of ET signaling may also occur in the IMCD, where ET_B receptor expression predominates.[15,16]

Recent studies in our laboratory have shown that V_2 mRNA is upregulated in the congestive heart failure model (cardiomyopathic hamsters), and bosentan, a mixed ET_A/receptor antagonist, could attenuate these changes in expression.[17] This report suggests that increased endogenous ET levels may effect increases in V_2 receptor expression and affect water reabsorption in congestive heart failure. The goal of this study was to investigate the effects of ET-1 on V_2 receptor expression, and identify the ET receptor mediating these effects.

MATERIALS AND METHODS

General IMCD Extraction and Culturing Procedures

Female Wistar rats (UBC Animal Centre, Vancouver, Canada) with an average mass of 300 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (Somnotol 65 mg/kg; MTC Pharmaceuticals, Cambridge, Canada). The kidneys were removed, longitudinally bisected, and the papillary tissue was excised. The IMCD was then coarsely homogenized, cultured in RPMI-1640 culturing medium (Sigma-Aldrich, Oakville, Canada) supplemented with 5% bovine calf serum (HyClone, Logan, UT), and incubated for 17 hours, at 37°C and 5% CO_2.

ET Receptor Specificity Determination

Depending on the experimental demands: 1×10^{-7} sarafotoxin (S6c), 1×10^{-7} ET-3, 1×10^{-7} ET-1 (Calbiochem, La Jolla, CA), ET-1, and 1×10^{-6} ET_A-specific receptor antagonist BQ-123 (Bachem, Torrance, CA), ET-1, and 1×10^{-6} ET_B-specific receptor antagonist BQ−788 (BachemA), or ET-1 and both BQ-123 and BQ-

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Submitted November 13, 2003; accepted February 13, 2004.

Supported by a grant from the Heart and Stroke Foundation of BC and Yukon to N.L.M.W. and Show Chwan Memorial Hospital.
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0026-0495/04/5309-0044$30.00/0
doi:10.1016/j.metabol.2004.02.022

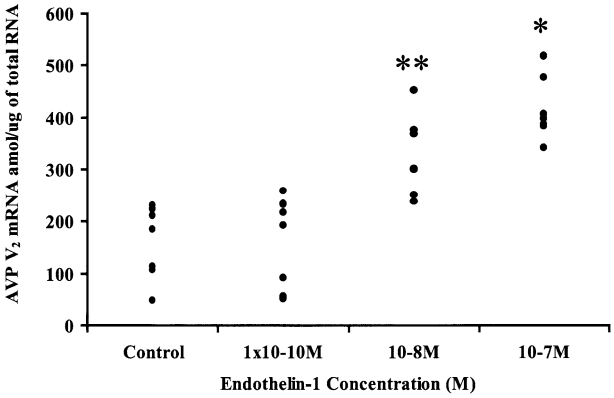

Fig 1. The effect of different concentrations of ET-1 on the expression of vasopressin V_2 mRNA expression in the IMCD of the rat. *$P < .001$, **$P < .003$, Student's *t* test.

788 were used for the determination of receptor specificity. ET receptor antagonists were added 1 hourr before ET-1 addition.

Competitive Reverse-Transcriptase Polymerase Chain Reaction

Competitive reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to quantify changes in the expression of V_2 receptor mRNA. Reverse transcription was performed on the samples followed by competitive PCR using a uniform quantity of RT product and a single set of primers to amplify both target and competitor. The 355-bp competitor was synthesized using the sense primer 5′-AGC AAC AGC AGC CAG GAG GAA C-3′ and antisense primer 5′-GGC CCA GCA ATC AAA CAC CCG CCA GGA TCA TGT AGG AGG-3′. The sense primer 5′-AGC AAC AGC CAG GAG GAA-3′ and antisense primer 5′-GGC CCA GCA ATC AAA CAC CC-3′ result in a 522-bp target product. All PCR reactions were performed using a Gene Amp PCR System 2400 thermocycler (Perkin Elmer, Norwalk, CT).

DNA Signal Quantification

DNA PCR products were separated using a 1.5% agarose gel (Invitrogen, Burlington, Canada), visualized using ethidium bromide (Sigma-Aldrich) staining, and quantified by computer densitometry (Multi-Image Light Cabinet; Alpha Innotech, San Leandro, CA).

Western Blot

Lysates were prepared and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes using the Semi-dry Transfer cell (Bio-Rad, Hercules, CA). Membranes were blocked in phosphate buffer containing 0.1% Tween-20 and 5% nonfat dry milk at room temperature for 2 hours. After blocking procedures, membranes were incubated in the primary and secondary antibodies for 1 hour and visualized using an enhanced chemiluminescence (ECL) system according to manufacturer's protocol (Amershan Baie d'Urfe, Canada). Anti-rat V_2 receptor antibody purchased from Alpha Diagnostic (San Antonio, TX) detects a band at 62 kd in the membranes from rat inner medulla collecting ducts. The bands were quantified by computer densitometry (Multi-Image Light Cabinet). The membrane was stripped and reprobed with β-actin to ensure equal loading.

Statistics

For statistical comparison, a single value of mRNA protein expression was determined for each incubation treatment condition, from an average of the values obtained from each treatment protocol. The value of (n) represents the number of rat kidneys for each treatment condition. Data are presented as means ± SE and the paired Student's *t* test was employed for statistical comparison. *P* values less than .05 were considered significant.

RESULTS

AVP V_2 Receptor mRNA Expression Following Incubation With Varying ET-1 Concentrations

Figure 1 illustrates the RT-PCR–determined changes in V_2 mRNA expression in rat IMCD tissue resulting from various incubation concentrations of ET-1. ET-1 concentrations of 1×10^{-10}, 1×10^{-8}, and 1×10^{-7}, resulted in V_2 mRNA expression levels of 217.4 ± 62.8 amol/μg total RNA (n = 7, difference not significant [NS]), 327.0 ± 29.0 amol/μg total RNA (n = 7, $P < .001$), and 387.1 ± 28.3 amol/μg total RNA (n = 7, $P < .003$), respectively, compared to control expression of 199.0 ± 28.3 amol/μg total RNA. Western blotting investigated changes in AVP V2 receptor levels. Relative levels of V_2 receptor expression increased from a control levels of 0.369 ± 0.075 to 0.709 ± 0.090 (n = 13, $P < .005$) when treated with 1×10^{-7} mol/L ET-1 as shown in Fig 2.

AVP V_2 Receptor Expression Following Incubation With ET-1 and ET-Specific Receptor Antagonists

The effects of ET-1 and ET-1 in combination with ET_A- and ET_B-specific receptor antagonists on V_2 receptor and receptor mRNA expression are shown in Figs 3 to 6. Seventeen hours incubation of rat IMCD cells with 1×10^{-7} mol/L ET-1 resulted in a 115% increase in V_2 receptor mRNA expression from a control expression of 186.8 ± 15.0 amol/μg total RNA to 430.7 ± 49.0 amol/μg total RNA, (n = 10, $P < .005$) as

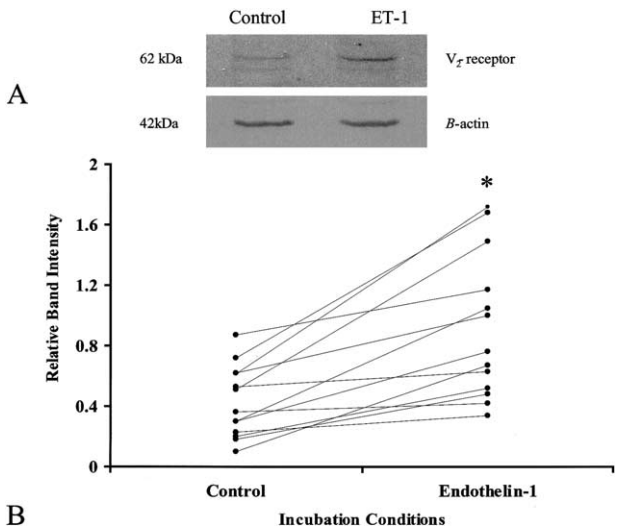

Fig 2. (A) A representative Western blot of control and 1×10^{-7}mol/L ET-treated IMCD tissue samples. (B) AVP V_2 receptor expression following $1 \cdot 10^{-7}$ mol/L ET-1 treatment. *$P < .005$.

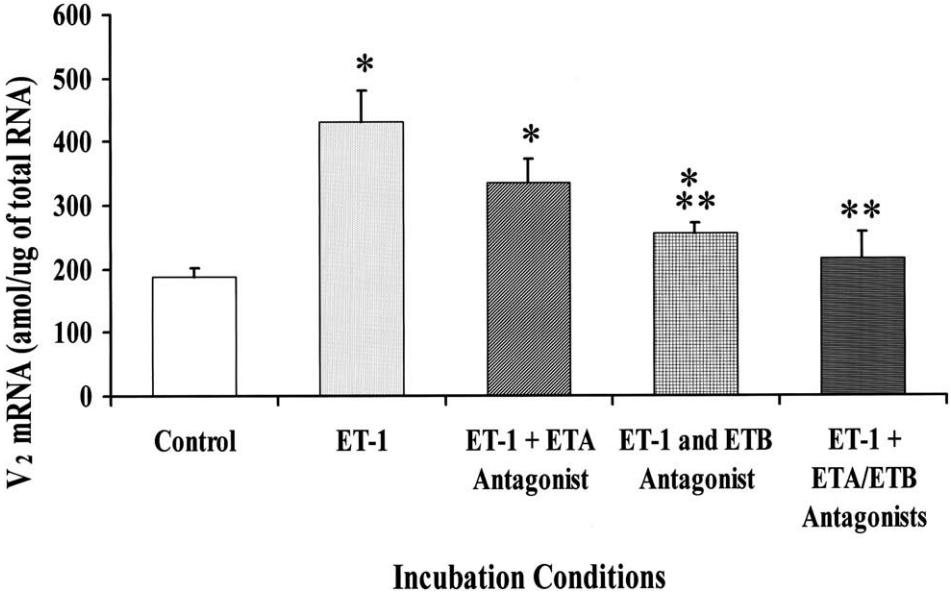

Fig 3. IMCD V_2 receptor mRNA expression in samples treated with ET-1 and ET-1 in combination with ET_B, and ET_A and ET_B receptor–specific antagonists. Significance *v* control samples: **P* < .003, and *v* ET-1–treated samples: ***P* < .002.

determined by RT-PCR (Fig 3). ET_A- and ET_B-specific receptor antagonists and ET-1 were then added in various combinations to investigate the identity of the ET receptor mediating ET signaling. Combined incubation of tissue with ET-1 and ET_A resulted in levels of V_2 mRNA expression that were not significantly different from ET-1 incubations, 335.0 ± 38.0 amol/μg total RNA (n = 10, P = .07) (Fig 3). Western blotting also revealed no significant differences in V_2 receptor expression compared to samples treated exclusively with ET-1 (Fig 4). ET-1/ET_B treatment resulted in the level of V_2 mRNA expression approaching controls, 256.0 ± 15.0 amol/μg total RNA (n = 10) (Fig 3). There was also a 24.8% relative decrease in protein expression, 0.46 ± 0.09, compared to paired ET-1–treated, 0.61 ± 0.10 (n = 6) (P = .05) (Fig 5). These results were similar to RT-PCR data from incubations using both the ET_A and ET_B receptor antagonists in combination with ET-1. V_2 receptor mRNA expression levels were found not to be significantly different from those of controls, 215.6 ± 42.3 amol/μg total RNA (Fig 3). Relative V_2 protein receptor expression decreased 24.9%, from 0.85 ± 0.11 to

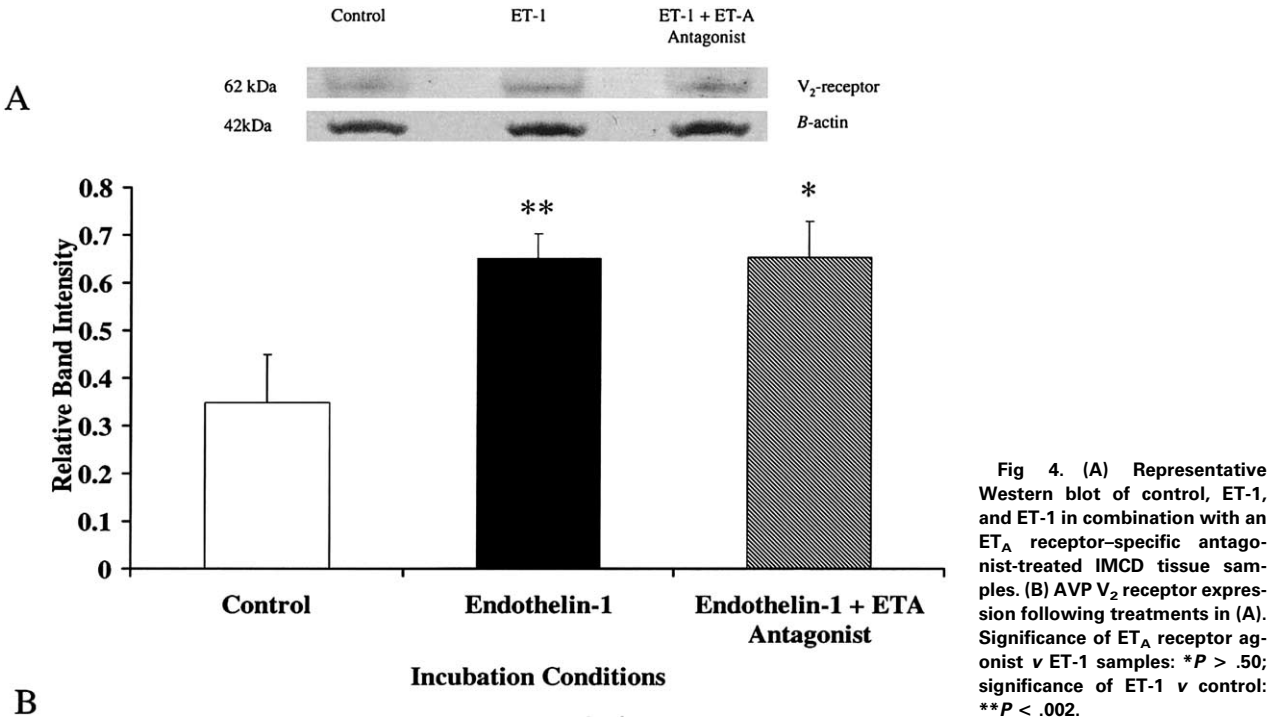

Fig 4. (A) Representative Western blot of control, ET-1, and ET-1 in combination with an ET_A receptor–specific antagonist-treated IMCD tissue samples. (B) AVP V_2 receptor expression following treatments in (A). Significance of ET_A receptor agonist *v* ET-1 samples: **P* > .50; significance of ET-1 *v* control: ***P* < .002.

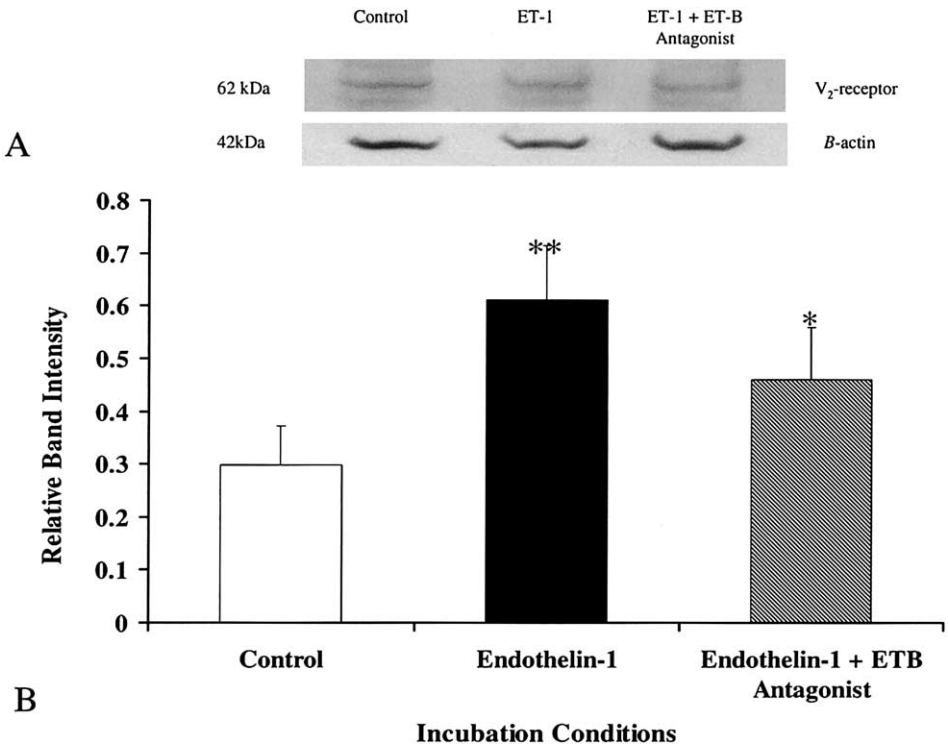

Fig 5. (A) Representative Western blot of control, ET-1, and ET-1 in combination with an ET_B receptor–specific antagonist-treated IMCD tissue samples. (B) AVP V_2 receptor expression following the treatments described in (A). Significance of ET_B receptor agonist v ET-1–treated samples: *$P = .05$; significance of ET-1 v control: **$P < .002$.

0.64 ± 0.06 (n = 6) ($P < .01$) with combined antagonist treatment (Fig 6).

Sarafotoxin and ET-3–Induced Changes in AVP V_2 Receptor mRNA and Protein Expression

Sarafotoxin c (S6c) is an ET_B receptor–specific agonist, allowing further investigation into the identity of the ET receptor mediating increases in AVP V_2 receptor expression. S6c was found to increase AVP V_2 receptor mRNA expression 58% from control levels of 199 ± 17.3 amol/μg total RNA, to 284 ± 42.1 amol/μg total RNA (n = 7) ($P < .05$) (Fig 7). Western blotting results were similar to RT-PCR data. Rat IMCD tissue samples incubated overnight with S6c increased V_2 expression to 2.69 ± 0.506 from control levels of 0.715 ± 0.226 (n = 4) ($P < .01$) (Fig 8). The equivalent affinity of the ET_B for both ET-1 and ET-3 were expected to result in equivalent effects to V_2 receptor expression. However, V_2 receptor mRNA expression demonstrated no significant changes in expression following ET-3 treatment. V_2 receptor mRNA expression following ET-3 treatment was found to be 364.7 ± 46.3 amol/μg compared to control levels of 384.6 ± 94.1 amol/μg total RNA (n = 6) ($P = .41$).

DISCUSSION

In previous studies, our laboratory reported that AVP V_2 mRNA levels were significantly elevated in cardiomyopathic hamsters compared to corresponding controls.[17,18] The increase in AVP V_2 mRNA in the IMCD of the kidney of cardiomyopathic hamsters correlates with the severity of heart failure. These observations suggest that upregulation of AVP V_2 receptors in the IMCDs of cardiomyoapthic hamsters contributes to excessive water reabsorption by the kidney that leads to fluid retention. The upregulation of AVP V_2 receptors in the kidney can be mediated by angiotensin II[18] or ET.[17] This notion was supported by studies with enalapril, an angiotensin-converting enzyme inhibitor, which reduced the upregulation of AVP V_2 receptors in cardiomyopathic hamsters to normal levels. Bosentan (a mixed ET_A/ET_B receptor antagonist) administered to cardiomyopathic hamsters also resulted in a decrease in AVP V_2 receptor expression.[17] This suggests that angiotensin and ET are involved in the control of AVP V_2 receptor expression. This relation is of particular interest given their possible interaction in congestive heart failure, where both increased levels of ET-1 and vasopressin have been demonstrated.[6,19,20,21] The bosentan studies identified an important role for ET in the pathogenesis of water retention in congestive heart failure. Therefore, the role of this study was to investigate the possible effect of ET-1 on AVP V_2 receptor expression and identify the ET receptor mediating these effects.

AVP plays a primary role in the maintenance of plasma osmolarity and hemodynamic control through the control of water reabsorption in the IMCD.[22] The activity of vasopressin system in congestive heart failure patients has been found to be disturbed at various levels of function. AVP levels and response have been found to be inappropriately high for given osmolalities in people with congestive heart failure.[23,24] Similarly, in congestive heart failure animal models, the renal response to AVP, as well as V_2 receptor expression, has been shown to be increased.[18,24] AVP induced cyclic adenosine monophosphate (camp) production; AQP-2 cell membrane insertion and AQP-2 water channel expression are also heightened.[4,5,18] Additionally, AQP-2 protein expression has been

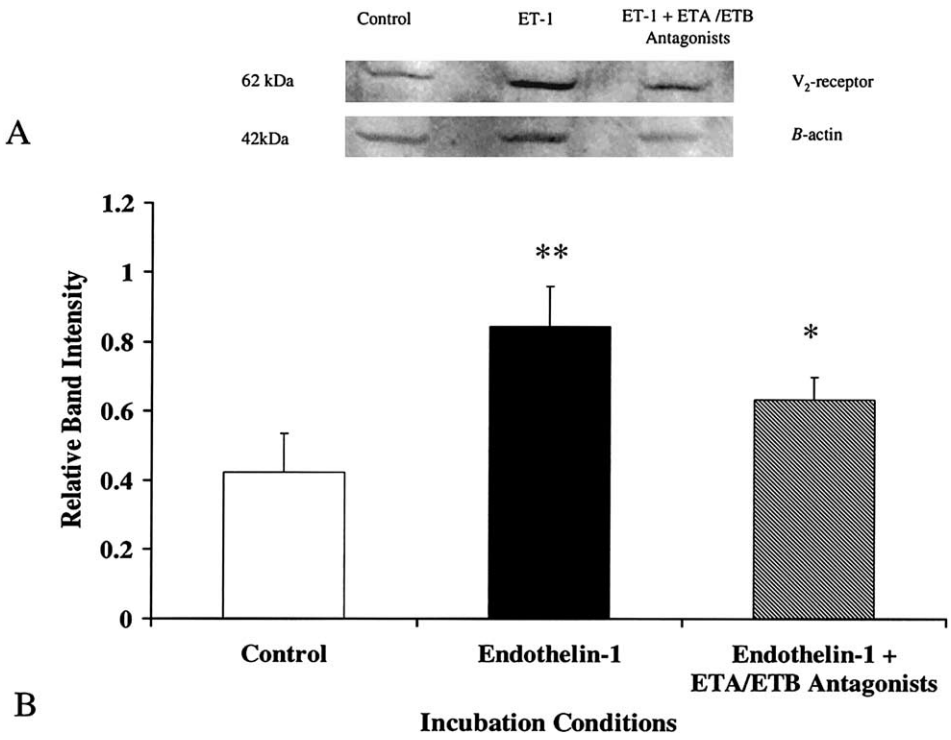

Fig 6. (A) Representative Western blot of control, ET-1, and ET-1 in combination with ET_A and ET_B receptor–specific antagonist-treated IMCD tissue samples. (B) AVP V_2 receptor expression following the treatments described in (A). Significance of ET_A and ET_B receptor agonist *v* ET-1–treated samples: *$P < .01$; significance of ET-1 *v* control: **$P < .002$.

shown to correlate with V_2 receptor expression.[3] Together, these observations suggest that V_2 receptor expression may account for the observed changes in AVP sensitivity.

ET-1 expression has been found to be an effective prognostic indicator, exhibiting a 2- to 3-fold elevation in expression in congestive heart failure patients, suggesting its possible role in congestive heart failure pathology.[20,21,25,26] ET-1 expression in the IMCD is greater than any other region of the kidney.[27-29] Two distinct endothelin receptors, ET_A and ET_B, are recognized to exist in mammals. ET_A receptors bind the N-terminus of ETs, and due to the N-terminus amino acid variability, demonstrate a differential binding affinity of ET-1 > ET-2 > ET-3 = S6c.[30,31] The ET_B receptor binds the invariable C-terminus, resulting in equal binding affinity for S6c and all endothelin isoforms. In 1993, Karne et al[31] cloned the ET_C receptor, which has high affinity for ET-3, from Xenopus

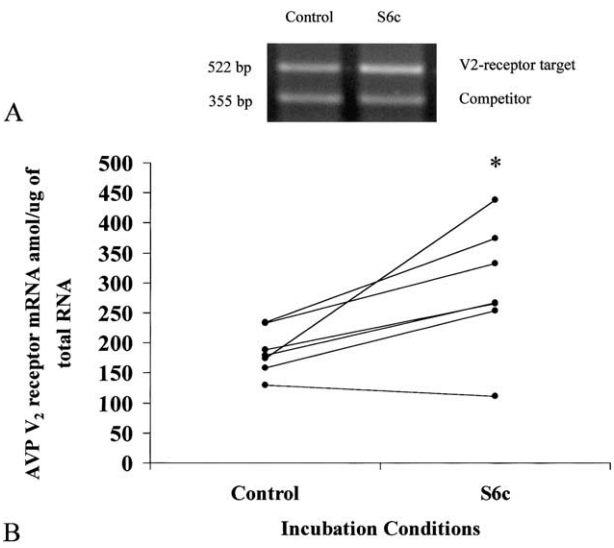

Fig 7. (A) Representative RT-PCR gel of control and S6c-treated samples. (B) Illustrates increased AVP V_2 receptor expression in S6c *v* control samples, *$P < .05$.

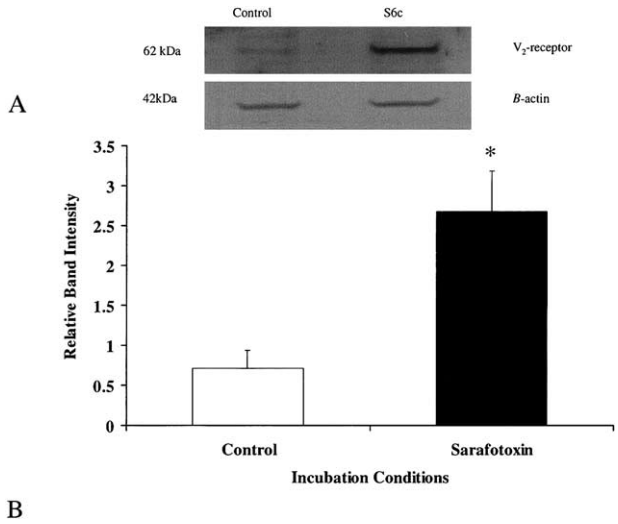

Fig 8. (A) Representative Western blot of control and S6c-treated samples. (B) Illustrates the AVP V_2 receptor expression following the treatments as described in (A). *$P < .01$.

dermal melanophores. Additionally, other mammalian ET-like receptors and ET_B receptor splice variants have been identified.[32-35] The ET_A and ET_B receptors are both expressed in the IMCD and the rest of the kidney; however, the ET_B receptor predominates in the IMCD, with ET_B expression approximately 4 times greater than that of ET_A within human kidneys.[15,17,36]

In the present experiment, incubation of rat IMCD tissue with ET-1 was found to significantly increase both V_2 receptor mRNA and protein expression. The ET_B receptor agonists ET-3 and S6c were used to investigate the ET receptor responsible for mediating these effects. As with ET-1, S6c treatment resulted in increases in V_2 receptor mRNA and protein expression. ET-3 treatment, however, unexpectedly resulted in no significant changes in V_2 receptor mRNA expression.

Bird et al[36] also reported differential effects of ET-3 and S6c on kidney function. One potential explanation for these discrepancies comes from recent studies by Taylor et al,[37] demonstrating the existence of an additional ET binding site within the IMCD of ET_B receptor knockout rats. The putative receptor was reported to have a high affinity for ET-3, but it is susceptible to an ET_A receptor antagonist. Therefore, in the ET-3 incubations the targeted ET_B receptor as well as an additional non-ET_A/non- ET_B receptor may have resulted in the inconsistent effects. Ligand-specific G-protein coupling is an additional explanation for the differential effects of S6c and ET-3 on V_2 receptor expression. However, Shraga-Levine et al[38] found similar ET_B receptor-G protein coupling following ET-3, S6c, and ET-1 treatments, suggesting that this is an unlikely hypothesis.

The ET_A and ET_B receptor antagonists, BQ-123 and BQ-788, respectively, were also used to investigate that identity of the ET receptor–mediating increases in V_2 receptor expression. Comparison of samples incubated with ET-1 to samples incubated with ET-1 and ET_A receptor antagonist shows that ET_A receptor antagonism had no significant effect on V_2 receptor mRNA expression. In contrast, incubations of ET-1 in combination with an ET_B receptor antagonist resulted in a significant reduction in V_2 receptor mRNA expression approaching that of control samples. Similarly, combined incubation of ET-1 with both the ET_A and ET_B receptor antagonists resulted in V_2 receptor mRNA expression levels approaching controls, which was significantly reduced when compared to the ET-1–incubated samples.

In conclusion, these experiments demonstrated that ET-1 upregulates V_2 receptor mRNA and protein expression. They also provide evidence that upregulation of V_2 receptor expression is mediated by the ET_B receptor. ET-1–induced increases in V_2 receptor expression provide a partial explanation of the role ET-1 in the pathology of congestive heart failure. Additional experiments are needed to elucidate the signaling pathways involved in ET control of V_2 receptor expression and function, as wells as further investigation into the ET binding site identified by Taylor et al.[37]

ACKNOWLEDGMENT

We acknowledge the expert technical assistance of Alice Fok.

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